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West Nile virus and wild bird populations

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WEST NILE VIRUS AND WILD BIRD POPULATIONS

A Thesis by

Thomas Robert Shelite

Bachelor of Science, Wichita State University, 2003

Submitted to the College of Liberal Arts and Sciences
and the Faculty of the Graduate School of
Wichita State University in Partial Fulfillment
of the Requirements for the Degree of
Master of Science

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WEST NILE VIRUS AND NATURAL BIRD POPULATIONS

I have examined the final copy of this Thesis for form and content and recommended that it be accepted in partial fulfillment of the requirement for the degree of Master of Science, with a major in Biological Sciences

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We have read this Thesis
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DEDICATION

To Bobbie Shelite friend, mentor, and father; we will see each other again some day.

A good gulp of hot whiskey at bedtime - it's not very scientific, but it helps.
~Alexander Fleming

Do or do not, there is no try.
~Yoda

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ABSTRACT

West Nile Virus (WNV) first appeared in the western hemisphere in 1999, and has since spread across the United States and into Mexico and the Caribbean. It has been hypothesized that WNV has spread rapidly via migratory birds, and that various avian species may facilitate viral amplification during winter months.

The goals of this research were to determine the role of American Tree Sparrows (*Spizella americana*) in the spread of WNV during their migrations and to determine the role of the Northern Cardinal (*Cardinalis cardinalis*) in winter survivorship and subsequent spring amplification of WNV. Additional wintering avian species were sampled to provide a general survey of the prevalence of WNV in winter in south-central Kansas. Blood samples were taken from the brachial vein of migratory and wintering birds captured using mist nets at four wintering feeding stations at the Wichita State University Field Station. Some samples were taken from retrapped birds within a single winter to determine if winter transmission occurs. Some birds were resampled in consecutive winters to monitor seroconversion rates. Analysis of serum samples were performed, in triplicate, using an epitope-blocking ELISA. The current study was conducted during the consecutive winters of 2003-04 and 2004-05.

It was concluded that resident species had an increased incidence of WNV exposure when compared to that of migratory species. This difference suggests that migratory species may not have as important a role in the dissemination of WNV as first hypothesized. Also, minimal, if any, winter transmission occurs on communal feeding grounds. Viral amplification during the winter was not demonstrated, although one individual seroconverted during a single winter.

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LIST OF ABBREVIATIONS / NOMENCLATURE

AMGO	American Goldfinch
ATSP	American Tree Sparrow
B	Background
BCCH	Black-capped Chickadee
BLJA	Blue Jay
C	Celsius
CS	Control serum
DEJU	Dark-eyed Junco
°	Degree
DOWO	Downy Woodpecker
EABL	Eastern Blue Bird
ELISA	Enzyme linked immunosorbent assay
ETTI	Eastern Tufted Titmouse
HASP	Harris's Sparrow
kDa	Kilodalton
MAb	Monoclonal antibody
mM	Millimolar
nm	Nanometer
NOCA	Northern Cardinal
PBS	Phosphate buffered saline
RBWO	Red-bellied Woodpecker
RNA	Ribonucleic Acid

LIST OF ABBREVIATIONS / NOMENCLATURE

RWBL	Red-winged Blackbird
SOSP	Song Sparrow
TS	Test serum
WNV	West Nile Virus
WTSP	White-throated Sparrow
χ^2	Chi square

CHAPTER I

LITERATURE REVIEW

STRUCTURAL DESCRIPTION

West Nile virus (WNV) is a Flavivirus in the family *Flaviviridae*. This family includes many different species of viruses. Japanese Encephalitis virus (JEV), St. Louis Encephalitis virus (SLEV), Kunjin virus, and Murray Valley Encephalitis (MVE) all belong to this family and are antigenically similar (Peterson and Roehrig 2001). All of these viruses, except Kunjin, are found in the United States. Kunjin virus is found only in tropical parts of Australia and is considered to be actually a subtype of WNV (Peterson and Roehrig 2001).

All of these viruses are maintained in epizootic transmission cycles with mosquitoes as vectors and birds as primary reservoirs (Peterson and Roehrig 2001). West Nile virus is a single-stranded, positive-sense, RNA virus, and its capsid is enclosed in a host-cell-derived envelope (Fig.1). It is made of a 30-35nm icosahedral core, which contains multiple copies of 12-kDa capsid proteins. West Nile virus has two integral membrane glycoproteins; E and prM. The E protein is the more immunologically important of the two in that it mediates virus-host cell binding. The protein prM is cleaved into M protein and is included into the mature virion; however, this protein is still poorly understood (Peterson and Roehrig 2001). The genome also contains seven nonstructural genes which play a role in intracellular replication.

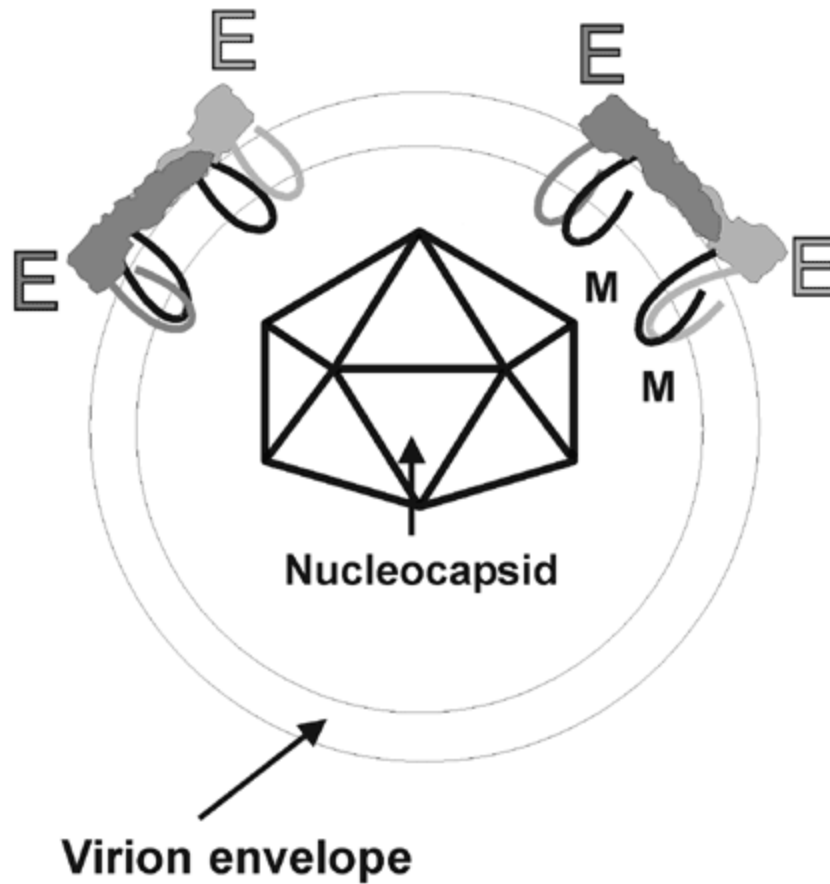


Figure 1. Structure of WNV (Peterson and Roehrig 2001).

HISTORY

West Nile virus was first isolated in 1937 from a febrile woman in the West Nile region of Uganda (Hayes 2001). Although it was first isolated in 1937, a recent paper speculates that the virus may have been around for at least two millennia (Marr and Calisher 2003). This paper considered the possibility that Alexander the Great may have died from WNV infection. This was suggested by the symptoms that were recorded from the scribes of that time. If true, then West Nile virus has been around considerably longer than originally thought, which could possibly account for the reduced pathogenicity

toward Old World hosts. The reason for not identifying it sooner was likely due to its ability to “mimic” other illnesses, but since 1937, there have been several outbreaks attributed to WNV including those in Israel during 1951-54 and 1957, South Africa during 1974, Romania & Morocco during 1996, Tunisia during 1997, Italy and Israel during 1998, as well as Israel, Russia, and the United States (US) during 1999 (Peterson and Roehrig 2001).

The outbreaks usually occurred around areas that had a greater amount of standing water, such as swamps and wetlands, that are important breeding grounds for mosquitoes. The outbreaks that occurred during the 1990s, showed disturbing new evidence that WNV may have become more pathogenic (Peterson and Roehrig 2001). Three new trends during these outbreaks were observed: 1) an increase in human and horse infections, 2) an increase in severe human disease, and 3) an increase in avian mortality, especially for those outbreaks occurring in Romania, Israel, and the US that accompanied human outbreaks indicating a possible increase in pathogenicity towards the virus’ host (Peterson and Roehrig 2001).

The US outbreak of 1999 was the first WNV outbreak to occur in the Western hemisphere. How WNV was introduced to North America is highly conjectural. Some hypothesize that it was a failed bioterrorist attack (Rappole and Hubalek 2003). Others believe that stowaway mosquitoes aboard planes introduced WNV into the New World. The most likely explanation of its introduction is via an avian introductory host (Rappole and Hubalek 2003). There are three possible ways this may have occurred; 1) normal migration 2) storm driven birds, or 3) importation, legally or illegally, of infected birds (Rappole and Hubalek 2003). Importation seems to be the most likely route. The reason

behind this is the fact that the WNV strain isolated in New York, the site of the original outbreak in the Western Hemisphere, is 99% homologous to the strain of WNV that caused an outbreak in Israel in 1998-99 (Hayes 2001).

TRANSMISSION CYCLE

As previously mentioned WNV is maintained in the environment via an epizootic transmission cycle. West Nile virus is an arthropod-borne virus. The primary vectors are various mosquito species (Hayes 2001). Specifically, *Culex (Cx.) sp.* is the main mosquito indicated in this transmission cycle. *Cx. pipiens* is the most ornithophilic, suggesting that it maintains the cycle within the avian species, while *Cx. modestus* has a wide range of hosts that could act as a bridge vector between avian species and humans (Andreadis et al. 2001). The reservoir for WNV is various bird species (McLean et al. 2001). The primary transmission cycle is between mosquitoes and avian hosts (Fig. 2).

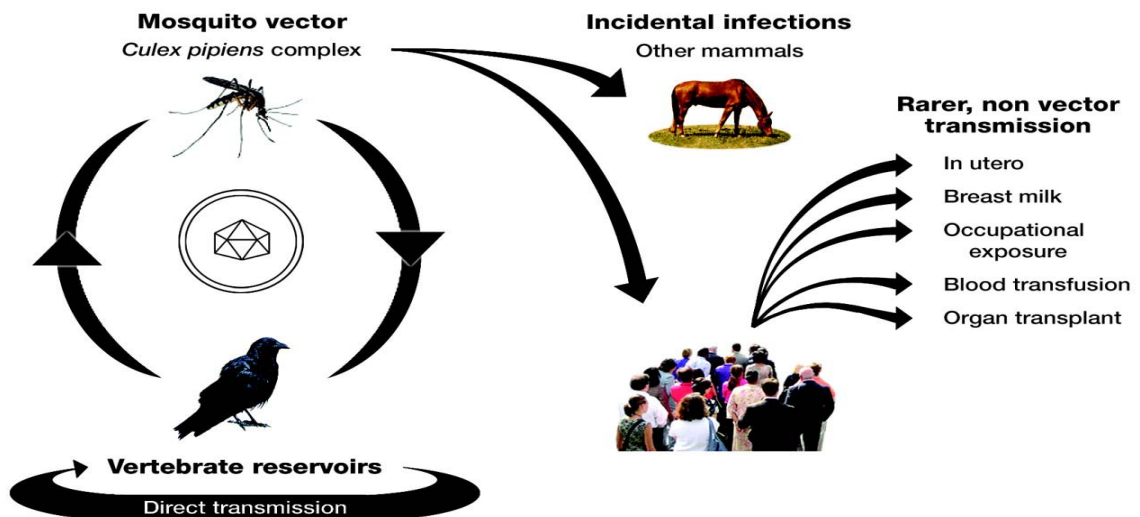


Figure 2. WNV transmission cycle (www.cdc.gov).

Humans and horses are only incidental hosts, although they may be killed by the virus. These hosts are unable to generate a viral titer high enough to infect new mosquitoes. Besides mosquito-to-bird and mosquito-to-human or –horse transmission, there are other means of transmission. In laboratory studies, female mosquitoes have been shown to vertically transmit the virus to offspring (Turell et al. 2001). Lateral transmission between mosquitoes has also been documented (Higgs et al. 2005). Lateral transmission was demonstrated from non-viremic hosts. An infected mosquito and a non-infected mosquito were allowed to feed adjacently on a non-viremic host. After seven days, the non-infected mosquito had viral titers high enough to infect new hosts (Higgs et al. 2005). Along with mosquitoes, other blood-feeding invertebrates may aid in the spread of WNV; Argas ticks and sandflies have also tested positive for WNV (Abassy et al. 1993).

Studies with captive American Crows (*Corvus brachyrhynchos*) showed the possibility of bird-to-bird transmission via saliva and/or fecal aerosols (Komar et al. 2003a, Rogers 2003). Ingestion of infected organisms has also been considered as a possible source of transmission (Garmendia et al. 2000, Peterson and Roehrig 2001, Miller et al. 2003). Blood transfusions and organ transplants became unlikely sources of transmission after 1999, since WNV wasn't one of the diseases originally screened for during donations (Iwamoto et al. 2003). There have been few documented cases of mother-to-fetus transmission, but there hasn't been a collective effort to keep track of potential cases. This remains a possible route of transmission that is being investigated further (Hayes et al. 2004). There have not yet been any documented cases of direct

animal-to-human infections, other than through a mosquito, although this may be possible.

The effects of WNV on birds are widespread; at least 208 North American species, most of which belong to the order Passeriformes (the perching birds), have been documented with WNV infections (Marra et al. 2004). After exposure to WNV, individuals will begin exhibiting symptoms in 3-14 days post infection (Komar et al. 2003). As with the number of species affected, a variety of systemic effects have been observed. Neurological symptoms of an infected bird consist of ataxia, tremors, abnormal head posture, circling, and/or convulsions. Pathology of the virus determined that it is nondiscriminatory as to which organs are infected. Brains, hearts, spleens, livers, kidneys, adrenals, intestines, pancreata, lungs, and ovaries all had numerous lesions due to infection and were all sites of replication of WNV. Nearly 100% of the brains and kidneys of infected birds, which represented eight orders and fourteen species, were infected and seropositive for WNV (Steele et al. 2000). Not all birds that are bitten become ill, some develop antibodies to WNV; species such as the domestic chicken have become useful as sentinels for WNV activity. The most susceptible birds are those belonging to the family Corvidae, especially American Crows (*Corvus brachyrhynchos*) and Blue Jays (*Cyanocitta cristata*) (Steele et al. 2000). These species are the most commonly submitted for testing; however, why these species seem to be so heavily affected has yet to be determined.

GEOGRAPHIC DISSEMINATION OF WNV

It has been hypothesized that the spread of WNV has been facilitated by migratory birds based on the dissemination of the virus in the Old World (Rappole et al. 2000, Malkinson et al. 2002). In Europe and the Middle East, WNV outbreaks often coincide with the arrival of migratory species from their wintering grounds in Africa, where WNV is endemic (Malkinson and Banet 2002). One such example is the migration of White storks (*Ciconia ciconia*) from Africa, passing through the Middle East, that preceded a WNV outbreak in 1998, and subsequent outbreaks in Israel (Malkinson et al. 2002). Excluding the continual outbreaks in Israel, the outbreaks of WNV that occur in Europe are sporadic and seem to require reintroduction of the virus via migratory birds (Fig. 3) (Hannoun et al. 1972, Hubalek 2000, Malkinson and Banet 2002).



Figure 3. WNV outbreaks in Europe (Hubalek and Halouzka 1999).

Upon introduction into the US, most researchers speculated that the virus would be spread in a similar fashion to that of the Eastern Hemisphere (Rappole et al. 2000). This model would predict that the virus would become endemic to tropical areas of the Western Hemisphere and consequently, those migratory birds that use these areas as wintering grounds would then introduce the virus to new locations during spring migration, thus coinciding with increased mosquito activity (Peterson et al. 2003). This would result in sporadic, annual outbreaks with occasional reoccurring outbreaks similar to that of Europe, but what actually happened is that the virus moved concentrically from the epicenter of the outbreak, New York City, and has reappeared in subsequent years (Figs. 4-6) (Rappole and Hubalek 2003). This pattern of concentric spread is not consistent with migratory bird introduction, as this should result in sporadic introduction of the virus due to the ability of migratory birds to travel great distances.

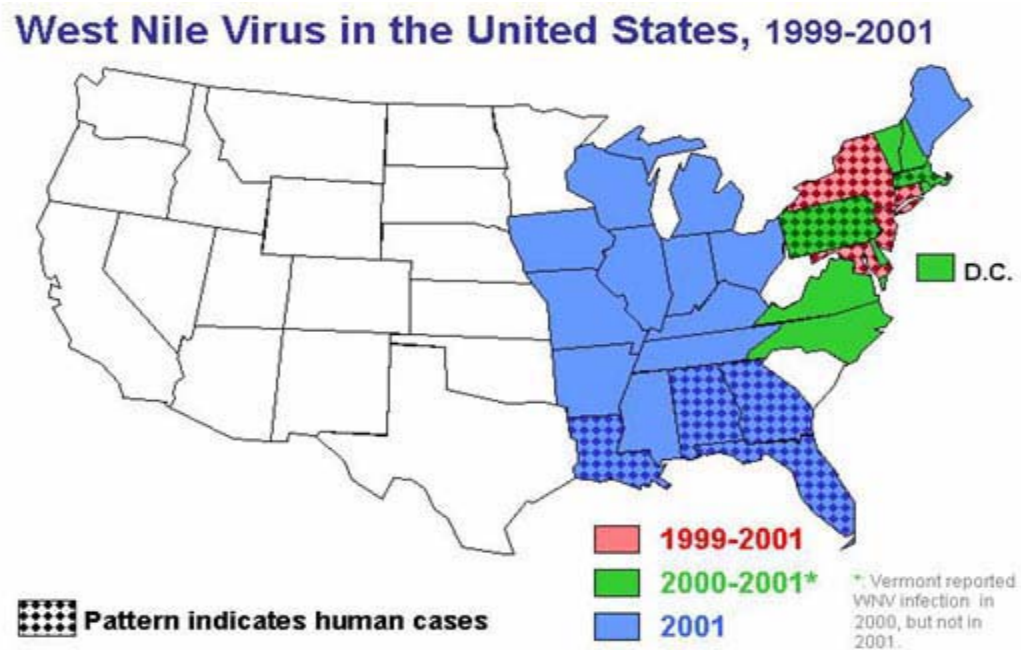


Figure 4. WNV infections 1999-2001 (www.cdc.gov).

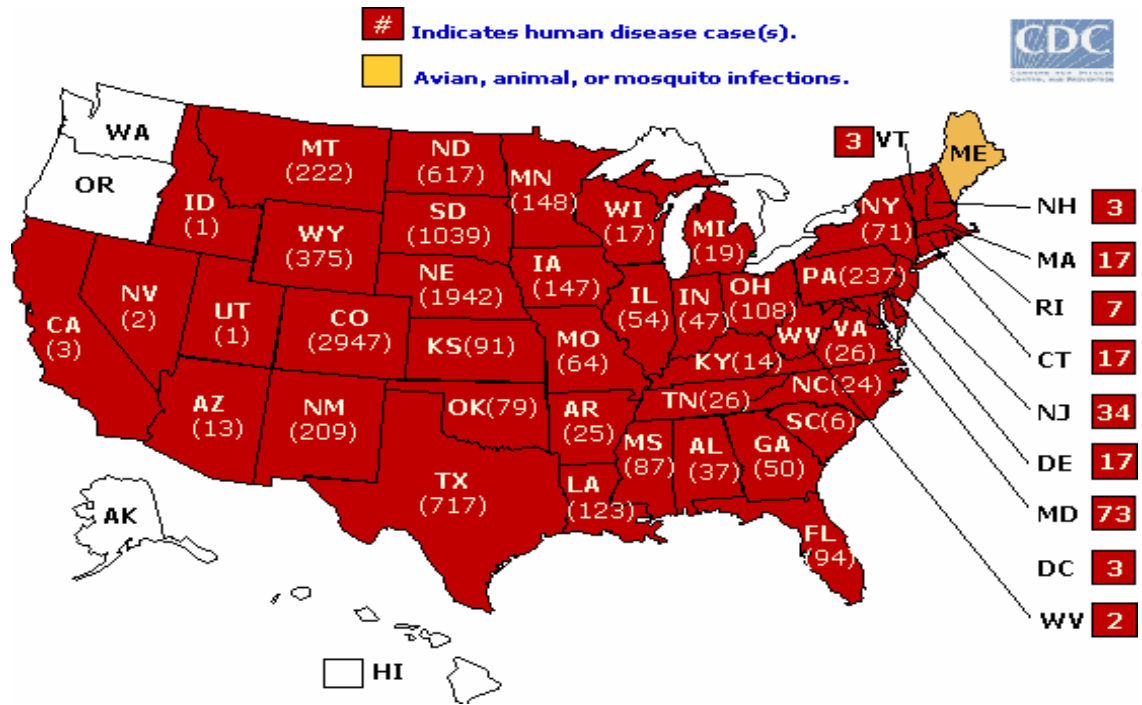


Figure 5. WNV infections 2002-2003 (www.cdc.gov).

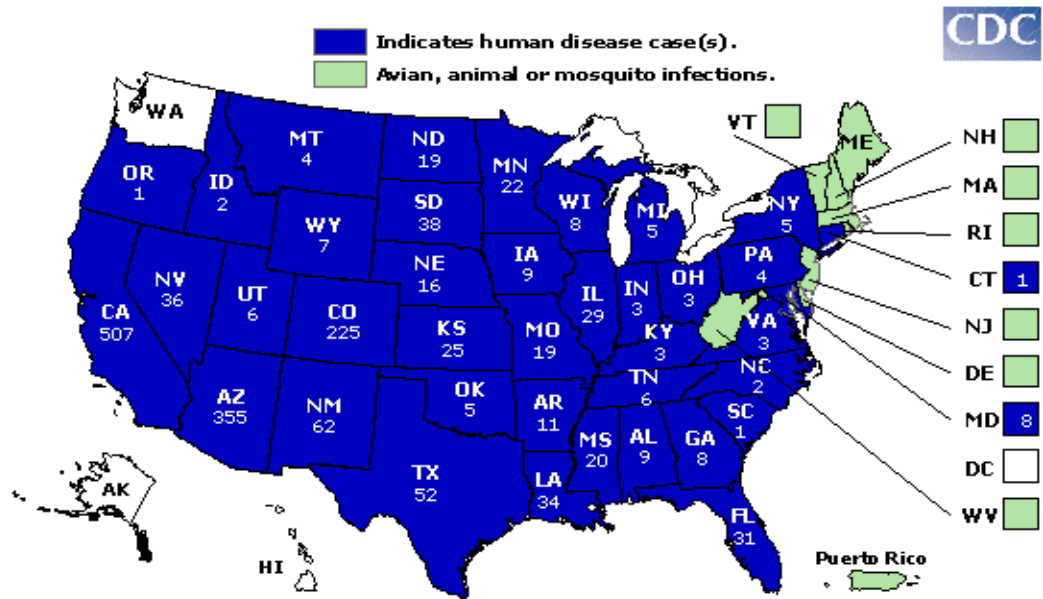


Figure 6. WNV infections 2003-2005 (www.cdc.gov).

Since its introduction into the US, WNV has spread across North America to 48 states and seven Canadian provinces as well as northern Mexico and several Caribbean nations (Fig. 7) (Komar et al. 2003b, Deardorff et al. 2006).

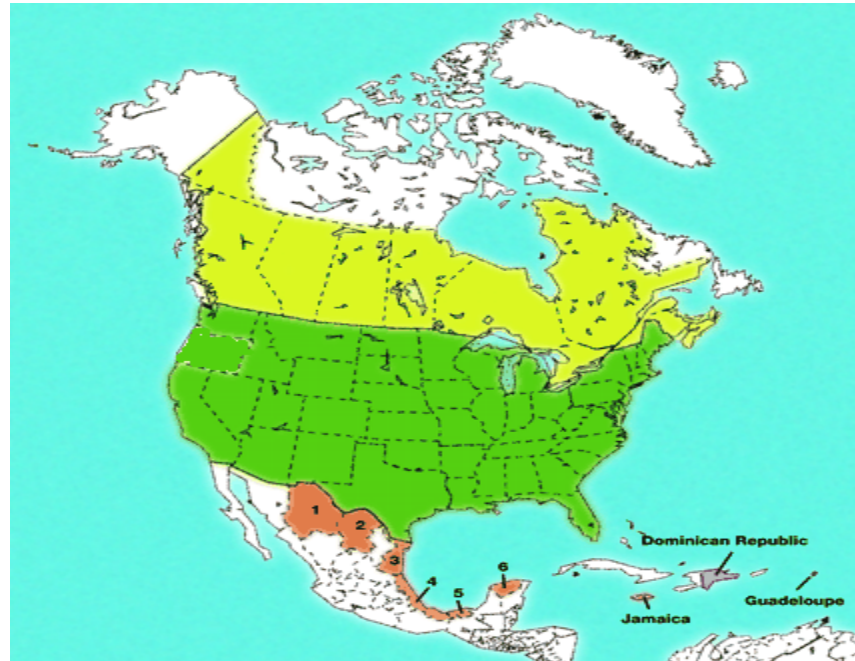


Figure 7. WNV distribution in North America as of 2005 (www.cdc.gov). Colors define areas infected with WNV.

HYPOTHESES AND RATIONALE

The goal of the current study was to develop a better understanding of the ecological factors affecting the spread of WNV, i.e. migration, viral winter survivorship, and spring amplification of WNV.

The first hypothesis of this research is that those common, medium-distance migrants such as American Tree Sparrows (ATSP) (*Spizella americana*), act as couriers

for WNV. The American Tree Sparrow is a 15-20 g medium-distance migrant. They breed in the northern tundra of Alaska and Canada and winter in central North America (**Appendix A**) (Naugler 1993). Their migratory path takes them through areas that may have experienced WNV activity the previous summer. Banding and sampling of ATSP in one winter, along with recapturing and sampling of uniquely banded birds in a second winter, was the mechanism used to determine if WNV is increasing in prevalence in this migratory population of birds, and what role, if any, they play in the potential spread of the virus to new locations during their migrations. This was addressed by comparing the number of infected individuals from a winter season with that from subsequent winters. If a greater number of banded birds test positive in the second winter, it will demonstrate a potential increase in WNV exposure in this migratory species, and therefore suggest they may have spread the virus to new locations over their migratory paths.

The second hypothesis behind this research is that relatively large, non-migratory birds play a role in WNV winter survivorship, as a reservoir or via lateral transmission, and play a role in spring amplification of WNV. Studies have shown that some individuals maintain latent infections that could result in subsequent spring amplification (Komar et al. 2003a). Study of Northern Cardinals (NOCA) (*Cardinalis cardinalis*) will allow a better understanding of the winter ecology of WNV. The Northern Cardinal is a large (45-55 g), non-migratory passerine (Halkin and Linville 1999) (**Appendix B**) that allowed for testing not only during the winter months, but during spring as well. This provided a way to observe increases in potential viral activity. Winter survivorship of the virus may be facilitated by the large size of this bird, and it has been implicated that some species of passerines maintain chronic, low-level infections that could result in fecal shed

of the virus in communal feeding areas, thus possibly resulting in lateral transmission (Komar et al. 2003a). Also, if low-level infections occur among NOCA, then it is possible that they may initiate the transmission cycle in spring when mosquitoes become active and reproductive stresses occur. If this species is a reservoir for WNV, reproductive stresses may act as a viral cue for amplification. Repeated seasonal sampling of uniquely banded individuals will allow for the observation of increased viral activity, assuming that low viral activity doesn't initiate an antibody response (Komar et al. 2003a).

Finally, a general survey of wintering birds, including various migrants and resident species, was conducted. Along with sampling of wintering ATSP and NOCA, Dark-eyed Junco (DEJU) (*Junco hyemalis hyemalis*), Harris's Sparrow (HASP) (*Zonotrichia querula*), Song Sparrow (SOSP) (*Melospiza melodia*), White-throated Sparrow (WTSP) (*Zonotrichia albicollis*), American Goldfinch (AMGO) (*Carduelis tristis*), Blue Jay (BLJA) (*Cyanocitta cristata*), Eastern Bluebird (EABL) (*Sialia sialis*), Red-winged Blackbird (RWBL) (*Agelaius phoeniceus*), Black-capped Chickadee (BCCH) (*Poecile atricapilla*), Eastern Tufted Titmouse (ETTI) (*Baeolophus bicolor*), Downy Woodpecker (DOWO) (*Picoides pubescens*), and Red-bellied Woodpecker (RBWO) (*Melanerpes carolinus*) were also sampled. This allowed for a better general understanding of the prevalence of WNV in wintering bird populations in south-central Kansas.

Understanding the role of migratory birds in the spread of WNV will aid in the potential identification of future outbreaks as well as act as a model for future introduction of arboviruses. Determining how the virus is maintained in the environment

when its primary vector is absent, or less active, will allow local- and state-funded agencies to better direct their efforts in controlling human outbreaks. It is the overall goal of this project to develop a better understanding of the ecological factors involved in the spread and maintenance of WNV in new areas.

CHAPTER II

RESEARCH DESIGN AND METHODS

COLLECTION OF SAMPLES

Species collection. Avian species were collected using standard mist nets, ranging 6-12m in length, at four feeding stations at the Wichita State University Field Station (Fig. 8), during the winters of 2003-04 and 2004-05 for sampling of ATSP, DEJU, HASP, SOSP, WTSP, AMGO, EABL, RWBL, BLJA, BCCH, DOWO, ETTI, NOCA, and RBWO. Serial sampling was implemented during winters 2004-05, with additional samples being acquired no sooner than 10-14 days after each previous sample of an individual, to evaluate if winter transmission had occurred. The delay was to remove the possibility of late winter seroconversions causing a false increase in WNV exposure in subsequent winters. Netting sites were chosen based on ecological habitats available, i.e. those locations ideally suited to the wintering avian community (Naugler 1993, Halkin and Linville 1999), water supply, and vehicle accessibility. Each individual bird was banded with a uniquely numbered aluminum U.S. Geological Survey numerical band on the left leg. Immediately after capture, a blood sample was acquired using a sterile 1cc insulin syringe with a 27 gauge 5/8 in. needle to puncture the left or right brachial vein of the bird. Whole blood was collected in 70- μ l micro-hematocrit capillary tubes (Fisherbrand). Three to five tubes were taken from each bird and allowed to coagulate for at least 10-15 minutes. Upon finishing collection of the blood sample, a piece of paper towel was applied to the puncture wound to promote clotting. When the wound stopped bleeding the bird was released and observed for obvious flight impairment. Individuals were in

captivity no longer than 30 minutes and were always kept warm to minimize shock. Associated IACUC animal protocols have been approved and were used.

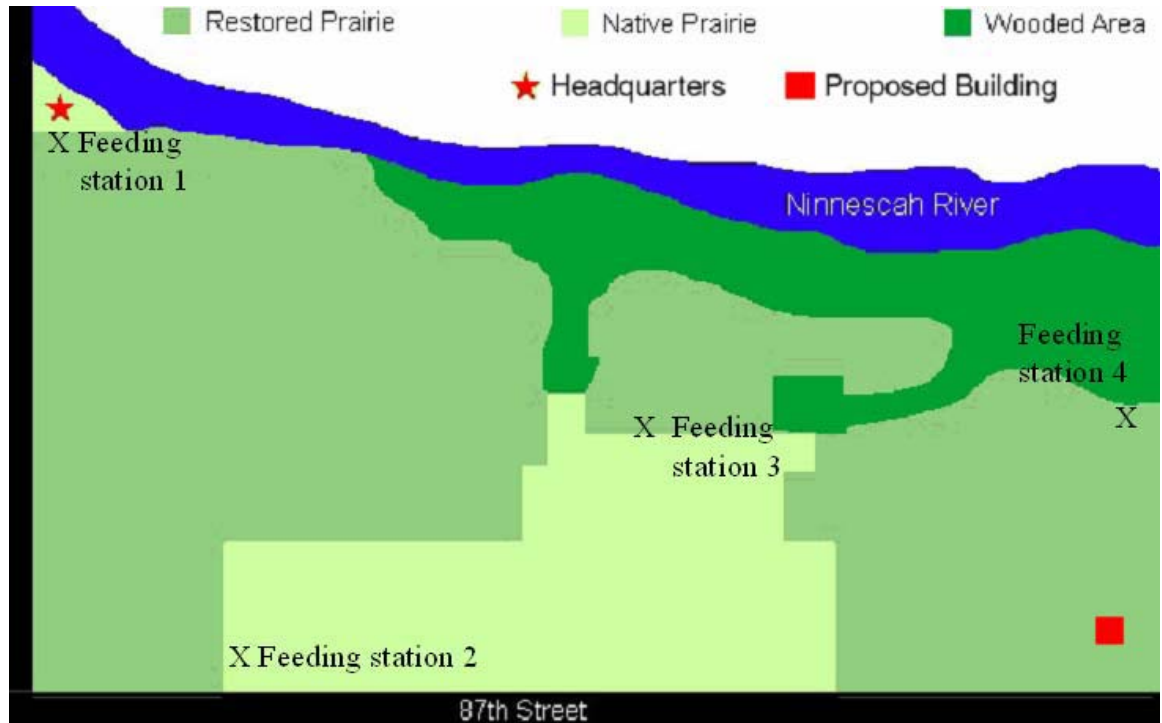


Figure 8. Wichita State University Field Station with feeding stations 1-4.

Serum collection. After coagulation occurred, the hematocrit tubes for each bird were centrifuged using an IEC MB micro-hematocrit centrifuge (Damon/IEC Division) for three minutes at 8xg and at room temperature. This was done to separate the blood cells and cell debris from the serum. The serum was collected using a syringe, and placed in externally threaded cryotubes as mandated by the Center of Disease Control and Prevention (CDC 2003). Samples were stored at 4 °C in a refrigerator, transported on ice from the field, and then stored at -70 °C until laboratory evaluation was conducted.

LABORATORY ANALYSIS OF SAMPLES

Epitope-blocking ELISA. Individual serum samples were tested for WNV antibodies using an epitope-blocking ELISA, as previously described (Hall et al. 1995, Blitvich et al. 2003a,b). This method is a rapid, sensitive, and inexpensive means to test multiple avian species for the presence of WNV antibodies. Briefly, two microtiter plates are prepared (Cat. No. 07-200-642 FISHER), one for each antisera, MAb 3.1112G and MAb 6B6c-1 (Chemicon); MAb 3.1112G is WNV specific and MAb 6B6C-1 is flavivirus group reactive (Hall et al. 1995, Blitvich et al. 2003a). Wells were coated using WNV antigen, prepared by the Arbovirus Infectious Disease Laboratory at Colorado State University, diluted to predetermined working dilutions in carbonate-bicarbonate buffer (50 mM sodium carbonate, 50 mM sodium bicarbonate at pH 9.6) for the number of samples being tested and incubated overnight at 4 °C (Blitvich et al. 2003b). Four negative controls, using uninfected chicken serum, and two positive controls, using WNV-positive horse serum, were included for a total of 60 coated wells per plate. Between every incubation of the ELISA, plates were washed four times with PBS (pH 7.4) containing 0.1% Tween 20 using an automated plate washer (BioTek Instruments Elx 50 Automated Stripwasher). Blocking buffer (PBS with 0.1% Tween 20 and 5% non-fat dry milk) was added after washing to each sample well and incubated for 40 minutes at 37 °C. After washing as above, a 1:10 dilution of serum samples in blocking buffer was added to the coated wells, with each sample ran in triplicate per plate, and incubated at 37 °C for 2 hours. After washing, two monoclonal antibodies were used, one per plate. Each MAb was diluted to its predetermined working dilution in blocking buffer. These were added to their respective plates and incubated at 37 °C for one hour. Both MAbs must be

labeled with horseradish peroxidase using horseradish peroxidase-conjugated rabbit anti-mouse IgG secondary antibody(Zymed Laboratories) diluted to 1:2000 in blocking buffer and then incubated at 37 °C for one hour. After washing as above, a ready-to-use ABTS solution (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma) was added to induce a color reaction by horseradish peroxidase. The optical density of each well was determined using an automated plate reader (Elx 800 BioTek Instruments Universal Microplate Reader) at a wavelength of 405nm (Blitvich et al. 2003b). The percent inhibition of MAb binding was calculated (Hall et al. 1995) as follows:

$$100-[(TS-B)/(CS-B)] \times 100$$

where TS is the mean optical density of the test serum, CS is the mean optical density of the control serum (from uninfected chickens), and B is the background optical density. This was calculated once the mean optical density of CS exceeded 0.3. For this method, a percent inhibition value of $\geq 30\%$ was considered the indicator of the presence of viral antibodies in the test sera (Blitvich et al. 2003b). All samples were assayed in triplicate to reduce investigator bias as well as procedural error. Mean values of the three test samples are reported. Statistical significance of migratory status and percent exposed was determined using a G-test to generate a chi square value to be compared to the critical chi square value at $p=0.05$ or less.

CHAPTER III

RESULTS

WINTER 2003-2004

Field Results. A total of 276 individual birds were captured and a blood sample taken from each for laboratory analyses. Of these 276 birds, 248 were migratory species and 28 were classified as resident species (Table 1).

Table 1

Winter 2003-04 Species Totals. Birds, listed alphabetically by migratory status, number of birds sampled

Species	Total Sampled	Migratory Status
American Tree Sparrow	192	Migratory
Dark-eyed Junco	14	Migratory
Harris's Sparrow	23	Migratory
Song Sparrow	16	Migratory
Blue Jay	3	Semi-migratory
Northern Cardinal	28	Resident
TOTAL	276	

Laboratory Results. For each sample collected, an epitope-blocking ELISA was done in triplicate, for both antibodies, to determine if the individuals sampled had been exposed to WNV prior to their capture in the winter of 2003-04 (Fig. 9). Sampling was conducted on several separate days throughout the winter, to determine whether the number of seropositive birds increased at anytime during the winter months (Figs. 10-11). The comparison of WNV exposure, with regard to migratory status using a G-test yielded a chi square value of $\chi^2 = 35.73$, $p = .0001$, and d.f. 1, indicating a significant difference based on migratory status of individuals testing positive for WNV exposure.

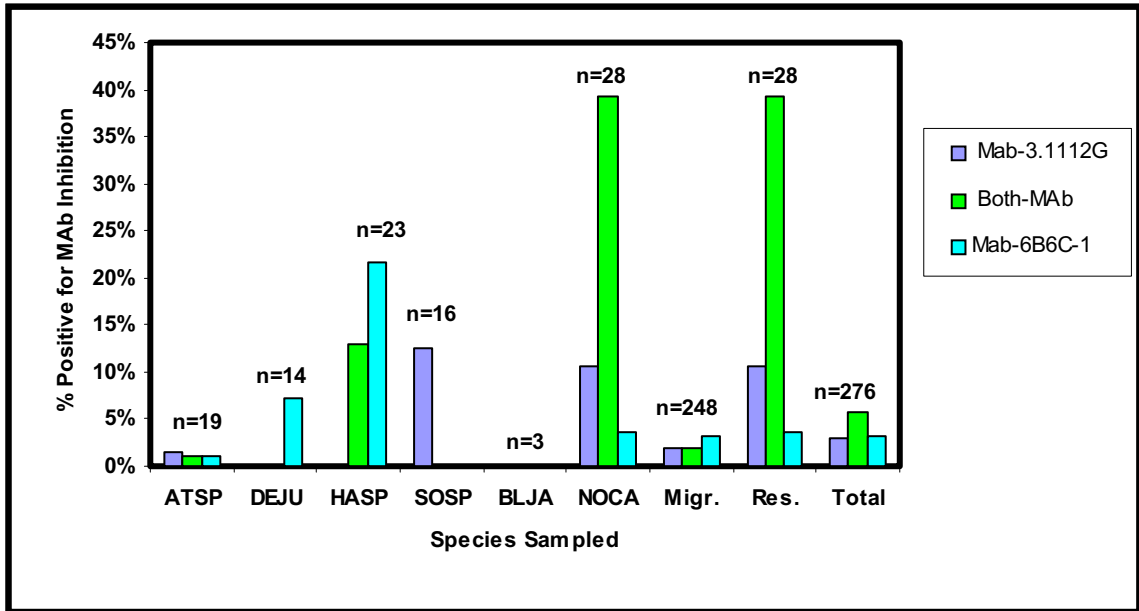


Figure 9. Percent positive for MAb inhibition of birds from winter 2003-04. Comparison of MAb inhibition by species and migratory status $\chi^2=35.73$, d.f. 1, $p<0.0001$. Individuals that inhibited binding of both MAbs were considered true positives for WNV exposure. American Tree Sparrow (ATSP), Dark-eyed Junco (DEJU), Harris's Sparrow (HASP), Song Sparrow (SOSP), Blue Jay (BLJA), Northern Cardinal (NOCA), Migratory (Migr.), and Resident (Res.).

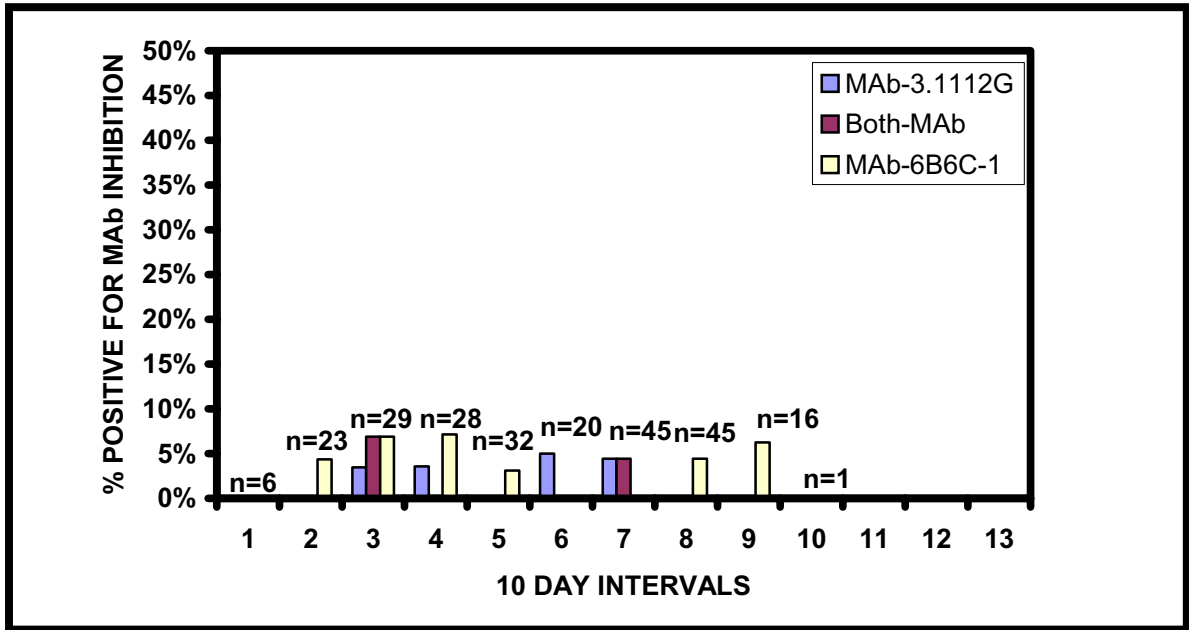


Figure 10. Percent positive for MAb inhibition in migratory birds from winter 2003-04 expressed as 10-day interval data. Comparison of MAb binding inhibition over time for migratory species starting with Dec. 1 and ending Apr. 16, 2004, occurring in ten-day intervals. Individuals that inhibited the binding of both MAbs were considered true positives for WNV exposure.

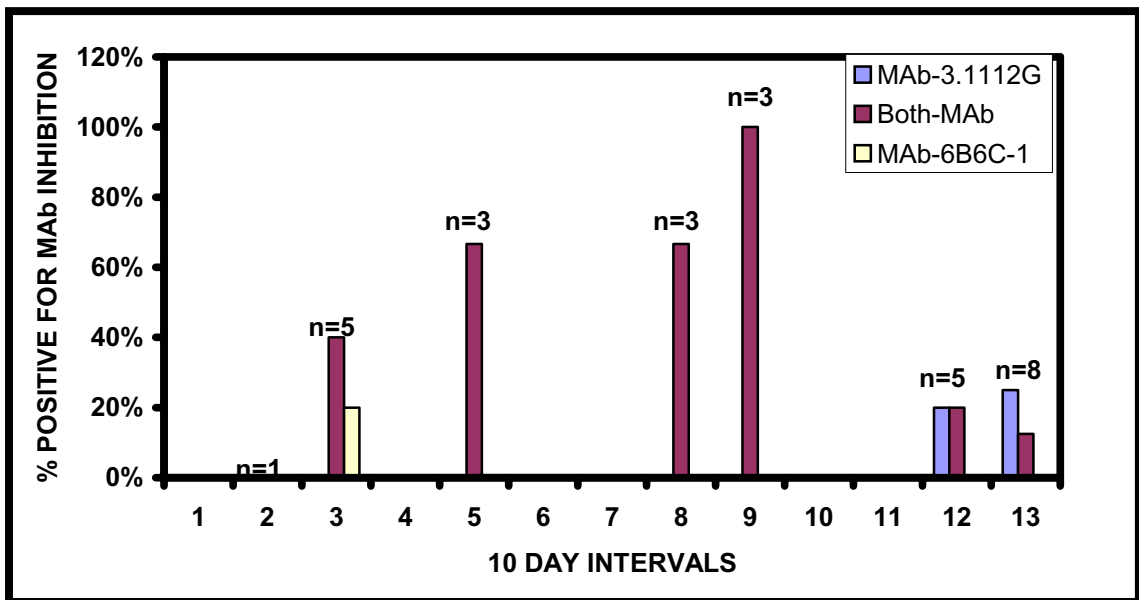


Figure 11. Percent positive for MAb inhibition for resident birds in winter 2003-04 expressed as 10-day interval data. Comparison of MAb binding inhibition over time for resident species starting with Dec. 1 ending and Apr. 16, 2004, occurring in ten-day intervals. Individuals that inhibited the binding of both MAbs were considered true positives for WNV exposure.

WINTER 2004-2005

Field Results. Throughout the course of this field season 340 new individuals were banded (Table 2). Species diversity was increased during this sampling season to include a greater number of resident species. There were 10 re-trapped individuals from the previous winter and 36 individuals who were serial sampled throughout winter 2004-05.

Table 2

Winter 2004-05 Species Totals. Birds, listed alphabetically by migratory status, number of birds sampled.

Species	Total Sampled	Migratory Status
American Tree Sparrow	128	Migratory
Dark-eyed Junco	52	Migratory
Harris's Sparrow	53	Migratory
Song Sparrow	21	Migratory
White-throated Sparrow	7	Migratory
American Goldfinch	1	Semi-migratory
Blue Jay	2	Semi-migratory
Eastern Bluebird	1	Semi-migratory
Red-winged Blackbird	2	Semi-migratory
Black-capped Chickadee	1	Resident
Downy Woodpecker	3	Resident
Northern Cardinal	68	Resident
Red-bellied Woodpecker	1	Resident
TOTAL	339	

Laboratory Results. Of the 340 new individuals sampled, 31 tested positive for WNV exposure using both antibodies (Fig. 12). Comparison of WNV exposure with regards to migratory status using a G-test generated a chi square value of $\chi^2=6.21$, $p<0.025$, and d.f. 1, indicating a significant difference based on migratory status for individuals testing positive for WNV exposure. Of those individuals that were sampled during winter 2003-04 and subsequently recaptured during winter 2004-05, there was no change in test results (Table 3). If the individual tested negative for WNV exposure the previous

winter, it tested negative in winter 2004-05 and those individuals that tested positive remained positive.

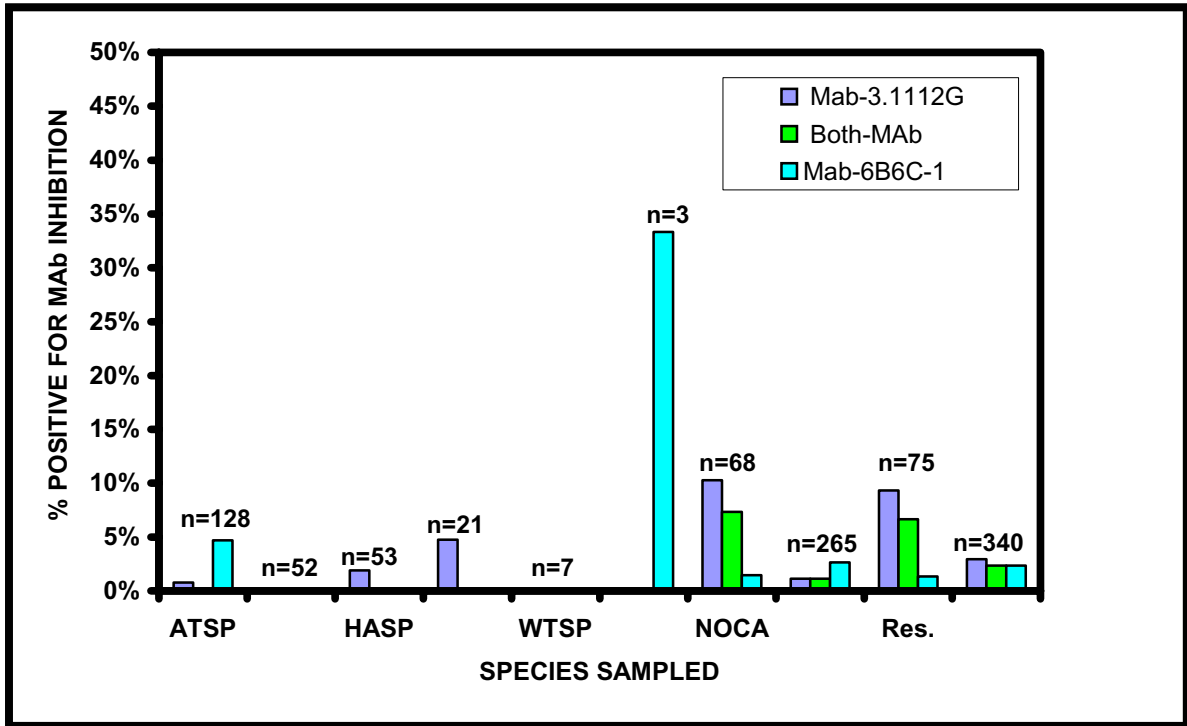


Figure 12. Percent positive for MAb inhibition of birds from winter 2004-05. Comparison of MAb inhibition by species and migratory status. Species with sample sizes lower than three are not listed individually, but are included in total and in migratory categories $\chi^2=6.21$, d.f. 1, $p<0.025$. Individuals that inhibited the binding both MABs were considered true positives for WNV exposure. American Tree Sparrow (ATSP), Dark-eyed Junco (DEJU), Harris’s Sparrow (HASP), Song Sparrow (SOSP), White-throated Sparrow (WTSP), Blue Jay (BLJA), Downy Woodpecker (DOWO) (*Picoides pubescens*), Northern Cardinal (NOCA), Migratory (Migr.), and Resident (Res.).

Table 3

Winter Retraps 2003-2005. Individual birds captured and tested for WNV exposure in winter 2003-04 were subsequently recaptured and tested in winter 2004-2005.

Band #	Species	Winter 2003-04	Winter 2004-2005
90584	American Tree Sparrow	neg	neg
90587	American Tree Sparrow	neg	neg
90657	American Tree Sparrow	neg	neg
90720	American Tree Sparrow	neg	neg
90757	American Tree Sparrow	neg	neg
56072	American Tree Sparrow	neg	neg
56090	American Tree Sparrow	neg	neg
90613	Dark-eyed Junco	neg	neg
35464	Northern Cardinal	pos	pos
35496	Northern Cardinal	pos	pos

Winter 2004-05 was the first field season that within-winter serial sampling of previously banded individuals was implemented. Of the migratory species that were sampled serially, no winter seroconversion occurred as the winter passed (Table 4). There was one resident bird, NOCA 35537, that experienced seroconversion between samplings. This individual was first sampled on 28 December 2004, at which time the sample tested negative for WNV exposure. The bird was then sampled again 19 February 2005, at which time the serum sample tested positive for WNV (Table 4).

Table 4.

Winter 2004-05: Within winter retraps. Individuals were serially sampled and tested for WNV exposure with 10 or more days between sampling.

Band #	Species	Sample 1	Sample 2	Sample 3
56096	American Tree Sparrow	neg	neg	
56097	American Tree Sparrow	neg	neg	
56098	American Tree Sparrow	neg	neg	
56233	Dark-eyed Junco	neg	neg	
56241	Dark-eyed Junco	neg	neg	
56247	Dark-eyed Junco	neg	neg	
56250	Dark-eyed Junco	neg	neg	
56267	Dark-eyed Junco	neg	neg	
56270	Dark-eyed Junco	neg	neg	neg
56279	Dark-eyed Junco	neg	neg	
56343	Dark-eyed Junco	neg	neg	
27653	Harris's Sparrow	neg	neg	
35498	Harris's Sparrow	neg	neg	
35522	Harris's Sparrow	neg	neg	
35541	Harris's Sparrow	neg	neg	
35552	Harris's Sparrow	neg	neg	neg
94566	Song Sparrow	neg	neg	
94570	Song Sparrow	neg	neg	
94575	White-throated Sparrow	neg	neg	
77780	Red-bellied Woodpecker	neg	neg	
35537	Northern Cardinal*	neg	pos	
35538	Northern Cardinal	neg	neg	
35544	Northern Cardinal	neg	neg	
35562	Northern Cardinal	neg	neg	
35587	Northern Cardinal	pos	pos	

*Green indicates that the individual seroconverted between samplings.

To determine if WNV transmission was occurring this field season, sampling dates were pooled into ten-day intervals and plotted versus the percent of the birds sampled that tested positive for WNV exposure (Figs. 13-14). There was no increase of WNV-positive birds as the season progressed.

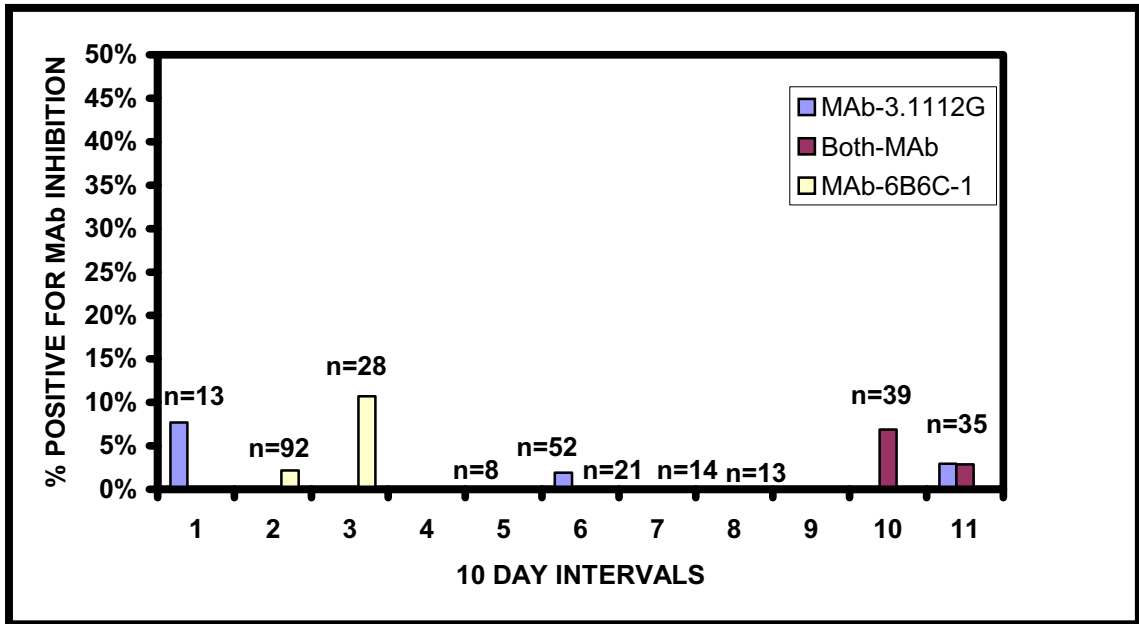


Figure 13. Percent positive for MAb inhibition in migratory birds from winter 2004-05 expressed as 10-day interval data. Comparison of MAb binding inhibition over time for migratory species starting with Dec. 1 and ending Mar. 19, 2005 occurring in ten-day intervals. Individuals that inhibited the binding of both MAbs were considered true positives for WNV exposure.

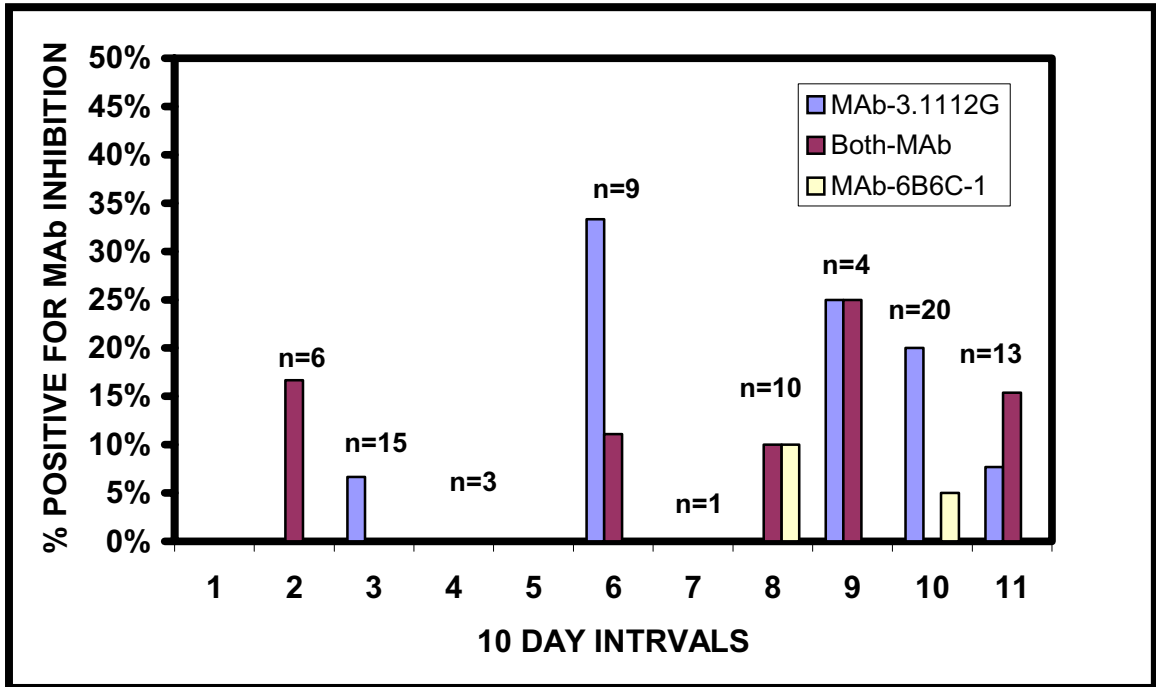


Figure 14. Percent positive for MAb inhibition in resident birds from winter 2004-05 expressed as 10-day interval data. Comparison of MAb binding inhibition over time for migratory species starting with Dec. 1 and ending Mar. 19, 2005, occurring in ten-day intervals. Individuals that inhibited the binding of both MAbs were considered true positives for WNV exposure.

CHAPTER IV

DISCUSSION

Since its introduction into the Western Hemisphere, WNV has spread across North America in six years (Fig. 7). The rapid dissemination of the virus has been speculated to have been facilitated by the movement of viremic, migratory avian species (Rappole et al. 2000, Peterson and Roehrig 2001). It was the goal of this research to develop a better understanding of the ecological factors affecting the spread of WNV, i.e. migratory birds' role and winter survivorship of WNV. The research design allowed comparison of WNV exposure prevalence for migratory and resident species, as well as determination of the occurrence of winter transmission and possible late winter amplification.

The hypothesis of migratory birds as the primary mover of WNV would suggest that exposure rates would be similar to, or greater than, that of resident species assuming that individuals of both migratory and resident species become viremic and recover. Analysis of the first winter's samples found a significant difference ($\chi^2= 35.73$, $p=0.0001$, $d.f.=1$) in WNV exposure between migratory and resident species, with resident species, i.e. NOCA, showing evidence of high exposure to WNV (39.3%). Migratory species experienced a low exposure rate (5.8%). Winter transmission and subsequent late winter amplification was examined at the population level for the first winter (Figs. 11-12). During the winter months, periods 1-11, there was no observable increase in birds testing positive for WNV exposure. Periods 12 and 13 showed what could be interpreted as an increase in WNV exposure, but the relatively low sample sizes confound interpretation. It is likely that if the sample size were increased, these periods would resemble the other

periods. Although this may be the case, it should be noted that periods 12 and 13 occur in late March when mosquito activity is on the rise and when breeding stresses begin to occur.

The second winter of this study used the same methodology as in the first winter, with the addition of serial sampling of individuals and retrapping individuals from the previous winter to determine if an increase in exposure occurred between winters. The former addition was done to observe the possible occurrence of winter transmission on an individual basis as well as the population as a whole. During this winter, the number of different species sampled was increased to develop a broader understanding of the roles of migrants and residents in the spread of WNV. As with the previous winter, there was a significant difference ($\chi^2=6.21$, $p<0.025$, $d.f.=1$) in WNV exposure between migrants and residents. Of the 265 migrants sampled, including retraps, only three (1.1%) tested positive for exposure to WNV. In contrast, 69 resident birds, predominately NOCA, were sampled, and of those 69, five (6.7%) tested positive for WNV exposure. This was greatly reduced from the previous winter demonstrating annual variation. These results suggest that migratory birds do not play as important a role in WNV dissemination as previously thought.

There were 10 individuals from the previous winter that were retrapped; eight were members of migratory species. All of the migratory individuals tested negative for WNV exposure in winter 2003-04 and again in winter 2004-05. This indicates that there possibly was no increase in WNV exposure among these migratory species and therefore no increased ability to spread the virus to new locations, although a greater sample size would more strongly support this statement. The two resident birds (NOCA) that were

sampled in 2003-04 and in 2004-05 tested positive both winters. This result is rather ambiguous. It could indicate either possible re-exposure or it is indicative of the longevity of circulating antibodies to WNV. Another potential explanation is the presence of chronic infection among this species that could aid in the annual reemergence of WNV in the same areas.

There were 36 individuals (30 migrants and 6 residents) who were serially sampled throughout winter 2004-05. Thirty-five individuals did not experience seroconversion. If the first sampling was either negative or positive, all additional samples showed the same result as the original. This would indicate minimal or no winter transmission at communal feeding sites. Northern Cardinal 35537 was the only individual that had seroconverted during the winter months. It was initially sampled on 28 December 2004 and resampled on 19 February 2005. The first sample tested negative for WNV antibodies, but the second sample tested positive for WNV antibodies. The sample's antibodies inhibited the binding of MAb 3.1112G which has a high specificity for the NS1 protein.

Seroconversion occurs when an organism is exposed to a particular antigen which generates an immune response. Assuming the organism survives this initial exposure, it will then have circulating antibodies to this antigen. There are three possible means of exposure which could lead to seroconversion. The least likely possibility is that the individual was bitten by a mosquito, during winter, between samplings. This is unlikely since there wasn't any observable mosquito activity at the time of the second sampling and winter temperatures between samplings were rarely above freezing. Although bird-to-bird winter transmission hasn't been documented, it is possible that individuals with

chronic, low-level infections shed viable virus during defecation (Komar et al. 2003a). If this were to occur on a communal feeding site, it is possible that an uninfected individual could have consumed contaminated food which could lead to viremia and eventual seroconversion. If this were a common means of WNV spread, the number of WNV-exposed birds would increase throughout the winter. From the samples collected during winter 2004-05 this was not the case (Figs. 13-14). As the winter progressed, there was no observable increase in WNV-exposed birds that would suggest that seroconversion during winter was the result of chronic, low-level infection. Thirdly, viral amplification in a chronically infected host would have to elicit an immune response that resulted in seroconversion. The low-level of seroconversion over winter, suggests that there is minimal, if any, winter transmission occurring.

A third hypothesis that was indirectly addressed by the current research was that no difference in WNV exposure exists between migratory and resident species. It could be that the large populations of migrant species may have equal exposure to that of resident species if population sizes are considered. This is unlikely because for both winter field seasons the migratory population sampled was at least five times greater than that of the resident population and the resident population exhibited at least a three times greater exposure rate. Also the migratory path of these individuals must be taken into consideration, since the species sampled migrate with the mosquito concentration gradient during fall migration. This would allow for the individuals to be exposed as they migrate and in turn increase the number of individuals exposed to WNV; yet they turned up negative in every retrap case.

The significantly lower rate of exposure for migratory species found in this study would suggest that migratory species may not play as large a role in the dissemination of WNV in the New World as originally expected. As mentioned previously, it was first thought that migratory birds in the New World would perform a similar role in the spread of WNV as their Old World counterparts, but the short distance the virus moved between years and the concentric pattern in which it appeared to spread would suggest otherwise (Rappole and Hubalek 2003). If migratory birds were the primary movers of WNV, it would be expected that the virus would appear in places sporadically as well as some distance apart, but this is not the case. The spread of the virus has occurred approximately at 70 km per month (Rappole and Hubalek 2003). This relatively slow rate of movement suggests that other, less mobile species may be responsible for the dissemination of the virus.

The results of the current study demonstrate that resident species, namely NOCA in this case, had a significantly higher rate of exposure. This suggests that abundant resident species play a potentially large role in the spread and maintenance of WNV. Species that do not migrate would have long contact with vectors in their area allowing for early spring amplification and subsequent outbreaks. The virus could be further amplified when individuals with chronic, low level infections come under breeding stress in early to late spring which coincides with the reemergence of competent vectors. This would allow for further infection of resident birds as well as migratory species passing through the area.

Resident or sedentary species with large home ranges and relatively large aggregations near urban areas (Ringia et al. 2004) would facilitate the spread of WNV as

has been observed. Candidate species could be the American Crow, Blue Jay, House Sparrow (*Passer domesticus*), and Northern Cardinal. Although the American Crow and Blue Jay have high mortality rates, they are still mobile while infected. This would allow for interactions with mosquitoes in new areas or even with other bird species which could be infected if the bird is highly viremic. It has been documented that crows at breeding roosts move an average of 7.6 km per day during spring and summer. As part of their daily movements they convene at communal feeding grounds where they interact with crows from other family groups (Yaremych et al. 2004). If a viremic crow is present at a communal feeding ground in an area where WNV was not previously active, the crow could contaminate food sources, via salival transfer or fecal matter, that could be subsequently fed upon by uninfected individuals that could become viremic and pass the virus to roost mates. Also, the infected individual could be fed upon by competent vectors, initiating a new epizootic cycle. It also is feasible that infected individuals may die away from the roost and be fed upon by other organisms and in turn infect them. American Crows are fairly abundant in rural areas where anthropogenic factors contribute to its success (Verbeek and Caffrey 2002). The home range of the American Crow encompasses the majority of North America (**Appendix C**). This extensive home range and susceptibility to the virus illustrate the possibility of the crow as a mover of the virus.

Blue Jays are another species that has benefited from urban sprawl (Tarvin and Woolfenden 1999). Like crows, it has been highly impacted by WNV and is one of the most submitted species for WNV testing (Ringia et al. 2004). Blue Jays develop high viral titers that can effectively infect vectors (Komar et al. 2003a). Combine this factor

with this specie's relative abundance and large home range, and Blue Jays would make for a competent mover of WNV (**Appendix D**) (Tarvin and Woolfenden 1999).

Although fewer House Sparrows have been submitted for WNV testing, probably due to their relatively small size and nondescript markings making them less noticeable, they have been shown capable of maintaining viral titers high enough to infect mosquitoes (Komar et al. 2003a). House Sparrows also have benefited from human development and have spread across the majority of North America (**Appendix E**) (Lowther and Cink 1992). Like the crow, this extensive home range would make for a competent courier of WNV.

The Northern Cardinal was the most abundant resident species sampled in the current study and also had the highest exposure rate among all species. The NOCA is abundant in both rural and urban areas (Halkin and Linville 1999). The high rate of exposure to WNV indicates that the individuals exposed to WNV survive unlike the American Crow and Blue Jay. It is possible that those exposed to WNV develop viral titers high enough to infect vectors and then recover. These birds may develop a chronic infection that could fuel the annual cycle as well as disperse it to new areas as fledglings disperse or individuals define new territories. The home range of NOCA encompasses many of the infected regions including those in Mexico (**Appendix B** and Fig. 7).

It is unlikely that any of these species are solely responsible for the spread of WNV. Nor is it conceivable that migratory birds are completely uninvolved in the dissemination of WNV. Migratory birds are most likely responsible for the introduction of WNV to the islands of the Caribbean in that it is unlikely that non-migratory species could reach them (Komar et al. 2003b, Dupius et al. 2003). The dissemination of WNV

is probably through a combination of mosquitoes, resident birds, and migratory movements that result in the introduction and maintenance of the virus in new and old areas. It is possible that the current spread of WNV in the Western Hemisphere is a model for the historical spread of the virus in the Old World. It could be that WNV introduction to Europe showed a similar pattern of spread, but after centuries of exposure outbreaks only occur in areas where new strains are introduced, availability of susceptible hosts, or a combination of both. The current study showed a 30% decrease in WNV exposure in NOCA between winters 2003-04 and 2004-05, subsequently, there were a decreased number of human cases reported between those years (www.cdc.gov). This could suggest that WNV is reaching equilibrium in the New World and future outbreaks may be heralded by the arrival of migratory birds from the tropics. This is assuming that WNV becomes endemic in the tropics of the Western Hemisphere as it is in the Eastern Hemisphere. More studies should be conducted to address these issues.

To address this uncertainty, survivorship curves should be established for species that seem capable of moving the virus short distances. Along with survivorship curves, determining the probability of an individual becoming chronically infected, and thus acting as a consistent reservoir for WNV amplification is important. Also, mapping of resident territories, examining ecological factors, and testing for the abundance of WNV positive mosquito pools within the territory will allow for a closer examination of the role of resident species in the dissemination of WNV.

It should be noted that mosquito trapping was not done during the current study. This leaves the possibility of winter seroconversion induced by mosquito infection an open question.

Conclusions

- I. It is concluded that resident species have an increased incidence of WNV exposure when compared to that of migratory species.
- II. This work suggests that migratory bird species role in the dissemination of WNV in the New World may not be as first hypothesized.
- III. We observed that minimal winter transmission occurs on communal feeding grounds.
- IV. Viral amplification as winter progresses was not adequately demonstrated, although one individual did seroconvert.

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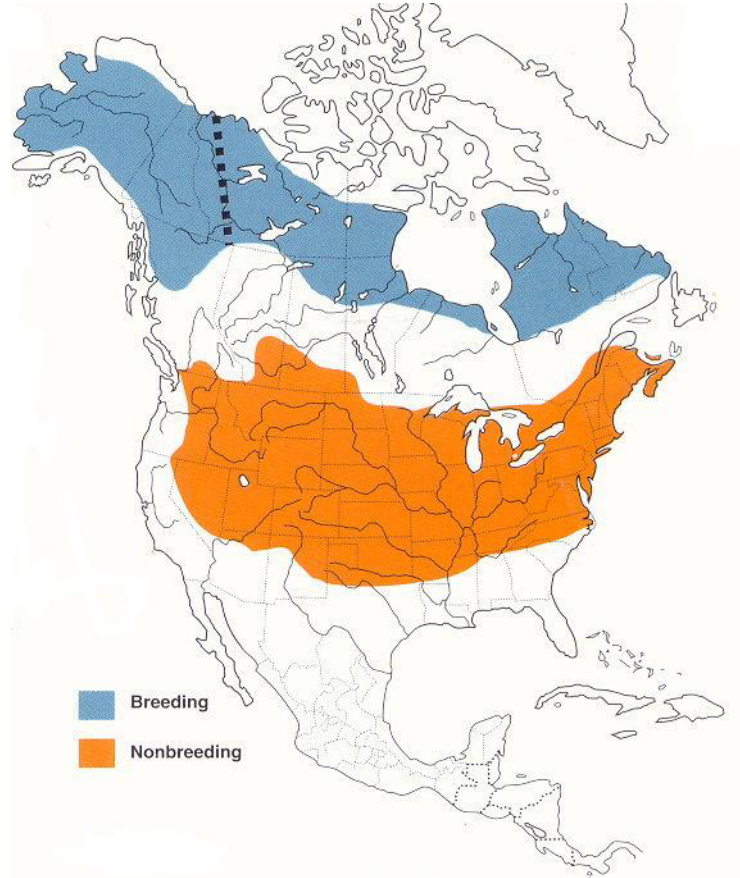
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APPENDICES

Appendix A

Breeding and wintering range of American Tree Sparrow (Naugler 1993).



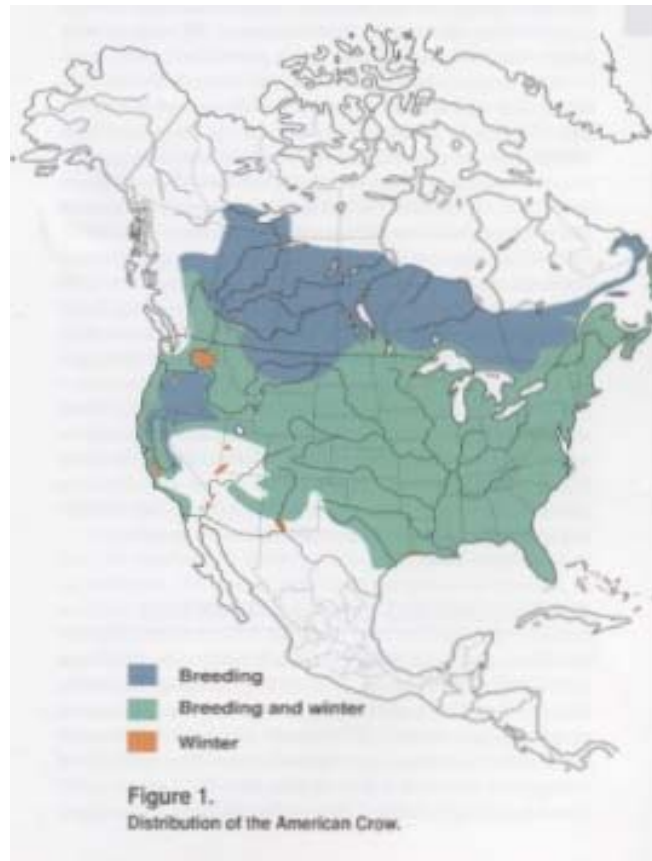
Appendix B

Geographic range of Northern Cardinal (Halkin and Linville 1999).



Appendix C

Geographic range of American Crow (Verbeek and Caffrey 2002)



Appendix D

Geographic range of Blue Jay (Tarvin and Woolfenden 1999)



Appendix E

Geographic range of House Sparrow (Lowther and Cink 1992)

