PERIDOMESTIC AVIAN SPECIES AS AMPLIFYING HOSTS AND SENTINELS OF WEST NILE VIRUS IN GEORGIA

by

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(Under the Direction of DAVID E. STALLKNECHT)

ABSTRACT

Blue jays (*Cyanocitta cristata*) are an effective indicator species for West Nile virus (WNV). The objectives were to describe the gross and microscopic pathology associated with natural WNV infection in blue jays, and determine the most appropriate tissues to be used for virus isolation, reverse transcriptase nested polymerase chain reaction (RT-nPCR), and immunohistochemistry (IHC) techniques. Brain, heart, and lung had the highest viral titers among the tissues. WNV antigen was most often detected by IHC in heart, kidney, liver, and lung. RT-nPCR proved to be the most sensitive diagnostic test applied in this study irrespective of the tissue type.

Wild caught rock pigeons (*Columba livia*) with WNV antibodies were monitored for 15 months to determine antibody persistence and compare results of three serologic techniques. Antibodies persisted for the entire study as detected by epitope-blocking enzyme-linked immunosorbent assay and plaque reduction neutralization test. Maternal antibodies in squabs derived from seropositive birds persisted for an average of 27 days.

Avian species that are locally involved as potential amplifying hosts of WNV could serve as indicators of WNV transmission over the physiographic and land use variation present in the southeastern United States. Avian serum samples (n = 14,207) from 83 species of birds captured throughout Georgia during the summers of 2000 through 2004 were tested by plaque reduction neutralization test for antibodies to WNV. Antibodies to WNV were detected in 869 (6.1%) of the samples, increased significantly throughout the study, and were species dependent. The highest antibody prevalence rates were detected in rock pigeons (*Columba livia*), northern cardinals (*Cardinalis cardinalis*), and common ground doves (*Columbina passerina*).

Geographic information systems (GIS) and logistic regression analyses were used to predict the distribution of WNV in the state of Georgia based on a wild bird indicator system, and to identify variables that are important in the determination of WNV distribution. Temperature, housing density, urban/suburban land use, and mountain physiographic region were important variables in predicting the distribution of WNV in the state of Georgia. The risks associated with WNV endemnicity appear to be increased in urban/suburban areas and decreased in the mountainous region of the state.

INDEX WORDS: West Nile virus, serology, avian, indicators, epidemiology, geographic information system, *Cyanocitta cristata*, blue jay, *Columba livia*, rock pigeon, *Cardinalis cardinalis*, northern cardinal, virus isolation, reverse transcriptase nested polymerase chain reaction (RT-nPCR), antibody persistence, maternal antibody, epitope-blocking enzyme linked immunosorbent assay, hemagglutination inhibition

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DEDICATION

This work is dedicated to my father and hero, Dr. E. Paul J. Gibbs, for his love, guidance, and infectious enthusiasm for animals, disease, and nature.

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CHAPTER 1

INTRODUCTION

New interest in an old disease was sparked in 1999 with the detection of West Nile virus (WNV) [*Flaviviridae, Flavivirus*] in North America. West Nile virus, in its most severe form, causes fatal encephalitis in birds, horses, and humans, and was first detected in a febrile woman in Uganda in 1937 (Smithburn, 1940). Outbreaks of WNV have occurred in humans and horses since the 1950's in Africa, Asia, and Europe. In 1998, WNV caused disease in white storks (*Ciconia ciconia*) and domestic geese (*Anser anser domesticus*) in Israel; this was the first time avian mortalities were associated with the virus (Hubalék and Halouzka, 1999; Malkinson, 2002). Avian mortality then followed the introduction of WNV to North America (Steele, 2000).

In Georgia, WNV was first detected in the summer of 2001, bringing with it concerns over how the virus would manifest itself in the warmer climate and different ecosystems of this and other southeastern states. Because the amplifying hosts and vectors are abundant throughout this region, the potential for WNV to become a significant human and animal health threat in this area was perceived to be high. Heightened surveillance therefore accompanied the southward and westward expansion of WNV in the hopes of better defining the regional epidemiology of this virus.

Avian mortality caused by West Nile virus across North America has been extensive. American crows *(Corvus brachyrhynchos*) are particularly susceptible to WNV, with mortality rates of 100% reported from experimental infection studies (Komar, 2003; McLean, 2001). Other

corvids, such as black-billed magpies (*Pica hudsonia*), blue jays (*Cyanocitta cristata*), and fish crows (*Corvus ossifragus*), are also susceptible to disease caused by WNV (Komar, 2000; Steele, 2000). For this reason, corvids have been utilized extensively for mortality-based WNV surveillance in the United States (Eidson, 2001; Kramer, 2001). Various approaches to detecting WNV, including virus isolation, viral RNA detection, and immunohistochemistry (IHC), have proven effective (Steele, 2000). In order to maximize the likelihood of detection, however, information on tissue-specific viral titers associated with WNV infection in avian species is needed. To date, such information is restricted to naturally and experimentally-infected American crows and experimentally infected blue jays.

During the summer of 2001, blue jays represented 30% of all dead bird submissions from Georgia while American crows represented 17%; 50% of all the avian WNV isolates were from blue jays while 43% were from American crows (SCWDS, unpublished data). These submission and infection rates indicate that blue jays are an effective indicator species for WNV and may be regionally important in surveillance efforts. This may be especially relavant in locations where American crows are not abundant or adequately represented in dead bird submissions.

In addition to dead bird surveillance, serologic surveillance of live bird populations also has utility in studying the epidemiology of WNV. This concept has been demonstrated with other arboviruses such as St. Louis encephalitis virus (SLE) and western equine encephalitis virus (WEE) (Gruwell, 2000; Reisen, 2000). The interpretation of serologic data is dependent upon information relating to antibody persistence, test performance, and the persistence and potential detection of maternal antibodies. Information on WNV antibody persistence in avian species is currently limited. Neutralizing antibodies in feral rock pigeons (*Columba livia*) were detectable over a nine week period post-inoculation, and in experimentally infected chickens for 28 days

(Komar, 2003; Langevin, 2001). No indication of antibody loss was observed in naturally infected wild birds in South Africa that were sampled twice over a two year period; however, these birds were not maintained in a mosquito-free environment (McIntosh, 1969). No information is currently available on persistence of maternal antibodies to WNV, but with SLE, maternal antibodies in the chicks of experimentally infected chickens were shown to last less than four weeks (Bond, 1965). The development of new serologic tests for WNV antibody detection has created a need to evaluate test performance especially as applied to naturally infected birds. Such information also is important for comparison of results with more traditional approaches such as hemagglutination inhibition tests and plaque reduction neutralization tests. Understanding the benefits and limitations of each technique provides a basis to choose the most appropriate method or combination of methods to meet specific study or surveillance objectives.

While dead bird testing has been the primary focus of avian-based surveillance across the U.S., this can be biased by varying degrees of public interest and surveillance infrastructure. Also, submission may be dependent on human population density and may not allow for collection of random and well distributed samples across all land use types. Many of these deficiencies can be avoided through active collection and testing of live birds; this approach has been used for detecting transmission of other flaviviruses (Monath and Tsai, 1987; Day and Stark, 1999). Antibody prevalence information from wild avian species serves to: 1) identify areas of virus transmission thus supporting field work related to mosquito vectors, 2) identify areas of potential human exposure to these viruses (a critical component of a human risk assessment), and 3) provide direction and field validation for more controlled experimental studies needed to determine specific host/vector/virus relationships. An ideal sentinel or indicator species should have several qualities including: 1) susceptible to infection; 2) a wide-spread

distribution within the study area; 3) resistant to disease; 4) development of a detectable antibody response; and 5) a temporal pattern of infection and seroconversion that precedes a human or domestic epidemic (Komar, 2001). Avian species in North America have proven to exhibit a wide range of susceptibility to infection and disease caused by WNV. As such, determination of the best indicator species will maximize the efficiency and effectiveness of future field studies and surveillance.

Georgia represents an ideal regional model in which to study the epidemiology of zoonotic wildlife diseases, such as WNV, for several reasons. Georgia is strategically located on the Atlantic flyway and has proven to be in the immediate path for the southern expansion of introduced and emerging diseases involving migratory birds. The state includes physiographic and land use diversity characteristics of most of the southeastern United States. Physiographic variation ranges from semi tropical barrier islands on the Atlantic coast to the Blue Ridge Mountains in the north, and with few exceptions, the physiographic diversity present throughout the southeast is reflected in this single state. Likewise, land use patterns also exhibit the regional diversity present and rapidly changing in the southeast. This ranges from large areas of natural and industrial forests, to areas of intensive animal and plant agriculture, to rapidly expanding urban and suburban environments. As such, the analysis of WNV serologic data in relation to land use and physiographic region allows for both the determination of risk factors associated with WNV transmission and information to increase the efficiency of target control programs. The three objectives included in this study are directed at defining the role that peridomestic avian species play as amplifying hosts and sentinels of WNV in Georgia.

Specific objectives of this study include:

1. Determine tissue tropism of WNV in the tissues of blue jays (*Cyanocitta cristata*), to facilitate their effective utilization as indicator species for WNV.
2. Determine the long-term antibody response to WNV in naturally infected wild caught rock pigeons ***(Columba livia)*** with specific emphasis on duration of antibody detectability.
3. Identify common peridomestic avian species that are potentially involved in the epidemiology of WNV in the southeastern United States and to utilize these species to identify broad scale regional physiographic and land-use patterns that contribute to WNV transmission.

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CHAPTER 2

LITERATURE REVIEW

History of West Nile virus

West Nile virus was first isolated in Uganda from a thirty-seven year old woman with no reported clinical illness other than a fever of 100.6° F (Smithburn, 1940). This discovery was made as part of a study attempting to isolate yellow fever virus from subjects at the edge of the yellow fever endemic zone. The serum from the Ugandan woman was inoculated intracerebrally into mice; this and subsequent passages caused 100% mortality. Further studies with the isolate, named West Nile virus, were conducted and the authors concluded that the virus was distinct from but related to Japanese encephalitis, louping ill, and St. Louis encephalitis viruses (Smithburn, 1940).

Epidemiologic studies conducted in Uganda, Kenya, and the Belgian Congo found a widespread seroprevalence of WNV antibodies in the human population (Smithburn and Jacobs, 1942; Smithburn, 1952). Arthropod transmission was demonstrated experimentally in *Aedes albopictus*, *Culex pipiens*, and *Cx. tritaenirohynchus* (Philip and Smadel, 1943; Kitaoka, 1950). Field studies in Egypt identified a WNV cycle involving birds as amplifying hosts and *Culex univittatus* as the primary vector; humans and horses were determined to be dead-end hosts (Taylor, 1956).

Following this initial work, epidemiologic surveys were conducted in Africa, Europe, the Middle East, and Asia, with the bulk of the surveillance taking place during the 1960's and

1970's (Murgue, 2002). Interest in the disease was maintained by sporadic cases and outbreaks in Algeria in 1994, Morocco in 1996, Tunisia in 1997, Democratic Republic of Congo in 1998, Israel in 1941 and from 1997-2000, Romania in 1996, Italy in 1998, Russia in 1999, and France in 2000 (Murgue, 2002). Throughout this time, avian species were used for WNV serologic surveillance (Murgue, 2002). While most of these outbreaks were characterized by illness only in humans and horses, the 1997-2000 outbreak in Israel differed in that avian mortality was detected. Acute neurological signs were reported in domestic geese infected with WNV, and the virus was isolated from dead white storks (*Ciconia ciconia*) and a lappet-faced vulture (*Anser anser domesticus*) (Bin, 2001; Malkinson, 2002b).

Observed avian mortality associated with WNV infection proved to be extremely important in the initial detection of this virus in North America. In 1999, cases of encephalitis in humans in the New York area and concurrent avian deaths in the Bronx heralded the arrival of WNV on the North American continent (CDC, 1999; Steele, 2000). When WNV was first introduced into North America, it was initially misdiagnosed as SLE based on human case findings. Further investigations were conducted however, because avian mortality did not fit the historical epidemiological pattern of SLE. In addition to dead crows, an unusually high mortality rate occurred in the avian collections at the Bronx Zoo in New York City; the cause was subsequently determined to be WNV (Ludwig, 2002). Fears that WNV would over-winter were confirmed with the finding of WNV in a dead hawk in New York in February of 2000, by finding live virus in overwintering mosquitoes, and the reappearance of avian and human cases later that year (Garmendia; 2000). Over the next five years, WNV became established in North America, Mexico, and the Caribbean (Reisen, 2004; CDC; 2005; Health Canada, 2004; Farfan-Ale, 2004).

The virus

WNV is a member of the Japanese Encephalitis antigenic complex within the genus *Flavivirus*, family *Flaviviridae*. Other important members of this complex are Murray Valley Encephalitis virus (MVE), Japanese Encephalitis virus (JE), St. Louis Encephalitis (SLE), and Kunjin virus. Kunjin virus is now considered a subtype of WNV based on sequence similarities (Heinz, 2000; Scherret, 2001). Flaviviruses are enveloped, icosohedral, with a single stranded positive sense RNA genome which is 10,000- 11,000 bases in length. The WNV genome is 11,029 bases in length and has a single open reading frame (Lanciotti, 1999). Virions are approximately 50 nm in diameter with a nucleocapsid of approximately 25 nm in diameter (Brinton, 2002). The genome codes for three structural proteins: the envelope, capsid, and premembrane proteins (Marfin and Gubler, 2001). Virus neutralizing antibodies are directed primarily towards the envelope protein (Chambers, 1998).

The distribution of WNV currently (2005) extends west from Eurasia, through Africa, and to the western extent of North and Central America as well as the Caribbean chain. The eastern extent of the WNV range in Eurasia overlaps with JE, while the western and southern distribution of WNV in the US overlaps with SLE (CDC, 2005). WNV currently represents the most globally widespread flavivirus; this distribution is likely to increase with the confirmation of WNV in Trinidad and expected spread to South America (Hubalék and Halouzka, 1999; CAREC, 2004).

Phylogenetic analyses of WNV have been conducted based on complete genome sequencing and partial sequencing of the nucleotide region encoding the premembrane and envelope proteins, or the envelope-glycoprotein nucleic acid (Davis, 2003; Lanciotti, 1999; Savage, 1999). The numerous strains of WNV and the subtype Kunjin virus fall into two

lineages (1 and 2). Lineage 1 viruses primarily originate in West Africa, the Middle East, Eastern Europe, Australia, and the introduced North American virus, while those in lineage 2 originate solely from the African continent (Lanciotti, 1999). Lineage 1 may be subdivided into three monophyletic clades: KUN viruses, the Indian WN viruses, and the European, African, and North American WN viruses (Lanciotti, 2002). Lineage 2 viruses appear to be maintained in an enzootic cycle and have not been involved in any outbreaks of human disease to date (Lanciotti, 1999). The first isolate of WNV, made from a Ugandan woman in 1937, is now classified as a lineage 2 virus (Lanciotti, 2002).

Phylogenetic analysis is also being used to investigate changes in viral sequence of WNV isolates and determine how these relate to phenotypic changes. Enhanced epidemic potential and increased virulence may occur as a result of virus evolution in the new host and vector species encountered in the Western Hemisphere. The pathology associated with avian WNV infections occurring since 1998 may reflect changes that occurred in the virus population prior to its introduction to North America. Experimental infection of American crows with WNV strains from Kenya, Australia (Kunjin), and North America (NY-99) showed a marked difference in phenotype between the three strains (Brault, 2004). This study suggests that avian mortality in North America is associated, at least in part, to the WNV strain which was introduced and is not solely the result of the introduction of WNV into a naïve ecosystem. Contrasting this study is the experimental infection of hooded crows (*Corvus corone sardonius*) with the Egyptian WNV strain (Eg-101) which caused mortality in 100% of the birds (Taylor, 1956).

Analysis of WNV phylogeny allows tracking of virus movement and estimates of the immediate origin of the strain. As an example, the complete genomic sequence of the New York strain from 1999 was determined to be most closely related to a WNV strain isolated from a dead

goose found in Israel in 1998 (Lanciotti, 1999). This information suggests that WNV was most likely introduced into the New York area from a source in the Middle East. The exact method of introduction into North America has not and probably never will be determined. Earlier work determined that the sequence of the virus strain which caused the 1996 WNV epidemic in Romania was identical to isolates from Senegal and Kenya, suggesting spread from sub-Saharan to northern Africa and then subsequent introduction into Romania (Savage, 1999).

Divergence of WNV strains has continued within the United States since its introduction and has been linked to changes in phenotype based on reduced replication in cell culture and decreased mouse neuroinvasiveness (Davis, 2004). As demonstrated by work conducted in Connecticut, these temporal and geographic genetic variations may represent microevolutinoary events (Anderson, 2001). Much work remains to be done on this topic.

Transmission and Maintenance of WNV

West Nile virus is maintained in a transmission cycle involving birds (amplifying hosts) and mosquitoes (primary vectors) (Komar, 2000). This basic mosquito/bird cycle is shared by other arboviruses which are present in North America such as SLE, EEE, WEE. Peak WNV transmission in North America (as determined by detection of avian, equine, and human cases) occurs in the late summer associated with sustained populations of several species of *Culex* mosquitoes (Rappole, 2000). Birds represent the only known WNV amplifying hosts, and while mammals can be extremely susceptible, their role in the epidemiology of the virus is as incidental dead-end hosts (Hayes, 1989). This is based on a low-level short-lived viremia that is below the threshold for significant transmission to mosquito vectors (Bunning, 2002). Transmission of WNV can occur in the absence of vectors and novel transmission routes have

included bird to bird transmission in captive American crows and domestic geese (McLean, 2001; Banet-Noach, 2003), oral transmission through consumption of infected hosts (Austgen, 2004; Miller, 2003), cofeeding of mosquitoes (Higgs, 2005), as well as human to human transmission through blood transfusions, organ donations, and transplacental and breast-milk transmission (CDC, 2004; Kleinschmidt-DeMasters, 2004; CDC, 2002a;Ognjan, 2002).

The factors influencing the maintenance of WNV from year to year are not fully understood. Hypotheses include over-wintering of WNV in mosquitoes, year-round transmission in warm climates, and viral persistence and recrudescence in avian hosts. West Nile virus was detected in field-collected overwintering *Culex pipiens pipiens* in Pennsylvania, and *Culex* spp. and *Culex pipiens* in New York, (Bugbee, 2004; CDC, 2000; Nasci, 2001). Vertical transmission of WNV in three experimentally infected California *Culex* species was also demonstrated, suggesting that progeny of mosquitoes infected in the fall could serve as an overwintering mechanism (Goddard, 2002). Vertical transmission of WNV also took place in experimentally infected *Culex pipiens* derived from mosquitoes collected in New York (Dohm, 2002). Evidence of year-round transmission of WNV was reported in Louisiana and Texas after the detection of the virus in eleven dead birds and pools of physiologically active adult mosquitoes (*Culex pipiens quinquefasciatus*) (Tesh, 2004). Virus was isolated from brain tissue of a red-tailed hawk (*Buteo jamaicensis*) found dead in New York in February of 2000 (Garmedia, 2000). This may have resulted from latent infection, ingestion of an animal with latent infection, or persistence of mosquitoes throughout the winter. Latent infection of WNV in avian species has also been suggested in rock pigeons (*Columba livia*) (Semenov, 1973).

Vertebrate Hosts

Avian

Wild birds have long been recognized as the critical vertebrate component in the epidemiology of WNV (Hayes, 1989), and through serologic testing have been utilized in epidemiologic studies in Africa, Europe, the Middle East, and Asia (Work, 1953; Work, 1955; Malkinson, 2002a). When WNV was introduced to North America, high levels of avian mortality provided an additional surveillance approach (Steele, 200). At this writing, nearly 300 species of free ranging North American avian species have been found positive for WNV antigen or antibodies (NWHC, 2004).

The role that each avian species may play in WNV transmission is dependent on factors such as susceptibility to infection, the level of viremia produced, geographic distribution, behavior in response to vectors, and population numbers. Peridomestic bird species have been suggested as a necessary link between areas where virus is naturally maintained and areas of potential human disease risk both for SLE and WNV (Holden, 1973; McLean, 1983; McLean, 1988; McLean, 1993; Gruwell, 2000). Further, avian species may be involved in different parts of the transmission cycle: 1. As introductory host, 2. As part of the sylvatic cycle in which virus is amplified among birds and ornithophilic mosquitoes, or 3. As part of an epidemic cycle in areas of human habitation that may or may not involve bridge vectors (Savage, 1999).

It has been suggested that the rapid spread of WNV through North America occured as a result of the long-range movement of infected migratory birds (Reed, 2003). Whether a viremic migratory bird is willing or able to travel hundreds or thousands of kilometers and arrive before the window of viremia closes (fewer than five days) to allow transmission to a vector is at question (Peterson, 2003; Rappole and Hubalek, 2003). In support of this theory are the findings

of viremic birds captured during migration of young white storks (*Ciconia ciconia*) which arrived in southern Israel in poor condition; WNV was isolated from the brains of four of these birds (Malkinson, 2002b). Older references to WNV isolations from migrating birds include the barred warbler (*Sylvia nisoria*) in Cyprus and the little tern (*Sterna albifrons*) in Tajikistan (Watson, 1972; Gordeeva, 1980). Evidence of WNV circulation down the Caribbean chain to Trinidad, part of the Atlantic migratory flyway, also supports the idea that migratory birds carry WNV over great distances (CAREC, 2004).

Mammal

Many vertebrates are susceptible to infection with WNV. Since the levels of viremia reached in these mammals are rarely high enough for re-infection of mosquitoes however, mammals serve as dead-end hosts in the WNV cycle (Augusten, 2004; Ratterree, 2004; Bunning, 2002). Wildlife species in which WNV viral antigen or antibodies to WNV have been found include: eastern fox squirrels (*Sciurus niger*) (Kiupel, 2003), gray squirrels (*Sciurus carolinensis*) (Heinz-Taheny, 2004), little brown bat (*Myotis lucifugus*) and northern long-eared bat (*Myotis septentriotalis*) (Pilipski, 2004), black bear (*Ursus americanus*) (Farajollahi, 2003), striped skunk (*Mephitis mephitis*) (Anderson, 2001), mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*), black-tailed prairie dog (*Cynomys ludovicianus*), and eastern chipmunk (*Tamias striatus*) (NWHC, 2004). Population impacts associated with WNV infections in these species, however, have not been suggested.

Non-human primate facilities in North America have also experienced WNV outbreaks in their collections. Rhesus macaques (*Macaca mulatta*), pigtail macaques (*Macaca nemestrina*), and baboons (*Papio* sp.) were all found to be seropositive at a breeding colony in southern

Louisiana, however no clinical signs were observed in any of the animals (Ratterree, 2003). Experimentally infected rhesus macaques developed subclinical infections with low viremia levels ($\leq$100 $TCID_{50}$/mL) lasting between one and five days (Ratterree, 2004). This serves as continued evidence that mammals are generally dead-end hosts. IgM and IgG antibodies in these animals lasted throughout the 63-day testing period, however IgM levels were dropping by the end of the study.

Domestic mammals reported to be infected with WNV include alpaca (*Lama pacos*) and llama (*Lama glama*)(Kutzler, 2004; Yaeger, 2004), sheep (Yaeger, 2004; Tyler, 2003), reindeer (*Rangifer tarandus*) (Palmer, 2004), domestic rabbit (*Oryctolagus cuniculus*), mule and donkey (NWHC, 2004), horse (OIE, 1999), and domestic dog (Lichtensteiger, 2003). Encephalomyelitis has been associated with infections in all of these species and as a result WNV has had a severe animal health and economic impact especially on the equine industry.

Reptiles and amphibians

Evidence of WNV infection in farm-raised American alligators (*Alligator mississippiensis*) and crocodiles has been reported. In Israel, 70% of farmed crocodiles were found to be seropositive for WNV; however no clinical signs or viremia were detected (Steinman, 2003). In the US, the farmed alligator industry suffered substantial economic losses as a result of high WNV mortality rates; WNV was isolated from the tissues of these cases (Miller, 2003; Jacobson, 2005). In one outbreak, the feeding of WNV infected meat was thought to be the route of transmission (Miller, 2003). An experimental infection study with juvenile American alligators revealed that these animals can sustain viremic titers greater than 5 $\log_{10}$ PFU/mL, a level high enough for transmission of the virus to mosquito vectors (Klenk, 2004).

Evidence of non-vectored WNV transmission was also seen in this study when non-inoculated cage-mates became infected.

Antibodies to WNV have also been detected in a captive crocodile monitor (*Varanus salvadorii*) (NWHC, 2004). Experimental infection of green iguana (*Iguana iguana*, Florida garter snake *(Thamnophis sirtalis sirtalis*), red-ear slider *(Trachymes scripta elegans*), and North American bullfrog *(Rana catesbeiana*) with WNV produced low titer viremias; virus was also detected in organs and oral and cloacal swabs of some of the animals (Klenk and Komar, 2003).

Arthropod Vectors

While mosquito species within the *Culex* genus are the most frequently reported to be naturally infected with WNV, isolations and/or RNA detection have also been made from species within *Aedes, Culiseta, Ochlerotatus, Anopheles, Mansonia, Coquillettidia, Mimomyia, Aedomyia, Deinocerites, Orthopodomyia, Psorophora, Uranotaenia* (Hubalék, 1999; Marfin and Gubler, 2001; CDC, 2005). Since its introduction into North America, 60 species of mosquito have tested positive for West Nile in the United States (CDC, 2005). Several conditions must be met before a species can be considered important as a vector of WNV however, including: repeated detection of the virus from that species from field caught mosquitoes; demonstration of vector competence; and an association in nature between the vector and the reservoir and susceptible species (Turell, 2005). Other ecological factors, such as the density of vectors and hosts, weather, temperature, feeding preference, and feeding habitat are also important in determining the importance of a vector species to WNV transmission. In Georgia, WNV has been isolated from *Culex quinquefasciatus, Cx. nigripalpus, Cx. Salinarius, Aedes albopictus, and Ae. Vexans* (SCWDS, unpublished data). These species vary in their vector competence but

all have significant potential to serve as enzootic vectors. *Cx. quinqufasciatus* appears to play a major role as an enzootic vector, and *Cx. salinarius* a major role as a bridge vector (Sardelis, 2001; Goddard, 2002). *Cx. nigripalpus* may play an intermediate role as enzootic and bridge vector (Turell, 2005).

In addition to mosquitoes, soft and hard ticks have also yielded WNV isolates (Hubalék, 1999). Ornithophilic argasid ticks have been the source of WNV isolates in the Caucasus, Azerbijan (*Ornithodoros capensis*) as well as in Egypt (*Argas reflexus hermanni*) (L'vov, 1975; Schmidt, 1964; Taylor, 1956). Experimentally infected *Ornithodoros moubata* were able to maintain the virus and transmit it to uninfected ticks co-feeding on unifected hosts (Lawrie, 2004). While ticks do not appear to play a major role in the epidemiology of WNV, especially in North America, it is important to consider these arthropods as potential reservoirs or bridge vectors in arid areas.

Public and Domestic Animal Health Implications

Historically, WNV in humans is associated with mild disease that is primarily associated with infants and young children (Taylor, 1956). As most people in the U.S. were not exposed to WNV during childhood, however, clinical cases in this country have also occurred in the elderly (Nash, 2001). Three and a half years after the introduction of WNV to the U.S., the WNV human case total has risen to greater than 4000, with 263 fatal cases (CDC, 2003). For 2003 and 2004, there were 9,862 and 2,470 human WNV cases respectively in the US; 264 deaths occurred in 2003 and 88 deaths in 2004 (CDC, 2005).

In humans, encephalitis with muscle weakness or flaccid paralysis may occur while milder signs include flu-like illness with associated fever, headache, sore throat, nausea,

diarrhea, conjunctivitis, fatigue and signs of respiratory disease (Ritchie, 2000; Petersen and Marfin, 2002). Of particular concern are the numerous iatrogenic forms of WNV transmission that have occurred in the US. As an example of the importance of this form of potential transmission, WNV was isolated from 199 blood donors in 28 states in 2004 (CDC, 2005; CDC, 2004). More intense screening methods have been developed for the blood banks to try and limit transmission. Work to develop a human WNV vaccine using chimera technology based on the Yellow Fever virus 17D vaccine is currently in progress (Monath, 2001).

Historically, WNV was associated with only mild disease in horses. Recently, however, there have been reports of severe equine illness involving high fever, ataxia, sudden tetraparesis or paraparesis progressing to tetraplegia, recumbancy and death (Cantile, 2001; Bunning, 2002; Durand, 2000). The 2002 WNV season in the U.S. was particularly bad for the equine industry, with the case total for the year rising to a high of 14,358. Equine losses have been curtailed to some extent by the availability of a killed vaccine for use in horses. While cases of WNV have been reported sporadically in other domestic species such as dogs, cats, llamas, and reindeer, these case numbers pale in comparison to those from the equine industry.

Temporal and Spatial Relationships

Understanding WNV epidemiology requires an in-depth knowledge of not only the biology of the virus and host/vector characteristics, but also temporal, geographic, and environmental trends associated with amplification events. These factors each complicate the path from an endemic maintenance cycle to human or animal disease (Day, 2001), and require some degree of synchrony for an epidemic to occur.

Temporal trends in WNV amplification are closely tied to seasonal fluctuations in mosquito populations and the availability of naïve hosts. The timing of temperature and rainfall events regulates the emergence of vector populations (Shaman, 2005; Ortiz, 2005), and the availability of naïve host populations is related to the timing of avian movement and breeding events. In temperate regions, the synchrony of vector abundance and naïve hosts occurs during the summer and early fall when mosquito populations are at their greatest and avian young have hatched. Subtropical and tropical regions may experience longer time periods during which WNV transmission can occur due to mild temperatures. In Georgia, which is temperate, the WNV "season" begins in late June and extends through the summer to October or November, mirroring the mosquito season. This temporal pattern has been documented over several years of virus isolation from dead birds and mosquitoes collected throughout the state (SCWDS, unpublished data). In Texas and Louisiana, parts of which are subtropical, year-round WNV transmission has been recorded (Tesh, 2004).

Spatial factors such as land use, elevation, and physiographic region also play a role in the maintenance and transmission of WNV. With the exception of the work by Brownstein (2002) which investigated the effects of vegetation on WNV risk, little information has been reported on the influence of other environmental factors on WNV transmission. In that study, spatial analysis of WNV case distribution in the New York City area in 1999 revealed that vegetation abundance was significantly and positively associated with human cases. This information was then used to predict areas of greatest human risk. The model constructed in the study showed that the less populated suburban regions were at greatest risk. An understanding of the biology of both the mosquito vectors and the avian reservoir hosts suggests that there are a variety of environmental conditions involved in the optimal amplification of WNV. Investigation

of these factors may aid in further describing the epidemiology of WNV and in changing human behavior to avoid risks associated with WNV.

Geographic information systems

Geographic information systems (GIS) are computer systems designed to capture, store, analyze, and display data that is related to a geographic location. Information from different sources or types of data is then related in a spatial context. Numerous layers of information, e.g. land use type, census data, elevation, can then be added for inclusion in the analysis. Because spatial data is unique, it can be linked to a geographic map for display. This mapping ability is invaluable because it converts extremely large quantities of information into an easily understood visual form.

The applications of GIS to epidemiology are immense. Not only can researchers use the system to create maps, but they can also perform statistical analyses of their data to determine the importance of relationships between disease and the environment. In addition, GIS can be used to identify space-time clusters, or “hot spots” of disease. Analysis of these disease clusters can then aid in decision making for control efforts and monitor the success of these efforts at different scales. Because data is inherently collected at discrete geographic locations and cannot cover a complete geographic area, an advantage of GIS is to be able to perform modeling. Modeling allows for the prediction of whether or not a disease is present in an untested area, creating a continuous risk map. Modeling also provides procedures for testing hypotheses about the causes of disease and the nature of disease transmission. As part of this model, regression analysis generates a linear combination of factors that best explain the spatial variation in disease prevalence.

GIS technology has been applied to research on a number of pathogens. Examples include: understanding spatial clustering of severe acute respiratory syndrome (SARS) (Lai, 2004); analyzing and managing an avian influenza virus (AIV) epizootic in Italy (Ehlers, 2003); describing the distribution of vector habitat and risk assessment for Ross River and Barmah Forest viruses in Australia (Dale, 1998); identifying areas of human tuberculosis transmission and incidence in the United States (Moonan, 2004); estimating the incubation period of raccoon rabies in Ontario (Tinline, 2002); determining landscape features associated with increased risk of EEE transmission in Massachusetts (Moncayo, 2000); and developing geographical approaches to the control of zoonotic parasitic diseases such as African trypanosomiasis (Mott, 1995).

Several studies, described in the following paragraph, have been conducting to evaluate the use of dead birds in WNV risk analysis. The initial bird population present in an area, the human population density, variation in size and shape of reporting areas, artificial boundaries of data formed by political boundaries, and the social variation in reporting interest all influence the dead bird reporting system. Geographic information systems (GIS) analyses provide a way to overcome some of these obstacles.

A study of the space-time interaction of dead birds as an indicator of WNV amplification showed that this information could predict areas of high risk in humans at least 13 days prior to the onset of illness in those areas (Theophilides, 2003). Another study conducted using dead bird clusters as an early warning system for WNV activity applied a spatial scan statistic to detect small area clustering of dead birds (Mostashari, 2003). This information could then be used to predict areas of high circulation of the virus and serve as a basis on which to target mosquito surveillance activities. A retrospective study was conducted of dead crow report data from

Chicago in 2002 (Watson, 2004). Spatial analysis of this data showed that human cases were three times more likely to occur inside areas of high early-season crow deaths.

Risk maps are an important product of GIS analysis. Models using human WNV case data as well as WNV positive dead birds and mosquito pools were constructed to provide an estimate of risk on a county level in the US (Brownstein, 2004). These models proved to be capable of serving as an early warning system based on early season disease maps. The study also found that mosquito surveillance was more sensitive to human risk than dead bird surveillance.

Pathology in Avian Species

With the exception of the 1997-1998 WNV outbreak in Israel involving domestic geese (*Anser anser domesticus*) and white storks (*Ciconia ciconia*), WNV had not been associated with clinical disease in avian species (Hubalék, 1999; Malkinson, 2002b; Work, 1953). When introduced to North America, however, a variety of bird species began exhibiting fatal neurological disease associated with WNV infection (Steele, 2000). Clinical signs reported in avian species include ataxia, abnormal head posture, circling, in-coordination, wing droop or convulsions, weakness, inability to perch or fly, depression, weight loss, and death (Steele, 2000; Weingartle, 2004; Swayne, 2001). Based on laboratory studies, some domestic species, such as domestic turkeys and chickens, do not appear to suffer clinical disease when experimentally infected with WNV (Senne, 2000; Swayne, 2000). Domestic geese, however, are susceptible to both naturally and experimental WNV infection and exhibit clinical signs of depression, weight loss, torticollis, opisthotonus and death (Swayne, 2001; Austin, 2004).

Gross lesions reported in WNV-infected birds include hemorrhage of the brain, splenomegaly, meningoencephalitis, myocarditis, and emaciation (Steele, 2000; Ellis, 2005). In several studies, gross lesions were reported as absent or rare in WNV positive birds (Fitzgerald, 2003; Wünschmann, 2004a). Studies of naturally infected American crows suggest that even in the absence of inflammatory gross and histopathologic lesions in these birds, WNV infection should be considered (Wünschman, 2004b). Because gross lesions observed in WNV positive avian species are not consistent or pathognomonic for the viral disease, further diagnostic tests must be performed to confirm the infection (Wünschmann, 2004b).

Microscopic lesions due to WNV are variable. Findings in naturally and experimentally infected corvids include necrosis of the spleen and bone marrow, necrosis of hepatocytes, myocarditis, necrosis of splenic lymphocytes, and vacuolation of cells in the lamina propria of respiratory capillaries (Weingartl, 2004; Wünschmann, 2004b). In naturally infected Cooper's hawks (*Buteo jamaicensis*) and red-tailed hawks (*Accipiter cooperi*), lymphoplasmacytic and histiocytic encephalitis or meningoenceohalitis were commonly observed and all birds had inflammatory lesions of the eye, heart, or brain suggestive of a viral infection (Wünschmann, 2004a). In naturally infected native North American owls, necrotizing myocarditis was the most common histological finding, however two owls in this study had no microscopic lesions in any tissue (Fitzgerald, 2003). Myocarditis was also the most common microscopic lesion found in naturally infected raptors in the state of Georgia (Ellis, 2005). In captive native and exotic birds naturally infected with WNV in the New York area at the start of the epidemic, meningoencephalitis and myocarditis were the most prominent microscopic lesions (Steele, 2000). Almost all of the birds in this study, which covered a variety of old and new-world species and included members of the *Corvidae* family, had histopathologic brain lesions that

included hemorrhage and infiltration of inflammatory cells into the cerebrum and cerebellum. Microscopic lesions of experimentally infected domestic geese included nonsuppurative meningoencephalitis, myocarditis, and vacuolation and apoptosis of pancreatic acinar cells (Swayne, 2001).

The pathologic findings in birds associated with WNV point to the difficulties in diagnosing this disease without diagnostic support either by virus isolation or antigen/ nucleic acid identification (IHC, RT-PCR techniques). Studying WNV pathogenesis in birds has also revealed that there are important species-specific differences in susceptibility to both infection and disease. These differences may lead to uneven avian population impacts of the disease in the Western Hemisphere.

Avian population impacts

Extensive avian mortality associated with WNV introduction into North America has generated concerns about possible effects on wild avian populations. Unfortunately, due to incomplete national data on avian populations prior to WNV introduction, evaluation of WNV impacts is not easy. Predictions based on avian biology and WNV epidemiology are inaccurate due to differences in immune response exhibited by different species (Male, 2003).A study following a radio tagged group of American crows (*Corvus brachyrhynchos*) in Illinois found that more than two thirds of the crows died of WNV infection (Yaremych, 2004). Similar studies of marked crow populations in New York State and Oklahoma are ongoing, however preliminary results suggest that declines are occurring in these populations as well (Marra, 2003). An assessment of the changes of American crow populations based on Christmas Bird Count data indicated that there was slowed population growth or declining abundance of crows after WNV

was detected in North America (Hochachka, 2004). In contrast, a broad-scale decline in avian populations has not been apparent in analysis of Christmas bird count (CBC) data (Caffrey and Peterson, 2003). In this study, CBC data from six northeastern states was pooled and examined for population changes over the period from 1989-2002. With the exception of American crows, general population trends in this study only weakly supported the hypothesis that WNV was impacting avian population levels.

Statistics from the dead-bird testing programs set up in each state allow a minimum estimate of the impact WNV has had on the avian populations in those areas. For the years 1999-2003, 39,190 birds tested positive for WNV and were reported to the CDC (CDC, 2005). For 2001-2003, 260,824 dead birds were reported. These numbers may just be the tip of the iceberg with regards to actual mortality numbers as many more birds die than are observed (McLean, 2004).

The species-specific differences in disease susceptibility are also complicating estimates of the impact WNV is having on the avian populations in North America. As an example, a study of WNV effects on a captive population of North American owls showed that there was a significant difference in mortality rates between owl species with northern native breeding ranges and those with southern breeding ranges. Species with northern native breeding range were at significantly higher risk for WNV associated mortality (Gancz, 2004). As a result of these findings, it is apparent that generalizations across family or genus lines cannot be made.

There are also apparent WNV disease susceptibility differences between age groups of birds. For example, in an outbreak of WNV in a flock of domestic geese in Canada, goslings were the most severely affected group; 25% of a flock of 2731 birds died due to WNV. Age differences were also apparent in the serologic data from this event, with 90% seroprevalence in

the juvenile and 15 month old cohorts, but only 10% in the 5 year and older cohort (Austin, 2004). Differences in juvenile behavior, feather coverage, and stocking density may play a role in these rates. In another example, a flock of 1,200 white storks (forced to land from exhaustion in Israel during migration and found to be clinically affected by WNV) was composed primarily of juvenile birds (Malkinson, 2002b).

Use of wild birds in WNV surveillance

As a critical component in the epidemiology of WNV, birds have naturally been a focus of WNV surveillance efforts. Traditionally, serology was used to monitor WNV in wild birds because, until 1998, clinical disease in avian species was not associated with the virus. WNV viremia is short-lived in avian species, lasting an average of 3-4 days, and therefore may be easily missed during serum testing (Komar, 2003). Targeting the antibody response to WNV is a more reliable method of detecting WNV exposure. Using antibody detection systems, retrospective studies of banked avian serum samples may also be conducted to establish a baseline and determine the time of WNV introduction into an area. As an example, testing of banked serum conducted at the Bronx Zoo in 2002 revealed that there was no evidence of WNV circulation within the park prior to 1999 (Ludwig, 2002).

Since the WNV strain introduced into North America causes mortality in avian species, surveillance efforts have gravitated towards dead bird monitoring or testing. While mortality may be high in some avian species, there is a wide range of species susceptibility to WNV (Komar, 2003). Among avian species found in the North America, birds in the family *Corvidae* seem to be the most susceptible to infection and disease associated with WNV. In contrast, experimental studies with WNV in chickens and turkeys demonstrated virus replication in these

species but no clinical disease associated with infection was observed (Swayne, 2000; Senne, 2000).

A diversity of techniques including antibody detection, virus isolation, RNA detection, and antigen detection have been utilized in the study of WNV epidemiology. Antibody detection has been a traditional method for surveillance of WNV in avian species in Europe, Asia, and Africa in live birds (Work, 1955; Taylor 1956). With the introduction of WNV to the U.S., the WNV detection methods most utilized for surveillance involving avian species have included virus isolation and RT-PCR of dead bird tissues.

Diagnostic tools

Antibody testing

Antibody assays for flaviviruses include plaque reduction neutralization test (PRNT), hemagglutination inhibition (HI), complement fixation (CF), and enzyme-linked immunosorbent assay (ELISA).

Plaque reduction neutralization tests remain the gold standard for serological testing for antibodies to WNV despite being time consuming, labor-intensive, and requiring BSL-3 facilities. Also, cross-reactions may occur between flaviviruses with PRNT. Due to the presence of SLE in the U.S., 90% neutralization titers must be performed in a dilution series against both WNV and SLE; a fourfold or greater titer for one of these viruses indicates that virus as the etiologic agent (Nasci, 2002).

Hemagglutination inhibition tests, while popularly used in the past for WNV antibody detection, are being replaced due to lower sensitivity and specificity than other tests, as well as increased automation and speed of tests such as ELISA. The Florida Department of Public

Health laboratories perform modified HI tests that are paired with IgM ELISA and serum neutralization tests.

Enzyme linked immunosorbent assays have in the past been limited when testing wild bird sera due to a broad species range. While the ELISA format is relatively quick, uses small volumes of serum and does not require BSL-3 facilities, the lack of a reliable "anti-wild bird" immunoglobulin and the necessity of further testing with PRNT initially limited its use in testing wild bird sera. Several studies have been conducted to try and develop an immunoglobulin for wild bird sera or an ELISA protocol that does not require the wild bird antibodies to be labeled. One approach uses a goat anti-wild bird immunoglobulin IgG that is able to detect anti-WNV antibody in sera of 23 avian species (Ebel, 2002). This test has a positive predictive value of 70% and needs to be followed by PRNT to determine whether the antibodies are anti-WNV or anti-SLE. The authors concluded that this test correlated well to HAI results and was more sensitive than neutralization. An early study focusing on domestic animals in Australia developed a blocking ELISA that is capable of differentiating between two closely related flaviviruses, Murray Valley encephalitis (MVE) and Kunjin (KUN) (Hall, 1995). This approach was successfully applied to a range of wild birds naturally infected with WNV in New York (Jozan, 2003). Further work was conducted with the epitope-blocking ELISA developed by Hall (1995) with the development and comparison of seven monoclonal antibodies in detecting and distinguishing WNV and SLE (Blitvich, 2003). This study found that using a pair of monoclonal antibodies, one which detects E protein epitopes and the other an NS1 epitope, was the most efficient combination for the detection of serum antibodies to WNV and SLE in wild birds. This allowed differentiation between antibodies to WNV and SLE, removing the necessity of follow-up PRNT.

Comparison of serologic studies conducted with different laboratory techniques presents difficulties. The HAI test has shown to be less sensitive than PRNT in several instances (Hayes, 1962; Gibbs, 2005; Farfan-Ale, 2004). It has been suggested that HAI antibodies reflect more recent infections while neutralizing antibodies persist for years up to the lifetime of the bird. Competitive ELISA results have thus far shown to closely reflect PRNT results (Gibbs, 2005; Weingartl, 2003). Further complicating serologic studies, the extent of antibody response mounted by different birds against different arboviruses may vary (McIntosh, 1969). All of these factors affect the interpretation of serologic results gained through live bird surveillance.

Virus Isolation

Isolation of flaviviruses from dead birds is possible by applying ground tissue samples from suspect cases to cell cultures made from chicken embryos, in porcine or hamster kidney cells, African green monkey kidney (Vero) cells, the MD-BK cell line, and mosquito cell lines (OIE, 2000). Cytopathic effect (CPE) is seen with some flaviviruses, but not all viruses and not all cell lines. West Nile virus produces CPE on Vero cells with cell rounding after three to five days of incubation and destruction of the monolayer. To confirm that CPE is caused by WNV, supernatant from cell cultures may then tested with RT-PCR using primers specific to the WNV envelope and/or pre-membrane protein.

The best tissues for virus isolation in American crows and blue jays are brain, heart, and lung tissues (Gibbs, 2005; Panella, 2001). The advantages of virus isolation include the high sensitivity of the test, the ability to detect viruses other than WNV during surveillance, and the ability to isolate virus for use in sequence analysis and further studies. Disadvantages of virus isolation include the need for BSL-3 facilities to work with live virus, and the length of time (up to 14 days) required to confirm a negative sample.

Nucleic acid detection

As nucleic acid detection systems are becoming less expensive and less time consuming, more emphasis is now being placed on these assays for the detection of WNV. TaqMan, RT-nPCR, Real time quantitative PCR, and Nucleic Acid Sequence Based Amplification (NASBA) techniques are all being used in WNV surveillance efforts.

The best avian tissue for use in nucleic acid detection techniques in American crows and blue jays appears to be the brain (Panella, 2001; Gibbs, 2005). Virus load in this tissue is high, and the tissue is easy to extract from carcasses and macerate for testing.

The advantage to nucleic acid detection techniques is that these methods allow fast determination of presence of flaviviruses, with some protocols producing results in a matter of hours. Also, these techniques do not require BSL-3 facilities. Several studies have been conducted to investigate the utility of newly developed nucleic-acid based techniques, and for the most part the assays have proven to agree well with virus isolation results with a few exceptions. TaqMan was shown to have greater sensitivity than traditional RT-PCR methods, but was slightly less sensitive than virus isolation (Lanciotti, 2000). A NASBA-ECL assay was demonstrated to have exceptional sensitivity and specificity when compared to virus isolation, TaqMan, and standard RT-PCR as it was able to detect 0.01 PFU of WNV (Lanciotti and Kerst, 2001). In contrast to these studies, in a comparison of cell culture and RT-PCR in detecting WNV in dead birds, the RT-PCR protocol used proved to be less sensitive than the virus isolation with only fair agreement between the two (kappa = 0.62) (Sirin, 2004).

Technology applying nucleic-acid based techniques is constantly advancing. With the subtle changes and improvements that are made to each protocol, comparison of all the

techniques is difficult. In addition, the tissues tested, species tested, and the genetic sequences targeted by the variety of available primers appear to influence the effectiveness of each test.

Antigen detection

Antigen detection assays for WNV include immunohistochemistry (IHC) and VecTest. Panella (2001) investigated the distribution of WNV within tissues of infected American crows and found that virus was present in highest titers within the brain. Immunohistochemical staining has shown WNV antigen to be highly localized within the brain tissue however, making sensitivity for WNV within brain tissue low with this test (Weingartl, 2004). For IHC, kidney, liver, and heart seem to be the most reliable tissues for testing (Ellis, 2005; Wünschmann, 2004b). The advantages of IHC include visualization of the antigen within the host tissue, lack of need for a BSL-3, as well as the ability to ship formalin-fixed paraffin-embedded tissues across international borders to labs with WNV IHC capabilities.

The VecTest WNV antigen capture assay (Medical Analysis Systems Inc, Camarillo, CA) has proven to be a simple and quick method for the diagnosis of WNV antigen in several different applications. This test uses a dipstick and a simple color indicator to reveal the presence of WNV antigen. The best samples for use in this test vary based on the avian species being tested. Oral swabs were adequate for use in American crows, blue jays, and house sparrows, but performed poorly in raptors (Stone, 2004). The sample being tested may also influence sensitivity and specificity of the test; differences in between oral (92.8% sensitivity) and cloacal swabs (58.3% sensitivity) have been demonstrated (Lindsay, 2003). Other samples, such as spleen and kidney tissue, also have demonstrated utility for use with VecTest in American crows and blue jays (Henson, 2004). The advantages of the VecTest include the little equipment and training needed, as well as the fact that a BSL-3 laboratory is not necessary to run the test. When

testing homogenates of mosquito pools, VecTest proved to be comparably sensitive and accurate to the RT-PCR assay and TaqMan RT-PCR (Chiles, 2004; Nasci, 2002). When applied to oral and cloacal swabs in dead corvids, VecTest results correlated well with TaqMan and virus isolation results from brain tissue (Lindsay, 2003; Siirin, 2004). In one study, accuracy of the VecTest varied widely according the avian species tested, however only blue jay and American crow samples were tested in high enough numbers to draw a sound conclusion (Stone, 2004). For these species, true positives were determined by VecTest 80% and 87% of the time, and false positives 1% and 2% of the time for blue jays and American crows respectively. These results suggest that for local determination of WNV activity in an area, VecTest may be appropriate if used with the right avian species; however positive results should be confirmed by more accurate virus detection methods.

Surveillance approaches

Passive surveillance

Prior to 1998, avian mortality associated with WNV infection had not been reported and as a result, surveillance efforts were focused at that time on serologic evidence of WNV in avian species and clinical disease in humans and horses (Taylor, 1956; Hubalék, 1999). Since then however, WNV has caused high mortality in the Western Hemisphere and the focus of surveillance has shifted to dead bird testing in that region. Dead bird testing programs rely on the state public health departments to collect dead birds submitted by the general public. These birds are then sent to participating laboratories for virus isolation and/or nucleic acid detection. In some states, an emphasis has been placed on highly susceptible species such as American crows (*Corvus brachyrhynchos*) and blue jays (*Cyanocitta cristata*), while in other states all dead bird

species are received and tested. Results are recorded for species, location, and diagnostic test used. These data are then collated and published in weekly reports (Morbidity and Mortality Weekly Report, CDC; ProMed Mail, International Society for Infectious Diseases) and posted on the CDC and NWHC web sites. Results from passive surveillance systems throughout North America have been made available with exceptional speed and are freely accessible.

The dead bird surveillance systems have been used in several ways. For example, testing of dead corvids was used to map the spread of WNV in the northeastern United States (Julian, 2002; Eidson, 2001). American crow mortality rates were also used as an indicator of human risk and as an "early warning" system to detect WNV transmission during initial amplification in the vector population prior to potential disease in humans and domestic animals (Eidson, 2001; Mostashari, 2003; Guptill, 2003). A retrospective study of dead crow sightings and their geospatial relationship to human WNV cases was conducted in Chicago (Watson, 2004). The authors found a significant relationship between the early-season crow deaths and the residences of WNV-infected patients.

There are several advantages to conducting passive surveillance systems for WNV including diagnosis of WNV epornitics as they occur (sometimes prior to human cases), providing carcasses for further understanding of WNV pathology, diagnosis of other infectious diseases amplified by avian species, and the opportunity to conduct avian mortality studies. As mentioned previously, dead bird testing programs have been applied with success to predict areas of high human risk due to WNV (Eidson, 2001; Mostashari, 2003; Guptill, 2003). Results are reported weekly to public health departments, allowing these offices to broadcast warnings to local residents in a timely fashion. This information may also be used to help direct mosquito control efforts.

Carcasses from passive surveillance programs have also been used to gain more insight into the pathogenesis of WNV. The variation in pathology and tissue tropism of WNV in corvids, raptors, and passerines has been demonstrated by several studies (Gibbs, 2005; Ellis, 2005; Wünschmann, 2004a and 2004b; Steele, 2000).

Programs which use virus isolation in their testing protocols have the additional advantage of identifying other diseases associated with wild birds. A study of WNV in American and fish crows, as well as other passerines, from Connecticut identified nine cases of EEE in addition to their WNV findings (Beckwith, 2002). Several isolates of Flanders, Highlands J, and EEE viruses have been obtained from dead bird submissions in Georgia (SCWDS, unpublished data; Gottdenker, 2003).

An inherent risk in relying solely on passive surveillance data for WNV monitoring is the lack of correlation between the true geographic range of WNV and the pattern of reported cases (Ruiz, 2004). Factors influencing this include socioeconomic and educational status of the people living in areas where dead birds are dying, extent of the public education campaign directed towards WNV, press coverage of local recent human and horse cases, public complacency about reporting dead birds, and density of human populations. In addition to these issues, after confirmation of the first WNV cases in a state, some public health authorities elected to discontinue dead bird testing. As a result of these factors affecting dead bird surveillance activities in different ways, nationwide data comparison is problematic.

Mathematical modeling of human WNV risk found that active surveillance was a more sensitive predictor than passive surveillance (Brownstein, 2004). These authors conclude that surveillance systems based only on dead birds are not as effective as active surveillance for early warning and targeted prevention efforts.

Active surveillance

Serologic studies of wild birds have been used in all regions where WNV is endemic, as well as in the Western Hemisphere where it has rapidly become endemic. Findings in each geographic area have added to the breadth of knowledge about WNV, as well as documenting changes in the epidemiology of the virus as it moves to new areas and encounters different species.

Africa: The initial work defining birds as important hosts of WNV took place in Egypt in early 1950's (Taylor, 1956). A combination of virus isolation, serologic data, and experimental infection confirmed the role wild birds play in the transmission cycle of WNV. High neutralizing antibody titer to WNV recorded in the hooded crow (*Corvus corone sardonius*) and house sparrow (*Passer domesticus*) suggested that these species could be used as natural sentinels for the virus. A group of studies conducted in South Africa in the 1960's further described the role of avian species in the maintenance and transmission of WNV. Of 2,022 wild birds tested in the first study, 252(12%) from 27 species were positive for antibodies to WNV (McIntosh, 1968). In this study, the birds with the highest antibody prevalence as determined by HAI tests were those associated with water: the red-billed teal (*Anas erythrorhynca*) 39% positive, the red-knobbed coot (*Fulica cristata*) 22% positive, the yellow-billed duck (*Anas undulata*) 17% positive, and the sacred ibis (*Threskiornis aethiopicus*) 14% positive. This same body of work also demonstrated that certain species of birds may have no or transient antibody responses to WNV despite known infection and viremia. Such findings admittedly complicate interpretation of serologic data, however, as negative results may represent naïve birds or exposed birds in which antibody could not be detected (McIntosh, 1969). As such, it is obvious that surveys relying on serology are only detecting a proportion of the immune population.

Europe: WNV outbreaks occurring across Europe from the early 1960's to the present have also been monitored through avian serologic surveillance. In most cases, humans, livestock, and mosquitoes were concurrently tested for virus and/or antibody. Wild birds were tested for antibodies to WNV in the 1995 and 1996 outbreaks in Poland and Romania (Juricova, 1998; Savage, 1999). In Poland, the focus was placed on house sparrows (*Passer domesticus*) and tree sparrows (*Passer montanus*). Of 179 house sparrows, 2.8% had antibodies to WNV while 12.1% of 33 tree sparrows had antibodies to WNV. In the Romanian study, only 12 passerines were tested and 1 of these, the European robin (*Erithacus rubecula*), was positive for antibodies to WNV. More recently in Russia, a combination of virus isolation and serologic tests were used to monitor WNV outbreaks and determine where the focus of activity was centered in the West Siberian migration way and the Volga delta (Ternovoi, 2004; L'vov, 2004). A study conducted in Israel monitored resident and migrant kestrel species (*Falco tinnunculus* and *Falco naumanni*) over a 12 month period and found that location and age of the birds significantly affected WNV serologic status, and that a small number of birds entering the country in April were previously infected (Banet-Noach, 2004). The authors suggested that this type of active surveillance could be used to forecast timing and dispersion of WNV in other areas.

While no disease associated with WNV has been reported in the United Kingdom, serological evidence has been found in resident and migrant wild birds (Buckley, 2003). In this survey, birds from three sites representing 30 species, including a few domestic free-range poultry species were tested. Of 353 serum samples, 52 (14.7%) were positive. Titers were fairly low in comparison to those reported from wild birds in the US, ranging from 10 to 40. This study illustrates how important serologic testing for WNV can be in studying its epidemiology, especially when clinical disease is not present in humans, domestic animals, or birds.

US and Canada: Precedence was set for the use of serology in wild birds for studying arboviruses in North America with research conducted on viruses of public health importance such as SLE, EEE, and WEE. The transmission cycles of these viruses, like that of WNV, involve birds as hosts and mosquito vectors (McLean, 2001). The house sparrow was shown to be a major host species for SLE and proved to be useful in detecting and predicting SLE epidemics (McLean and Bowen, 1980; Mclean, 1983). Serologic data gathered from birds in South Carolina and Georgia in 1995 and 1996 suggested that several wild birds were potential reservoir hosts for encephalitis viruses in coastal Georgia (Durden, 1997). In Florida, sentinel chickens seroconverted to SLE 10 weeks earlier than wild birds, and decreased amplification of transmission was associated with hard winter freezes resulting in an abundance of seronegative birds (Day and Stark, 1999). Researchers used serologic data from wild and sentinel birds to detect temporal and spatial changes in WEE and SLE activities in California (Reisen, 2000). Also in California, a ten year study was conducted to evaluate house finches as an early warning system for SLE and showed that seroconversion to SLE in these birds preceded those of sentinel chickens (Gruwell, 2000). This study also demonstrated year-round SLE transmission. During the same time period, a five-year study conducted in upstate New York demonstrated that song sparrows (*Melospiza melodia*) were the primary amplifying host of both Highlands J and EEE viruses, while the gray catbird (*Dumatella carolinensis*) was most likely involved in yearly virus reintroduction into the area (Howard, 2004). These are just a few examples describing the research environment into which WNV was introduced in 1999.

After WNV was discovered in the US, several wild bird serological surveys were begun. The first of these surveys centered on the site of the first epizootic in Queens, New York and areas peripheral to Queens (Komar, 2001c). This study determined house sparrows and pigeons

would be good candidates for wild bird WNV sentinels, and also served to identify geographic locations where WNV transmission was high. A second study was conducted on nearby Staten Island, New York (Komar, 2001b). Species with the greatest seroprevalence in this study were the northern cardinal (*Cardinalis cardinalis*) 69.2%, and the rock pigeon (*Columba livia*) 54.5% while the house sparrow (*Passer domesticus*) had 8.6% seroprevalence. The concentration of infections in nonmigrant species suggested that these birds might play a role in the local transmission cycle. At this time, scientists were unsure which avian species were susceptible to disease caused by WNV; these results indicated that northern cardinals and rock pigeons could survive infection. In 2003, after WNV had reached California, a serosurvey of sentinel chickens and free-ranging birds in Imperial and Coachella valleys was conducted (Reisen, 2004). This study found that rock pigeons and white-winged doves had the highest seroprevalence for flaviviruses among the wild birds tested.

Mexico, the Caribbean, and Central America: Active surveillance has been used to follow WNV as it moves into Mexico and the Caribbean, where dead bird surveillance is not as practical. Serologic evidence of WNV was detected in the Cayman Islands in 2001 in a human, throughout Mexico in 2001 in birds, horses, and livestock, in Jamaica, Dominican Republic, and Guadeloupe in 2002 in resident birds and livestock, Puerto Rico in 2004 in resident and migratory birds, and in Belize in 2004 in a horse (CDC, 2002b; Dupuis, 2003; Komar O, 2003; Quirin, 2004; Dupuis 2004 unpublished data; Fernandez-Salas, 2003; Estrada-Franco, 2003; Ulloa, 2003; Farfan-Ale, 2004; OIE, 2003). Farfan-Ale (2004) found a low seroprevalence (0.09%) for WNV among wild resident and migratory birds tested between 2000 and 2003 in Mexico. Birds testing positive in this study were the blue bunting (*Cyanocompsa parellina*),

brown-crested flycatcher (*Myiarchus tyrannulus*), gray catbird, indigo bunting (*Passerina amoena*), and rose-breasted grosbeak (*Pheucticus ludovicianus*).

There are several advantages to using active rather than passive surveillance. Active surveillance, though more costly and time consuming, allows the investigator to ensure adequate numbers of samples, even distribution of collection points, and unbiased collection methods. The data collected gives the investigator information on the avian species potentially involved in the epidemiology of the disease, the locations where the virus is circulating, the ecological and temporal distribution of the virus, and insight into the age of reservoir hosts targeted by the vector. Risk factors associated with virus transmission can then be determined. The ability to design active surveillance systems with a multifaceted approach, the fact that these have utility in the absence of clinical disease, and the use of live wild birds as natural sentinels are also advantages to using active surveillance for WNV studies.

The importance of an active surveillance approach is demonstrated by two examples. In the Tuscany region of Italy in 1998 and the Camargue region of France in 2000, disease occurred in horses but was not reported in humans; seroconversion in sentinel birds was low in the Camargue (Autorino, 2002; Murgue, 2001; Zeller and Schuffenecker, 2004). In this case, active surveillance was important in describing the full extent of the outbreak by applying serologic studies to a variety of hosts. In a second example, clinical disease in humans, birds, and horses was not a prominent finding as WNV spread south into Mexico and the Caribbean (Dupuis, 2003). Despite this, active surveillance efforts indicated the presence of the virus through equine and avian serosurveys (Fernandez-Salas, 2003; Farafan-Ale, 2004; Estrado-Franco, 2003).

Active surveillance also has more utility than passive surveillance in the absence of clinical disease. Since the discovery of WNV, surveillance for the virus has focused on human and avian serologic testing (Smithburn, 1952; Taylor, 1956.) The availability of human serum samples from projects investigating other viral diseases in Africa, as well as the lack of avian mortality associated with WNV led to this initial preference. This approach continued to be used widely until the introduction of the virus to North America. As WNV becomes more widespread in North America and avian populations become resistant to clinical disease however, this approach will again gain greater utility.

Another advantage to active surveillance systems lies in the ability to use live wild birds as natural sentinels of WNV. The use of free ranging sentinels has been valuable in detecting flavivirus transmission (Monath and Tsai, 1987; Day and Stark, 1999). While finding antibodies to WNV in a bird does not imply that it is an active host and involved in the propagation of the virus (Taylor, 1956), it does provide information on where the virus is circulating. Serologic studies may also be combined with virus isolation attempts from blood samples to determine the role each species may play in maintenance and transmission of WNV (Allison, 2004).

A program using wild birds is effectively monitoring a larger region than would be examined with captive sentinels (Komar, 2001a). This approach also avoids the difficulties of knowing where domestic sentinel birds, e.g. chickens, are best placed in the environment and allows for coverage of a greater variety of canopy height and habitat types.

Some of the disadvantages inherent in active surveillance systems stem from the interpretation of serologic data. Antibody persistence, maternal antibody transfer, and mortality all affect the interpretation of serologic surveyresults. Antibody prevalence estimates may not accurately reflect exposure if birds are antibody positive for only a short time, or are removed

from the testing pool as a result of mortality caused by WNV. In cases of long-term antibody persistence, prevalence estimates may not reflect recent exposure. This can be partially corrected by determining antibody prevalence in hatch year birds to document recent exposure. In order to detect temporal and spatial trends, testing must cover several virus amplification seasons and a large geographic area. This necessitates a large sample size and multiple years of testing. In addition, a major disadvantage involves the cost associated with bird capture and sampling.

West Nile virus surveillance involving birds in the U.S., to date, has relied on both passive and active surveillance strategies. The research presented here investigated several components of current surveillance approaches and sought to improve our understanding of WNV epidemiology in the U.S. This work focused on refining testing strategies, exploring the extent and timing of the avian immunologic response to WNV and how that information affects live bird surveillance, searching for a good avian indicator of WNV, as well as determining land use and physiographic region variables affecting WNV distribution. The information gathered through these projects will improve future WNV surveillance and provide a basis for further investigation.

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CHAPTER 3

West Nile virus detection in the organs of naturally infected Blue Jays (*Cyanocitta cristata*)[1]

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[1]Gibbs SE, Ellis AE, Mead DG, Allison AB, Moulton JK, Howerth EW, Stallknecht DE. 2005. *Journal of Wildlife Diseases* 41(2): *In Press*. Reprinted here with permission of publisher.

Abstract

Blue jays (*Cyanocitta cristata*) are an effective indicator species for West Nile virus (WNV) and may be regionally important in surveillance efforts. The sites of WNV replication and sensitivity of virus detection techniques are undefined for blue jays. The objectives of this study were to describe the gross and microscopic pathology associated with natural WNV infection in blue jays, as well as determine the most appropriate tissues to be used for virus isolation, reverse transcriptase nested polymerase chain reaction (RT-nPCR), and immunohistochemistry (IHC) techniques. Blue jays were collected in the state of Georgia, USA, between May and September of 2001. Initial screening by virus isolation indicated that 36 of 59 blue jays chosen for evaluation were WNV positive. From this group, 20 positive and five negative birds were chosen to compare virus detection techniques. Six positive and five negative birds were selected for histopathology examination. Splenomegaly and poor body condition were the most consistent gross findings among positive birds. The most consistent histopathologic findings in the tissues of WNV positive blue jays were mononuclear leukocytosis and epicarditis/myocarditis. Brain, heart, and lung had the highest viral titers among the tissues. WNV antigen was most often detected by IHC in heart, kidney, liver, and lung. RT-nPCR proved to be the most sensitive diagnostic test applied in this study irrespective of the tissue type. Brain tissue could be used effectively for both virus isolation and RT-nPCR, and this tissue is simple to remove from the carcass and homogenize. The success of IHC is highly dependent on the tissues tested and the use of multiple tissues including heart, kidney, liver, or lung is recommended.

Introduction

Since its initial discovery in the United States during the summer of 1999, West Nile virus (WNV) has caused extensive bird mortality across North America. American crows *(Corvus brachyrhynchos*) are particularly susceptible to WNV with mortality rates of 100% reported from experimental infection studies (Komar et al., 2003; McLean et al., 2001). Other corvids, such as black-billed magpies (*Pica hudsonia*), blue jays (*Cyanocitta cristata*), and fish crows (*Corvus ossifragus*), are also susceptible to disease caused by WNV (Komar, 2000; Steele et al., 2000;). For this reason, corvids have been utilized extensively for mortality based WNV surveillance in the United States (Eidson et al., 2001; Kramer et al., 2001).

Various approaches to detecting WNV, including virus isolation, viral RNA detection, and immunohistochemistry (IHC), have proven effective (Steele et al., 2000). In order to maximize the likelihood of detection however, information on tissue specific viral titers associated with WNV infection is needed. To date, this work has been restricted to naturally and experimentally infected American crows and experimentally infected blue jays. One study of naturally infected American crows used TaqMan reverse transcriptase polymerase chain reaction (RT-PCR) and plaque assay to compare WNV titers in six different tissues and found brain tissue to be the most sensitive target organ (Panella et al., 2001). A second study using TaqMan RT-PCR on tissues of naturally infected American crows found kidney, and secondarily brain, to be infected most consistently (Kramer and Bernard, 2001). Virus isolation and RT-PCR performed on kidney and heart tissue from naturally infected birds in the New York area were consistently positive (Steele et al., 2000). In this same study, brain was the least sensitive tissue for IHC in American crows, fish crows, and black-billed magpies (Steele et al., 2000). Experimental infection studies involving blue jays reported highest viral titers in lung at 9.2 $\log_{10}$ PFU/0.5cm^3

(Komar et al., 2003). Immunohistochemistry, virus isolation, and RT-PCR performed on replicate tissue samples from a variety of naturally infected avian species revealed that heart, kidney, and spleen were consistently positive by all three diagnostic tests (Steele et al., 2000).

Studies on the pathology of WNV in avian species have described a range of gross and microscopic lesions present in infected birds. Gross hemorrhage of the brain, splenomegaly, meningoencephalitis, and myocarditis were the most prominent lesions noted in birds found dead at the beginning of the WNV epidemic (Steele et al., 2000). Subsequent studies noted similar lesions in experimentally infected blue jays, crows, chickens, and turkeys, as well as naturally infected owls (Weingartl et al., 2004; Wünschmann et al., 2004; Swayne et al., 2000; Senne et al., 2000; Fitzgerald et al., 2003). No pathoneumonic lesions for WNV have been described however, and lesions between species are not consistent.

WNV was first detected in the state of Georgia in the summer of 2001. During that year, blue jays represented 30% of all dead bird submissions from Georgia while American crows represented 17%; 50% of all the avian WNV isolates were from blue jays while 43% were from American crows (SCWDS, unpublished data). These submission and infection rates indicate that blue jays are an effective indicator species for WNV and may be regionally important in surveillance efforts. This may be especially true in locations where American crows are not abundant or adequately represented in dead bird submissions.

The objectives of the study were to describe the gross and microscopic pathology associated with natural WNV infection of blue jays, and to determine the most appropriate blue jay tissues to be used for virus isolation, reverse transcriptase nested polymerase chain reaction (RT-nPCR), and IHC techniques. The current study builds on the knowledge of the pathogenesis of WNV infection in birds by describing in detail the lesions associated with WNV infection,

identifying sites of viral replication, and determining appropriate diagnostic tests for blue jays naturally infected with WNV.

Materials and Methods

Bird Selection and Sample Collection

Fifty-nine of 475 blue jays submitted to the Southeastern Cooperative Wildlife Disease Study (SCWDS) as part of a statewide WNV surveillance program conducted from May to September 2001 were selected for evaluation. Those birds with the least post mortem autolysis were chosen. Necropsies were performed in a Class II safety cabinet. The birds were handled and samples collected by procedures approved by the Animal Care and Use Committee at the University of Georgia (A2000-10071-M2). Brain stem/cerebellum and heart were aseptically collected for initial screening by virus isolation. In addition, duplicate 0.5 cm^3 samples of heart, lung, liver, kidney, brain, spleen, flight muscle, and bursa were taken aseptically from each bird. One set of these samples, for use in virus isolation and quantification as well as RNA detection by reverse transcription nested polymerase chain reaction (RT-nPCR), was placed in separate microcentrifuge tubes containing 0.5 ml BA-1 medium (minimal essential medium, 0.05M Tris ph 7.6, 1% bovine serum albumin, 0.35 g/L sodium bicarbonate, 100 units/mL penicillin, 100 mg/mL streptomycin, 1 mg/mL Fungizone) and stored at -70° C until analyzed (Langevin et al., 2001). The second set of samples was placed in 10% buffered formalin for 24 hours and embedded in paraffin for histopathology and IHC.

Initial screening indicated that 36 of the 59 blue jays chosen for evaluation were WNV positive. Twenty WNV positive and five WNV negative birds were then randomly chosen to compare virus isolation and quantification, RT-nPCR, and IHC techniques. Tissues from six of

the positive birds and all five negative birds used for comparison of techniques were also evaluated for microscopic pathology.

Gross and Microscopic Pathology

Gross examination was performed on brain, heart, lung, liver, kidney, spleen, bursa, gastrointestinal tract, skeletal muscle, pancreas, reproductive tract, and skin. Gross examination findings were categorized into six groups: calvarial hemorrhage, presence of myocardial lesions, presence of splenomegaly, pulmonary congestion, body condition, and presence of trauma. Myocardial lesions included pallor and hemorrhage. Body condition was categorized as emaciated, thin, or good. A Chi square test was used to determine if differences in the prevalence of gross findings in the 36 positive and 23 negative birds were significant.

Sections of formalin-fixed paraffin embedded tissues (3-5 μm thick) were stained with hematoxylin and eosin. Five negative and six positive birds with minimal autolysis were chosen for microscopic evaluation, since autolysis can obscure subtle lesions. Tissues evaluated histologically and immunohistochemically from these birds included brain, heart, lung, liver, kidney, and spleen.

Initial screening using virus isolation and RT-PCR

Virus isolation was performed on brain stem/cerebellum and heart tissues to screen the 59 sample birds for WNV infection. All virus work was performed under BSL-3 conditions. Tissues were macerated with a plastic tissue grinder in BA-1 and centrifuged at 7,200 x g for five minutes. Supernatant of homogenized samples (100 μL) was placed on three day old Vero cell (African green monkey kidney) monolayers in 12-well cell culture plates (Corning, Acton, MA) containing 2 ml per well of maintenance medium consisting of 2% antibotic solution (100 units/mL penicillin, 100 mg/L streptomycin, and 100 μg/mL Fungizone,) 3% fetal bovine serum,

95% minimum essential medium with non-essential amino acids. The monolayers were incubated at 37º C and observed daily for cytopathic effect (CPE) for 7 days. If no CPE was observed at 7 days, the sample was passaged to new cells and observed for an additional 7 days.

Reverse transcription polymerase chain reaction (RT-PCR) was performed to confirm WNV in samples with CPE. Total RNA was extracted from the supernatant using a QIAamp viral RNA Mini Kit (Qiagen Inc., Valencia, CA) following the manufacturer's protocol. Reverse transcription of RNA to cDNA and subsequent primary amplification were carried out in a single-tube reaction. Briefly, 2 µL of each RNA sample was added to individual 0.5 ml thin-walled PCR tubes (USA Scientific Inc., Ocala, FL) containing 48 µl of a premix which contained 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, 1.6 mM beta-mercaptoethanol, 0.25 mM dNTPs, 200 ng primers (WNV 897-F 5 '-GCYGTCATTGGWTGGATG and WNV 1195-R 5' - TCRTTGTGRGCTTCWCCCAT), 3 U AMV reverse transcriptase and 1.5 U *Taq* polymerase (Promega Corp., Madison, Wisconsin). Negative controls to detect possible cross-contamination consisted of 2.0 µL RNase-free water as template and 48 µL premix. Positive reaction controls consisted of RNA extracted from the Eg101 WNV isolate.

Reaction mixtures were incubated at 43^{o}C for 15 minutes then subjected to 40 cycles of 94^{o}C for 45 s, 50^{o}C for 60 s, and 72^{o}C for 75 s using a PTC-100^{TM} Thermal Cycler (MJ Research, Inc., Waltham, MA). Reaction products were separated by electrophoresis through a 1.5% agarose gel, stained in ethidium bromide, and visualized under ultraviolet light for qualification and quantification. Visualization of a 298-bp product indicated that WNV RNA was present in the original sample.

Virus Isolation and Quantification

End point titrations were performed on tissues from birds testing positive by virus isolation during the initial screening process. Frozen tissues were thawed and macerated in BA-1 with a glass tissue grinder and centrifuged at 7,200 x g for five minutes. Virus titrations were performed with homogenized tissue in 96-well plates containing Vero cells with eight replicated wells per dilution (Lennette et al., 1995). Titrations were begun at 10^{-2}, as toxicity was encountered at lesser dilutions. Plates were incubated at 37º C for seven days, at which time the 50% tissue culture infective dose ($TCID_{50}$) was determined using the Spearman-Kärber method (Finney, 1964).

RNA Extraction and Reverse Transcription Nested Polymerase Chain Reaction (RT-nPCR)

RT-nPCR was performed on all tissues of the 20 positive and five negative blue jays. Total RNA was extracted from 125 µL of ground tissue in an RNase free environment using an RNeasy Mini Kit (Qiagen Inc., Valencia, CA) following the manufacturer's protocol. The reverse transcription and primary reaction methods were the same as those used for initial screening, however WNV-310F 5'-TSAACAAACAAACAGCRATGAA and WNV-1637R 5'-AGGTTSAGRTCCATRAACCA forward and reverse primers were used for the primary reaction, and primers WNV 897-F 5 '- GCYGTCATTGGWTGGATG and WNV 1195-R 5' – TCRTTGTGRGCTTCWCCCAT were used for the nested reaction. Methods and materials for the nested reaction were the same as for the initial screening RT-PCR with the following exceptions: 1.0 µL of the first-stage amplification products was added to individual 0.5 ml thin-walled PCR tubes containing 49 µl of the premix, and the reaction mixtures were subjected to 35 cycles of 94^{o}C for 45 s, 52^{o}C for 45 s, and 72^{o}C for 60 s using a PTC-100^{TM} Thermal Cycler (MJ Research, Inc., Waltham, MA).

The RT-nested PCR (RT-nPCR) protocol used in this project was designed to accurately detect WNV RNA in avian tissues. Published sequences from representative WNV isolates (GenBank accession numbers AF260968, AF260967, AF202541, and AF196835) were aligned using Sequencher version 4.1 (Gene Codes Corp., Ann Arbor, MI). The degenerate primers were designed to amplify a fragment spanning the premembrane gene from conserved regions of these alignments.

The sensitivity of the RT-nPCR was determined by comparing the endpoint titration of WNV stock (Georgia isolate DES-01-107) in Vero cell culture with the endpoint dilution at which viral RNA was detected by RT-nPCR. Tenfold dilutions of virus were prepared. The endpoint titration in cell culture was $10^{6.45}$ $TCID_{50}$/25 µL. Endpoint dilutions for detection of viral RNA by RT-nPCR were $10^{5.2}$ $TCID_{50}$/25 µL after first-stage amplification, and $10^{8.0}$/25 µL after nested amplification. Specificity was examined by testing viral RNA extracted from related Flaviviruses (St. Louis encephalitis virus and Dengue viruses 1-4) and from unrelated North American arboviruses that are found in avian species (Eastern equine encephalitis virus, Western equine encephalitis virus, and Highlands J virus). The RT-nPCR procedure performed on these samples did not result in observable amplification. RNA from other Flaviviruses was not available for testing.

Immunohisotochemistry

Immunohistochemistry was performed on paraffin embedded tissues as previously described (Gottdenker et al., 2003). The primary antibody was a rabbit α-WNV polyclonal, which was diluted 1:500 (Bioreliance, Rockville, MD) and applied for one hour at room temperature.

Results

Gross Pathology

Splenomegaly and poor body condition were the most consistent gross findings among positive birds (Table 3.1).

Microscopic Pathology

Histopathologic findings are summarized in Table 3.2. The most consistent histopathologic findings in the tissues of WNV positive blue jays were mononuclear leukocytosis and epicarditis/myocarditis. Although leukocytosis was most easily observed within the lung, similar large mononuclear leukocytes were noted within vessels in all organs.

Cardiac lesions were mild. The most common changes were the presence of lymphoplasmacytic infiltrates with fewer macrophages within the myocardial interstitium and the presence of macrophages with fewer lymphocytes and plasma cells in the epicardium. Epicardial lesions were most severe around the base of the heart and at the junction of the atria and ventricles. Endocardial inflammation, when present, was very mild.

Vascular and perivascular lesions were observed in the great vessels of the heart and in a few smaller pulmonary vessels. Inflammation was present in the tunica media, tunica adventitia, and surrounding adipose tissue. The pulmonary vasculitis was associated with a focal area of air sacculitis, and was probably not directly due to WNV infection.

In spleen, reticuloendothelial cells were hyperplastic and diffusely swollen. Lymphoid tissue was also hyperplastic with numerous large plasma cells containing Russell bodies. Other findings included marked, multifocal lymphoid apoptosis and large deposits of hemosiderin within reticuloendothelial cells.

Kidneys from four of five WNV positive birds had degeneration of single or multiple tubular epithelial cells within scattered proximal tubules. Inflammation was not a prominent feature in any of the kidneys examined.

Lung lesions were inconsistent. Changes included increased numbers of peribronchial and perivascular lymphocytes and plasma cells and scattered, small, necrotic foci.

The most common lesion observed in the livers of WNV positive birds was a lymphoplasmacytic infiltrate within perivascular and periportal areas. However, two WNV negative birds had similar inflammatory infiltrates. Similarly, hemosiderin deposition within hepatocytes and Kupffer cells was noted in both WNV positive and negative birds.

No abnormalities were noted in any of the brains from the six WNV positive birds.

Virus Isolation and Quantification

Maximum virus titers are shown in Fig. 3.1. The minimum detectable titer for all samples was $10^{3.1}$ $TCID_{50}$/ml. Ranked virus quantification results are shown in Figure 1. Brain, heart, and lung had the highest viral titers among the tissues. Liver, kidney, and muscle had the lowest viral titers, while the viral titers of spleen and bursa fell between these two groups. Infectious virus was not detectable in tissues from the five negative control blue jays.

Reverse Transcriptase Nested Polymerase Chain Reaction (RT-nPCR)

All tissues from WNV positive blue jays were positive by RT-nPCR. RT-nPCR results were negative for all tissues in the five negative control blue jays.

Immunohistochemistry

Results of IHC by individual tissues are given in Table 3.3. All 20 WNV positive birds were also positive by IHC in at least one tissue. WNV antigen was most often detected in heart, kidney, liver, and lung. In heart, staining ranged from multifocal to diffuse with antigen present

in decreasing order in interstitial cells, myocardial fibers, and infiltrating macrophages. Staining was especially common within macrophages infiltrating epicardial adipose tissue. Antigen was also detected in the great vessels of the heart in a few birds within infiltrating macrophages and rare endothelial cells. In kidney, antigen was present most often within resident interstitial cells and infiltrating macrophages and rarely within tubular epithelium; staining in the kidney was most often multifocal but sparse. In liver, antigen was observed in virtually all Kupffer cells. Hepatocytes were never observed to contain antigen. A similarly generalized staining of splenic reticuloendothelial cells was observed. Staining was sparse but multifocal in lung, with antigen observed in tissue macrophages and circulating monocytes. Staining was highly variable in brain. Seven birds had staining of capillary associated cells in the brain which ranged from mild and scattered (three birds) to diffuse (four birds). The identity of these cells is not known, but possibilities include astrocytes, microglia, or extravasating macrophages. Antigen was also observed in neurons, particularly in and around the optic lobe, and glial cells. In four birds, the only WNV positive cells in the brain were intravascular and/or perivascular monocytes and macrophages in the choroid plexus.

Discussion

Detectability of WNV varies by tissue and is dependent on the diagnostic test employed. Lung, brain, and heart tissues of naturally infected blue jays in this study contained the highest WNV titers of the eight tissues examined. For blue jays, dead bird surveillance efforts utilizing virus isolation can be limited to one or more of these tissues. The authors have found brain tissue to be the most practical for use with virus isolation as it is simple to remove from the carcass and is easily homogenized.

Gross and microscopic pathology were the least effective procedures for WNV diagnosis in blue jays. Although all of the gross lesions observed by Steele et al. (2000) were seen in one or more of the blue jays in this study, only splenomegaly and body condition were significantly correlated with WNV infection. These nonspecific lesions are not diagnostic for WNV infection, however, as other infectious agents may cause similar gross lesions. In short, WNV cannot be accurately diagnosed based on gross pathology in blue jays.

WNV antigens were most often detected in kidney, heart, liver, and lung tissue by IHC, making them the most optimal tissues for use with this procedure. As many labs do not have BSL-3 facilities, IHC is an appropriate alternative to virus isolation. IHC may also be employed for relatively fast turn around of results as a diagnosis may be reached within 48 hours of tissue collection. The success of IHC was shown in this study to be highly dependent on the tissues tested. Weingartl et al. (2004) noted that viral antigen in experimentally infected crow and blue jay brains was not easily detected by IHC; this is consistent with our findings. Care must be taken in interpreting IHC of the heart tissue as staining of myofibers often has a paintbrush appearance that could easily be misinterpreted as background. Renal tubular epithelium often stains nonspecifically but this is not difficult to differentiate from true staining. Also, due to variability in staining between tissues, the authors recommend using at least three of the optimal tissues when performing surveillance to increase sensitivity.

Nested RT-PCR proved to be the most sensitive diagnostic test applied in this study, and did not detect viral RNA in any of the tissues from five WNV negative blue jays. The nested protocol allows results to be determined within 24 hours of tissue collection. The equipment required for RT-nPCR is expensive, however the speed and sensitivity of the test exceeded the capabilities of both virus isolation and IHC. This advantage is even more apparent with currently

used real-time PCR formats that can provide both rapid diagnosis and quantitative results (Lanciotti et al., 2000). As with virus isolation, brain tissue may be the most practical tissue for PCR due to ease of collection and maceration.

In other studies of WNV infection in birds, a triad of lesions (histiocytic to lymphoplasmacytic epicarditis/myocarditis, lymphoplasmacytic encephalitis, and renal tubular degeneration) has been described as being highly suggestive of WNV infection (Steele et al., 2000; Swayne et al., 2001; Garmendia et al., 2000). However, histopathologic lesions of encephalitis were not observed and renal lesions were uncommon in the blue jays in this study similar to reported findings in blue jays, crows, chickens and turkeys (Weingartl et al, 2004; Swayne et al., 2001; Senne et al., 2000),.

One lesion that was commonly noted in the blue jays was the presence of numerous large mononuclear cells in blood. This finding has not been previously reported in any avian species with WNV infection. Mild hepatocellular necrosis, observed in two WNV positive blue jays in this study, has been described by Steele et al. (2000) and Weingartl et al. (2004). Several lesions observed in the blue jays, including hepatitis and hemosiderosis in liver and spleen, were interpreted to be unrelated to WNV infection as they were seen in both WNV positive and negative birds. Urates were noted in the kidney of one WNV positive bird, but this is merely indicative of dehydration.

Immunohistochemistry results highlight the fact that gross and histopathologic lesions are poor indicators of presence of viral antigen. Viral antigen can be, and often is, present in large amounts in the absence of corresponding gross or histopathologic lesions. Even in cases where inflammation was present in a tissue such as heart, the majority of the staining occurred in histologically normal areas with only small amounts of antigen noted in inflamed areas.

The high WNV titers present in these blue jay tissues reinforces the need for personal protection when handling the birds and performing post-mortem exams. The high titers also illustrate the potential for blue jay tissues to be a source of WNV infection if ingested by scavenger species.

Immunohistochemistry, virus isolation, and RT-nPCR are all useful techniques in WNV surveillance. We have shown, however, that when employing these techniques, tissue selection is critical for immunohistochemistry (heart, kidney, liver, and lung were best) and virus isolation (based on tissue titer, brain, heart, and lung were best). With RT-nPCR however, it seems possible to detect viral RNA in a variety of tissues of positive birds, allowing the investigator to choose the tissue based on ease of collection.

Acknowledgments

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Table 3.1. Prevalence of various gross findings in birds from which WNV was and was not isolated on initial screen.

	Virus isolation positive birds with lesion #(%), n= 36	Virus isolation negative birds with lesion #(%), n=23
Calvarial hemorrhage	13 (36.1)	10 (43.5)
Myocardial lesions	5 (13.9)	2 (8.7)
Splenomegaly*	17 (47.2)	2 (8.7)
Pulmonary congestion	2 (5.5)	11 (47.8)
Poor body condition *	22 (61.1)	3 (13.04)
Trauma	3 (8.3)	4 (17.4)

*Splenomegaly and poor body condition were statistically significant lesions in WNV positive birds ($p \leq 0.005$) using a Chi square analysis

Table 3.2. Microscopic pathology results from blue jay tissues with no autolysis

	Virus isolation positive birds with lesion	Virus isolation negative birds with lesion
Heart		
Endocarditis	3/6*	0/5
Myocarditis	5/6	0/5
Epicarditis	6/6	0/5
Myofiber degeneration	1/6	0/5
Ganglioneuritis	1/6	0/5
Liver		
Hepatitis		
Perivascular	1/6	0/5
Periportal	0/6	2/5
Random	0/6	1/5
Mixed	3/6	0/5
Piecemeal necrosis	2/6	0/5
Hemosiderosis		
Mild	1/6	1/5
Moderate	0/6	2/5
Severe	2/6	0/5
Congestion	2/6	0/5
Hemorrhage	1/6	0/5
Atrophy	0/6	1/5
Kidney		
Tubular degeneration	4/5	1/5
Urates	1/5	0/5
Ureteritis	0/5	1/5
Lung		
Peribronchial lymphocytosis	1/6	1/5
Congestion	0/6	1/5
Necrosis (mild)	1/6	0/5
Spleen		
Swollen RE cells**	4/4	0/3
Hypercellularity	4/4	0/3
Plasmacytosis	0/4	1/3
Lymphocyte apoptosis	2/4	0/3
Hemosiderosis	1/4	0/3
Brain		
Histopathologic lesions	0/6	0/5
Blood/Blood vessels		
Leukocytosis (mononuclear)	5/6	0/5
Vasculitis	2/6	0/5
Perivasculitis	2/6	0/5

*number of tissues with lesion/number examined

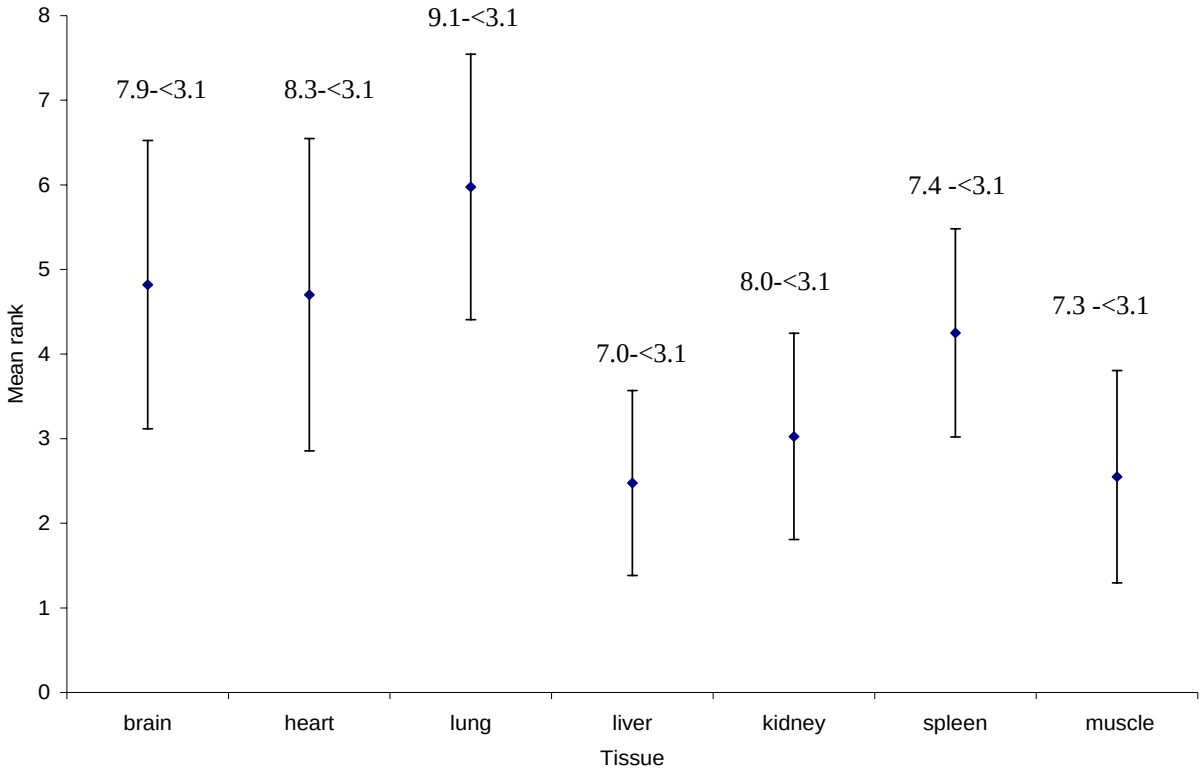
**reticuloenothelial

Table 3.3. Immunohistochemistry (IHC) and reverse transcriptase nested polymerase chain reaction (RT-nPCR) results for eight tissues from 20 blue jays naturally infected with WNV as detected by virus isolation (brain and heart).

	IHC	RT-nPCR
	IHC pos/screen pos	PCR pos/screen pos
Brain	10/20	20/20
Heart	18/20	20/20
Lung	15/20	20/20
Liver	16/20	20/20
Kidney	18/20	20/20
Spleen	12/20	20/20
Muscle	Not done	20/20
Bursa*	Not done	5/5

* For bursa, n=5

Figure 3.1. Comparison of mean rank and standard deviation for West Nile virus titers in seven different tissues of blue jays. Tissues were ranked according to West Nile viral titer within each bird, with the highest viral titer obtaining the highest rank. Values above standard deviation bars represent the range of viral titers for each tissue in Log_{10} $TCID_{50}$/ ml.



CHAPTER 4

Persistence of antibodies to West Nile virus in naturally infected rock pigeons (*Columba livia*)[1]

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[1]Gibbs SEJ, Hoffman DM, Stark LM, Marlenee NL, Blitvich BJ, Beaty BJ, Stallknecht DE. 2005. *Clinical and Diagnostic Laboratory Immunology* 12(5):665-7. Reprinted here with permission of the publisher.

Abstract

Wild caught rock pigeons (*Columba livia*) with antibodies to West Nile virus were monitored for 15 months to determine antibody persistence and compare results of three serologic techniques. Antibodies persisted for the entire study as detected by epitope-blocking enzyme-linked immunosorbent assay and plaque reduction neutralization test. Maternal antibodies in squabs derived from seropositive birds persisted for an average of 27 days.

Introduction

West Nile virus (WNV) (*Flaviviridae* family, *Flavivirus* genus) is maintained in a bird-mosquito transmission cycle, and wild bird surveillance has proven effective in tracking the spread of this virus in North America. Since extensive avian mortality has been associated with WNV infection in North America, much of this surveillance has concentrated on dead bird testing (16). As demonstrated with other arboviruses, such as St. Louis encephalitis virus (SLEV), eastern equine encephalitis virus, and western equine encephalitis virus, serologic testing of birds represents another tool for further investigating WNV epidemiology (7, 8, 14). The duration of the antibody response, test performance, and persistence of maternal antibodies can complicate interpretation of serologic results. Information on the persistence of antibodies to WNV in avian species is currently limited. Experimentally, persistence of neutralizing antibodies to the North American strain of WNV in rock pigeons (*Columba livia*) was demonstrated over a 9-week period postinoculation and in chickens over a 28-day period postinoculation (9, 11). Pigeons inoculated with an African strain of WNV maintained antibodies for 16 months (12). Recaptured naturally infected wild birds in South Africa with initial WNV antibody titers of > 40 lost demonstrable antibody by hemagglutination inhibition (HAI) in as few as 3 weeks (13).

The objectives of this study were the following: (i) to determine the long-term persistence of antibodies to WNV in naturally infected rock pigeons, (ii) to compare the long-term utility of commonly used WNV serologic techniques (plaque reduction neutralization test [PRNT], HAI, and epitope-blocking enzyme-linked immunosorbent assay [ELISA]), and (iii) to determine the persistence of maternal antibodies to WNV in squabs derived from these naturally infected birds.

Materials and Methods

Thirty rock pigeons, 20 seropositive for WNV and 10 negative controls, were captured in April 2003 in Atlanta, Georgia. All birds were banded and housed in a mosquito-free facility for 60 weeks. Venipuncture was performed on each bird upon entry and at 3-week intervals by wing vein. Serum samples were stored at _70°C. Using WNV (Georgia isolate DES-107-01) and SLEV (strain TBH-28), PRNTs were performed following standard protocols (1, 10). Titers were expressed as the reciprocal of serum dilutions reducing the number of plaques_90% (PRNT90). Samples with PRNT90 titers to WNV which were fourfold greater than titers to SLEV were considered seropositive for WNV.

HAI assays were performed at the Florida Department of Health using a published protocol (5). The antigen used for HAI, SLEV (TBH-28), was prepared by following the sucroseacetone procedure (4). Epitope-blocking ELISAs were performed using the WNV-specific monoclonal antibody (MAb) 3.1112G (Chemicon International, Inc., Temecula, CA) and the flavivirus-specific MAb 6B6C-1 (provided by the Centers for Disease Control and Prevention, Fort Collins, CO) as previously described (2). MAb 3.1112G detects an NS1 protein epitope; MAb 6B6C-1 detects an envelope protein epitope.

All serum samples collected over the 60-week period were tested by PRNT using WNV to determine persistence and antibody titers. Serum samples collected on day 0 were also tested by PRNT using SLEV. To compare antibody persistence as measured by PRNT, HAI, and epitope-blocking ELISA, a subset of samples collected from five positive birds on day 0 to week 45 were tested with all three serologic tests. To compare performance of serologic assays, a second subset consisting of samples collected from all birds on weeks 3, 24, and 45 were tested by PRNT, HAI, and epitope-blocking ELISA.

Concordance of results was determined using the Kappa statistic (15). Persistence of maternal antibodies to WNV was determined in five squabs that hatched during the study. Blood samples were taken from all squabs 8 days after hatching and every several days thereafter until 6 weeks of age. PRNT was used to test all samples.

Results

The 20 birds that had antibodies to WNV at the time of capture remained antibody positive during the 60-week study period; the 10 control birds that had no detectable antibody toWNV remained antibody negative (data not shown). From the first subset of samples, antibodies to flaviviruses were detected in two of the five PRNT-positive birds by HAI and in all five birds by ELISA at all time points tested (Table 1).

The initial WNV $PRNT_{90}$ titers ranged from 40 to 640 for the 20 birds that had antibodies to WNV. $PRNT_{90}$ titers for 16 of these birds did not vary by more than a twofold dilution throughout the 60-week testing period. The titers for the remaining four pigeons varied only fourfold (two dilutions). The 60-week $PRNT_{90}$ titers for 18 birds were within a twofold dilution

of the day-zero titer. The two HAI-positive samples in the first subset remained positive, with a steadily decreasing trend in HAI titer.

Comparative results for serologic assays are shown in Table 2. HAI results were inconsistent with PRNT results (kappa = 0.14) (a kappa value of 0.8 to 1.0 indicates almost perfect agreement between tests). While good agreement was observed between ELISA (when positive with both MAbs) andPRNT results (kappa = 0.91), agreement improved slightly (kappa = 0.95) when results were considered positive by either MAb.

Neutralizing maternal antibodies to WNV in the squabs lasted for an average of 27 days (Table 3).

Discussion

The pigeons used in this study were naturally infected field-collected birds. The dates of WNV infection are therefore unknown, and an absolute estimate of antibody persistence could not be determined. This study has shown, however, that the minimum duration of persistence of antibody to WNV in rock pigeons is 15 months, there is little long-term variation in antibody titers, and there is no serological evidence of viral recrudescence. Based on these findings, the population immunity to WNV can be expected to increase as WNV establishes itself in North America.

The consistency of antibody titers observed over time during this study contrasts with the findings for experimentally infected pigeons (9). The antibody responses of those birds reflect an acute post-infection immunologic response, while the present study most likely reflects older infections. As a result, a direct comparison cannot be made between the two studies.

The persistence of antibodies to WNV in an avian species for more than a year complicates interpretation of multiyear studies involving serologic surveillance of wild bird populations. Because the antibody titers in this study remained at high levels, it suggests that pigeons maintain neutralizing-antibody titers to WNV for several years. Seroprevalence of WNV in avian populations may therefore increase while transmission of the virus in an area remains stable over time. Because species variation in the persistence of antibodies to WNV may exist, antibody persistence in other avian species should be evaluated.

The results in this study proved to be highly test dependent, and serologic results should be interpreted with this in mind. The HAI test was not as effective as the PRNT or ELISA in this study. Neutralizing antibodies are generally considered to persist longer than HAI antibodies, however, so the results of this study may reflect differences in the timing of infection in individual birds (3). Those pigeons positive by HAI in this study potentially represent more-recent infections. Additionally, the HAI assay was performed using SLEV antigen as a flavivirus group reactive antigen, rather than a specific WNV antigen, which may have affected test sensitivity.

To our knowledge, this is the first report detailing the persistence of avian maternal antibodies to the North American strain of WNV. Columbiformes are unique in that in addition to the maternal antibodies transferred through the egg yolk, they receive both maternal and paternal antibodies through crop milk after hatching. Immunoglobulin A and immunoglobulin G antibodies are present in the crop milk and are absorbed by 1-day-old squabs; further transfer of antibodies past day 1 appears to be limited (6).

The role of nestlings in WNV amplification cycles may be reduced by maternal-antibody persistence. In the case of pigeons, the additional opportunity for transfer of passive immunity

from not only the hen but also the cock increases the proportion of squabs with resistance to WNV infection. How maternal antibody persistence in pigeons compares to that in indigenous North American avian species is unknown. When determined, this information will help to elucidate variations in WNV disease resistance among avian populations.

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Table 4.1. Persistence of antibodies to WNV in naturally infected feral pigeons: reciprocal antibody titers determined by plaque reduction neutralization test (PRNT). Samples from five birds were also tested with hemagglutination inhibition (HI) and epitope-blocking enzyme linked immunosorbent assay (ELISA).

Bird ID		Week																			
		0	3	6	9	12	15	18	21	24	27	30	33	36	39	42	45	48	51	54	60
13	PRNT	80†	160	80	160	160	160	160	80	160	160	160	160	160	160	160	160	160	160	160	80
	ELISA	+/+*	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+				
	HAI	<10†	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10				
31	PRNT	40	80	40	80	40	80	80	80	80	40	40	80	80	80	80	80	40	80	80	40
	ELISA	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+				
	HAI	80	80	80	80	40	40	40	40	80	40	40	40	40	20	40	20				
42	PRNT	640	640	640	1280	1280	1280	1280	640	640	640	320	640	640	320	640	640	640	640	640	640
	ELISA	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+				
	HAI	20	20	20	10	20	20	10	10	10	10	10	10	10	10	10	10				
192	PRNT	80	160	160	160	160	80	80	160	160	160	160	160	160	160	160	160	160	160	160	160
	ELISA	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+				
	HAI	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10				
200	PRNT	80	160	160	160	160	160	160	160	160	80	80	80	80	160	160	160	80	160	80	160
	ELISA	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+				
	HAI	<10	10	10	10	10	10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10				
15	PRNT	80	160	160	160	160	160	160	160	160	160	160	320	160	160	160	160	160	160	160	160
24	PRNT	80	320	320	320	320	160	160	160	160	320	160	160	160	80	160	160	80	80	80	80
34	PRNT	160	160	320	320	320	160	160	160	160	80	160	160	160	160	80	80	80	80	80	160
43	PRNT	320	320	640	640	640	640	640	640	640	640	320	640	640	320	320	320	640	640	320	320
179	PRNT	640	640	640	640	640	320	640	640	640	640	160	640	640	640	320	640	640	640	640	640
180	PRNT	40	40	80	80	80	80	80	80	80	80	80	40	80	80	40	40	40	40	40	40
181	PRNT	320	320	160	640	320	320	160	160	320	320	160	160	160	320	320	160	160	160	160	160

185	PRNT	320	320	320	320	320	320	320	320	320	320	160	320	320	320	320	320	320	320	320	320
186	PRNT	320	320	320	320	320	160	160	160	160	160	160	160	320	160	320	320	160	160	320	320
187	PRNT	320	320	640	640	640	320	320	320	640	320	640	640	320	320	640	640	320	320	320	320
189	PRNT	40	40	40	80	160	40	40	40	40	20	20	10	20	20	20	10	20	20	10	10
190	PRNT	80	160	160	160	160	80	80	80	160	80	320	320	160	320	320	320	320	320	320	640
194	PRNT	320	320	320	320	160	160	160	320	320	320	640	640	640	320	320	320	320	320	160	320
196	PRNT	80	160	320	320	320	160	160	80	80	80	160	80	80	80	80	80	160	160	80	160
199	PRNT	80	160	80	80	80	80	80	160	160	160	80	160	160	160	160	160	80	160	160	160

† PRNT (90%) and HI titer

***** Results for monoclonal antibodies: 6B6C-1 / 3.1112G

Table 4.2. Comparison of serologic assays: plaque reduction neutralization test (PRNT), hemagglutination inhibition (HI), and epitope-blocking enzyme linked immunosorbent assay (ELISA) in determining flavivirus antibody titer or status of naturally infected feral pigeons

Bird ID	**Week**								
	3			**24**			**45**		
	PRNT	**ELISA**	**HI**	**PRNT**	**ELISA**	**HI**	**PRNT**	**ELISA**	**HI**
1	-†	-/-*****	-†*	-	-/-	-	-	-/-	-
5	-	-/-	-	-	-/-	-	-	-/-	-
13	160	+/+	-	160	+/+	-	160	+/+	-
15	160	+/+	-	160	+/+	-	160	+/+	-
24	320	+/+	-	160	+/+	-	160	+/+	-
26	-	-/-	-	-	-/-	-	-	-/-	-
31	80	+/+	80	80	+/+	80	80	+/+	20
34	160	+/+	-	160	+/+	-	80	+/+	-
37	-	-/-	-	-	-/-	-	-	-/-	-
39	-	-/-	-	-	-/-	-	-	-/-	-
41	-	-/-	-	-	-/-	-	-	-/-	-
42	640	+/+	20	640	+/+	10	640	+/+	10
43	320	+/+	-	640	+/+	-	320	+/+	-
178	-	-/-	-	-	-/-	-	-	-/-	-
179	640	+/+	10	640	+/+	10	640	+/+	-
180	40	+/+	-	80	+/+	-	40	+/+	-
181	320	+/-	-	320	-/-	-	160	+/-	-
185	320	+/+	80	320	+/+	40	320	+/+	20
186	320	+/+	-	160	+/+	-	320	+/-	-
187	320	+/+	-	640	+/+	10	640	+/+	0
188	-	-/-	-	-	-/-	-	-	-/-	-
189	40	+/+	-	40	+/+	-	-	+/-	-
190	160	+/+	-	160	+/+	-	320	+/+	-
192	160	+/+	-	160	+/+	-	160	+/+	-
194	320	+/+	-	320	+/+	-	320	+/+	-
196	160	+/+	-	80	+/+	-	80	+/+	-
197	-	-/-	-	-	-/-	-	-	-/-	-
199	160	+/+	-	160	+/+	-	160	+/+	-
200	160	+/+	10	160	+/+	-	160	+/+	-

† PRNT and HI titer <10,

***** Results for monoclonal antibodies: 6B6C-1 / 3.1112G

Table 4.3. Persistence of maternal antibodies to WNV in squabs of naturally infected feral pigeons.

Bird ID	Date of birth	Bleed date								Duration of antibody persistence
		6/10	6/14	6/18	6/21	6/24	6/29	7/2	7/12	
56	6/2	10†	10	10	10	<10	<10	<10	<10	19
57	6/2	20	20	20	20	10	<10	<10	<10	22
61	5/30	20	40	20	20	20	20	20	<10	33
62	5/30	40	20	20	20	10	10	<10	<10	30
58	6/2	40	40	40	40	20	10	10	<10	30

† 90% plaque reduction neutralization test (PRNT) titer

CHAPTER 5

West Nile virus antibodies in avian species of Georgia, USA: 2000 – 2004[1]

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[1]Gibbs SEJ, Allison AB, Yabsley MJ, Mead DG, Wilcox BR, Stallknecht DE. To be submitted to *Vector Borne and Zoonotic Diseases*.

ABSTRACT

West Nile virus (WNV) was first isolated in the state of Georgia in the summer of 2001. Since then, combined surveillance that has included avian, human, and equine cases, and live bird serology has illustrated a nearly complete distribution of WNV across the state. As amplifying hosts of WNV, avian species play an important role in the distribution and epidemiology of the virus. The objective of this study was to identify avian species that are locally involved as potential amplifying hosts of WNV and can serve as indicators of WNV transmission over the physiographic and land use variation present in the southeastern United States.

Avian serum samples (n = 14,077) from 83 species of birds captured throughout Georgia during the summers of 2000 through 2004 were tested by a plaque reduction neutralization test for antibodies to WNV and St. Louis encephalitis virus. Over the five year period, WNV neutralizing antibodies were detected in 869 (6.2%) of the samples. The seroprevalence rates increased significantly throughout the study and were species dependent. The highest antibody prevalence rates were detected in rock pigeons (*Columba livia*), northern cardinals (*Cardinalis cardinalis*), and common ground doves (*Columbina passerina*). Northern cardinals, in addition to having high geometric mean antibody titers and seroprevalence rates, were commonly found in all land use types and physiographic regions. Rock pigeons and common ground doves, although also having high seroprevalence rates and high antibody titers against WNV, were more restricted in their distribution and therefore may be of more utility when attempting to assess exposure rates in specific habitat types. Of all species tested, northern cardinals represent the best potential avian indicator species for widespread serologic-based studies of WNV throughout Georgia due to their extensive range, ease of capture, and high antibody rates and titers. Due to

the great geographic area covered by this species, their utility as a WNV sentinel species may include a large proportion of the United States.

KEY WORDS: avian, indicator, Georgia, land use, physiographic region, plaque reduction neutralization test, PRNT, serology, West Nile virus, WNV

INTRODUCTION

While dead bird testing has been the primary focus of avian-based West Nile virus (WNV) surveillance across the United States (U.S.), this approach can be biased by varying degrees of public interest, human population density, and surveillance infrastructure (Komar, 2001a; Ward et al. 2005). Many of the deficiencies inherent in dead bird surveillance systems can be avoided through active collection and testing of live birds for serum antibodies. As demonstrated with other North American arboviruses such as St. Louis encephalitis virus (SLEV) and western equine encephalitis virus (WEEV), serologic testing of avian species represents an important approach for investigating virus ecology and epidemiology (Gruwell et al. 2000; Reisen et al. 2000; Monath and Tsai, 1987; Day and Stark, 1999).

Wild birds have been used in serologic-based studies of WNV in all global regions where this virus is present. In Egypt, high WNV neutralizing antibody titers found in hooded crows (*Corvus corone sardonius*) and house sparrows (*Passer domesticus*) suggested that these species could be used as natural sentinels for monitoring virus transmission (Taylor et al. 1956). In South Africa, the utility of this approach was demonstrated by the finding that birds associated with water had the highest antibody prevalence as determined by hemagglutination inhibition tests (HAI) (McIntosh et al. 1968). In Europe, from the early 1960's to present, serologic testing of wild birds for WNV antibodies has been used to support outbreak investigations and to better understand the epidemiology of this virus (Juricova et al. 1998; Savage et al. 1999; Ternovoi et al. 2004; L'vov et al. 2004; Banet-Noach et al. 2004, Buckley et al. 2003).

After WNV was discovered in the U.S., several serological surveys of wild birds were conducted. The first centered on the site surrounding initial human cases in and adjacent to Queens, New York (Komar et al. 2001c). In this study, it was determined that house sparrows

and rock pigeons (*Columba livia*) would be good candidates for WNV sentinels based on a relatively high prevalence of neutralizing antibodies and may be used to identify areas where WNV transmission was high. A second study was conducted on nearby Staten Island, New York in the summer of 2000 (Komar et al. 2001b). Species with the highest prevalence of WNV antibodies in this study were the northern cardinal (*Cardinalis cardinalis*) 69.2% (*n*=13), and the rock pigeon 54.5% (*n*=55), while only 8.6% (*n*=93) of house sparrows tested seropositive. The high antibody prevalence in nonmigrant species suggested that these birds might play a role in the local transmission cycle and indicated that these species could survive WNV infection.

Serologic-based surveillance was also used to follow WNV as it moved into Mexico and the Caribbean, where dead bird surveillance is not as practical. Antibodies to WNV have been detected in resident and migratory birds in Mexico and Guadeloupe (Farafan-Ale et al. 2004; Quirin et al. 2004).

Serologic surveillance for WNV antibodies in wild avian species (especially non-migratory birds) can be used to: 1) identify areas of virus transmission, either in support of or in the absence of other WNV surveillance strategies such as dead bird or mosquito testing, 2) identify areas of potential human exposure to these viruses (a critical component of a human risk assessment), and 3) provide direction and field validation for more controlled experimental studies needed to determine specific host/vector/virus relationships. Avian species in North America exhibit a wide range of susceptibility to infection and disease caused by WNV (Steele et al. 2000; Komar et al. 2003). Consequently, identifying effective indicator species would serve to maximize the efficiency and effectiveness of future field studies and surveillance work designed to enhance understanding of the long-term temporal and spatial patterns of WNV occurring in North America.

Georgia represents an excellent location in which to study the regional epidemiology of WNV. Georgia is strategically located on the Atlantic flyway and has proven to be in the immediate path for the southern expansion of WNV by migratory birds. The state includes physiographic (coastal, coastal plain, piedmont, and mountain) and land use (agriculture, forested, urban suburban, and wetland) diversity characteristic of most of the southeastern U.S. Physiographic variation ranges from subtropical barrier islands on the Atlantic coast to the Appalachian mountains in the north, and with few exceptions, the physiographic diversity present throughout the southeast is reflected in this single state. Likewise, land use patterns also exhibit the regional diversity that is present (and rapidly changing) in the southeast. This ranges from large areas of natural and industrial forests, to areas of intensive animal and plant agriculture, to rapidly expanding urban and suburban environments.

The objective of this study was to identify avian species that are potentially involved in the epidemiology of WNV and that may serve as indicators for WNV over the physiographic and land use variation present in Georgia with evaluation of applicability to the southeastern United States.

MATERIALS and METHODS

Site selection

Study sites were chosen with the purpose of achieving state-wide coverage, including all land-use types and physiographic regions. Specific study sites were selected based on landowners' permission, opportunistic sampling (e.g. in coordination with nuisance bird removal programs), and areas with documented high concentrations of birds. Study sites ranged from

private suburban residences to national forests, agricultural lands, municipal dumps, urban industrial sites, and wildlife management areas.

Bird capture and sample collection

Birds were captured by standard methods including mist net, rocket net, whoosh net and walk-in trap. All birds were temporarily marked with India ink applied to the feathers on the head to avoid re-sampling. Blood was collected by jugular, brachial, or medial metatarsal venipuncture. All birds were released after blood was drawn. Blood samples were transferred to Microtainer serum separator tubes (Becton Dickinson, Franklin Lanes, New Jersey, USA) and allowed to sit at ambient temperature for at least 30 minutes. Tubes were then centrifuged at 5,000 G and the serum was removed and stored on ice in the field and then transferred to -20˚C until testing.

During the year 2000 and part of 2001 (i.e. prior to detection of WNV in Georgia), bird capture, sample collection, and serologic testing were performed as part of the Georgia WNV surveillance program. For these two collection periods, only species and location data were recorded for the samples. In 2002-2004, the species and age (hatch year vs. adult) of each bird, based on morphologic characteristics (Sibley, 2000; Pyle, 1997), were recorded in addition to global positioning system (GPS) coordinates and a classification of the local land use type and physiographic region for each site. Birds tested in all five years were captured between May and October to maximize sampling of non-migrants.

Antibody testing

Plaque reduction neutralization tests (PRNT) were performed to determine the presence of WNV neutralizing antibodies. Using WNV (Georgia isolate DES-107-01) and SLEV (strain TBH-28), PRNTs were performed as described by Allison (2004). Titers were expressed as the

reciprocal of serum dilutions reducing the number of plaques >90% ($PRNT_{90}$). Samples with $PRNT_{90}$ titers to WNV which were fourfold greater than titers to SLEV, and samples with a WNV titer $\geq$ 10 with no SLEV titer, were considered seropositive for WNV.

Statistical analysis of data

Variables included physiographic region (coastal, coastal plain, piedmont, and mountain), land use (agriculture, forest, urban/suburban, and wetland), county, year collected, age of the bird, species, weight, avian family, habitat, flocking behavior, and feeding habits. Variables potentially affecting seroprevalence were analyzed for significance using Chi square and t-test analyses. Logistic regression was then performed with SAS 8.2 (SAS Institute, Inc., Cary, North Carolina, USA) using the serologic test result (positive or negative for WNV antibodies) of each avian sample as the dependent variable. A forward stepwise procedure with a $p<0.05$ was used to determine which of the variables maximized the fit of the model. Models were first constructed using all data from 2002-2004, then each year was analyzed individually. Finally, data for northern cardinals and rock pigeons were evaluated individually for all three years. To provide a finer scale related to physiographic region, the mountain region was divided into east and west sections to determine whether the differing geologic characteristics (such as elevation and depth of valleys) between these areas influenced WNV antibody prevalence.

RESULTS

Seroprevalence

From 2000 to 2004, a total of 14,077 serum samples were collected from birds captured in 151 counties in the state of Georgia. Locations for samples collected in 2000 (n = 1,450), 2001 (n = 1,762), 2002 (n = 2,703), 2003 (n = 4,009), and 2004 (n = 4,153) are shown in Figure 5.1.

The statewide WNV antibody prevalence for all species combined increased from 0.8% in 2000 to 10.1% in 2004. Of the 83 avian species represented in the collection over the five years, antibodies to WNV were detected in 37 (44.6%).

Antibody prevalence rates for selected peridomestic species as well as for all species combined are shown in Table 5.1. The species in this table were selected for presentation based on their peridomestic behavior or high antibody prevalence. Northern cardinal samples represented 29.8%, 50.0%, and 83.2% of the total positive bird samples and 19.3%, 24.3%, and 31.8% of the total bird samples collected in 2002, 2003, and 2004 respectively. Rock pigeons, northern cardinals, and common ground doves (*Columbina passerina*) had the highest seroprevalence rates for WNV during those three years. These results led us to further explore the suitability of these three species as indicators of WNV.

Three samples were positive for SLEV antibodies; two of these were from rock pigeons (one in 2003 and one in 2004) and the third was from a little blue heron *(Egretta caerulea)* sampled in 2004.

Species data for 2000-2001

Species positive for antibodies to WNV and the months seropositives were obtained in 2000 and 2001 include (number positive/ number sampled/month -year): American coot (*Fulica americana*) (3 /60, November 2000); American crow (*Corvus brachyrhynchos*) (6/230, May, July, and August 2000; May and July 2001); boat-tailed grackle (*Quiscalus major*) (2 /110, August 2000); cattle egret (*Bubulcus ibis*) (1 /101, July 2001); house sparrow (1/15, July 2001); laughing gull (*Larus atricilla*) (2 /93, May 2001); northern cardinal (1 /182, August 2001); ring-billed gull (*Larus delawarensis*) (1 /92, December 2000); and rock pigeon (2 /317, June and August 2000).

Species negative for antibodies to WNV in 2000 and 2001 that are not listed in Table 1 include: American goldfinch (*Carduelis tristis*) (*n*=1), American oystercatcher (*Haematopus palliates*) (*n*=9), American robin (*Turdus migratorius*) (*n*=3), barn swallow (*Hirundo rustica*) (*n*=1), black vulture (*Coragyps atratus*) (*n*=3), blue grosbeak (*Guiraca caerulea*) (*n*=1), blue jay (*Cyanocitta cristata*) (*n*=17), brown headed cowbird (*Molothrus ater*) (*n*=54), Carolina wren (*Thryothorus ludoviciantus*) (*n*=3), chipping sparrow (*Spizella passerina*) (*n*=1), common grackle (*Quiscalus quiscula*) (*n*=13), domestic chicken (*Gallus gallus*) (*n*=10), eastern bluebird (*Sialia sialis*) (*n*=5), eastern phoebe (*Sayornis phoebe*) (*n*=1), eastern screech owl (*Otus asio*) (*n*=1), eastern towhee (*Pipilo erythropthalmus*) (n=6), Eurasian collared dove (*Streptopelia decaocto*) (*n*=4), European starling (*Sturnus vulgaris*) (*n*=6), fish crow (*Corvus ossifragus*) (*n*=39), great-crested flycatcher (*Myiarchus crinitus*) (*n*=4), great egret (*Ardea alba*) (*n*=1), hermit thrush (*Catharus guttatus*) (*n*=1), herring gull (*Larus argentatus*) (*n*=7), indigo bunting (*Passerina cyanea*) (*n*=1), little blue heron (*n*=1), loggerhead shrike (*Lanius ludovicianus*) (*n*=1), mallard (*Anas platyrhnchos*) (*n*=8), marbled godwit (*Limosa fedoa*) (*n*=3), mourning dove (*Zenaida macroura* (*n*=28), red-bellied woodpecker (*Melanerpes carolinus*) (*n*=4), red-winged blackbird (*Agelaius phoeniceus*) (*n*=14), red-eyed vireo (*Vireo olivaceus*) (*n*=2), scarlet tanager (*Piranga olivacea*) (*n*=1), summer tanager (*Piranga rubra*) (*n*=1), Swainson's thrush (*Catharus ustulatus*) (*n*=1), turkey vulture (*Cathartes aura*) (*n*=1), white-throated sparrow (*Zonotrichia albicollis*) (*n*=16), wood duck (*Aix sponsa*) (*n*=1), wood stork (*Mycteria americana*) (*n*=15), wood thrush (*Hylocichla mustelina*) (*n*=1), yellow-bellied sapsucker (*Sphyrapicus varius*) (*n*=2), and yellow-breasted chat (*Icteria virens*) (*n*=2).

Species data for 2002 through 2004

Species seropositive for WNV that are not included in Table 1 due to low seroprevalence or sample size are (number positive/number sampled): American crow (3/31), American robin (11/150), blue jay (14/323), boat tailed grackle (1/77), brown headed cowbird (9/494), Carolina wren (1/83), common grackle (1/182), Cooper's hawk (*Accipiter cooperii)* (1/1), eastern bluebird (3/126), eastern meadowlark (*Sturnella magna*) (1/1), eastern towhee (*Pipilo erythrophthalmus)* (19/197), Eurasian collared dove (1/5), fish crow (3/12), great crested flycatcher (1/50), loggerhead shrike (1/9), mourning dove (30/243), orchard oriole (*Icterus spurious*) (3/76), pileated woodpecker (*Dryocopus pileatus*) (1/5), red bellied woodpecker (3/165), red winged blackbird (3/253), scarlet tanager (2/71), summer tanager (6/68), white breasted nuthatch (*Sitta carolinensis*) (1/35), white throated sparrow (1/23), wood thrush (5/32), yellow breasted chat (2/28), and yellow-billed cuckoo (*Coccyzus americanus*) (1/1).

Individuals of the following species were negative for WNV antibodies: American oystercatcher (*n*=28), barn swallow (*n*=6), barred owl (*Strix varia*) (*n*=1), blue grosbeak (*n*=45), cedar waxwing (*n*=1), dark eyed junco (*n*=4), downy woodpecker (*Picoides pubescens*) (*n*=41), eastern kingbird (*Tyrannus tyrannus*) (*n*=8), eastern phoebe (*n*=18), eastern wood-pewee (*Contopus virens*) (*n*=2), european starling (*n*=116), great horned owl (*Bubo virginianus*) (*n*=2), guinea fowl (*Numida meleagris*) (*n*=1), hairy woodpecker (*Picoides villosus*) (*n*=1), hermit thrush (*n*=5), killdeer (*Charadrius vociferous*) (*n*=3), little blue heron (*n*=1), Louisiana waterthrush (*Seiurus motacilla*) (*n*=1), mallard (*n*=3), marbled godwit (*n*=5), muscovy duck (*Cairina moschata*) (*n*=4), northern bobwhite (*Colinus virginianus*) (*n*=3), northern flicker (*Colaptes auratus*) (*n*=5), northern waterthrush (*Seiurus noveboracensis*) (*n*=1), ovenbird (*Seiurus aurocapillus*) (*n*=3), prothonotary warbler (*Protonotaria citrea*) (*n*=1), purple martin

(*Progne subis*) (*n*=3), red-eyed vireo (*n*=4), red-headed woodpecker (*Melanerpes erythrocephalus*) (*n*=12), red-shouldered hawk (*Buteo lineatus*) (*n*=1), ring-billed gull (*n*=3), rose-breasted grosbeak (*Pheucticus ludovicianus*) (*n*=1), song sparrow (*Melospiza melodia*) (*n*=60), veery (*Catharus fuscescens*) (*n*=1), willet (*Catoptrophorus semipalmatus*) (*n*=1), wood duck (*n*=1), and yellow-bellied sapsucker (*n*=1).

Antibody titers

Antibody titers against WNV ranged from 10 to >640 (Figure 5.2). The geometric mean titer for WNV antibodies was 133.6 in rock pigeons, 91.1 in northern cardinals, and 68.4 in common ground doves.

Land use

Birds with antibodies to WNV were found in each land use type (Table 5.2). The urban/suburban and agriculture land use types were most highly represented in the sampling process, while wetland was the least represented. Northern cardinals were well-distributed across agriculture, urban/suburban, and forest land use types, with fewer caught in wetlands; rock pigeon samples were heavily concentrated in the urban/suburban areas; and common ground doves were captured in a variety of land use types depending on the year of the study. Seroprevalence rates were highest for rock pigeons in the urban/suburban landscape. For the northern cardinal, antibody prevalence significantly differed across land use types only in 2002 ($p \leq 0.025$). Seroprevalence was significantly higher in northern cardinals than all species combined for 2002-2004 in agriculture, forested, and urban/suburban land use types ($p \leq 0.001$).

Physiographic region

Birds with WNV antibodies were captured in counties in each physiographic region of Georgia (Fig 5.1). Prevalence of WNV antibodies for 2002-2004 in northern cardinals was dependent upon physiographic region ($p \leq 0.01$) (Table 5.3, Fig 5.3b); however, for all avian species combined, physiographic region was significant only in 2002 ($p \leq 0.01$) (Table 5.3, Fig 5.3a). Prevalence of WNV antibodies was significantly higher in northern cardinals than all species combined for coastal plain ($p \leq 0.001$), mountain ($p \leq 0.001$), and piedmont ($p \leq 0.001$) physiographic regions, but differences were not detected for the coastal region ($p \leq 1$). The seroprevalence for all bird species combined in the eastern section of the mountain region for 2002-2004 was significantly lower than the rest of the state and significantly lower than the western section of the mountain region for 2003 and 2004 ($p < 0.001$).

Age and behavior of birds

Prevalence of WNV antibodies in hatch year and adult birds for 2002-2004 are listed in Table 5.4. In northern cardinals, the adult to hatch year ratios were 2:1, 2:1, and 1:1 for 2002, 2003, and 2004, respectively (not listed in table).

Weight, family, habitat, flocking behavior, and feeding habits were not significant factors in determining WNV seroprevalence.

Logistic regression analysis

Logistic regression analysis revealed a strong relationship between WNV antibody prevalence and year, age, and land use variables (Table 5.5). Age and species were important variables in 2002-2004, and land use was significant but less important than age and species in 2002 for determining serologic status. Year, age, and region were all highly correlated with

positive WNV serologic status in northern cardinals, however, the association was stronger with year and age than with region. For the rock pigeon, there was a moderate relationship between positive samples and age, land, and county variables.

DISCUSSION

Northern cardinals meet all of the criteria for an excellent avian indicator. As applied to serologic-based epidemiologic studies, characteristics defining a reliable avian indicator for WNV would include: 1) a widespread distribution ; 2) a range that includes and allows for detection of potentially important ecological variables such as physiographic regions and land use types; 3.) a close association with humans (i.e. peridomestic); 4) an abundance and behavior that facilitates sampling; 5) a detectable antibody response following infection; and 6) an ability to survive WNV infection. Similar guidelines have been suggested for studies conducted in New York (Komar, 2001a). Northern cardinals are distributed throughout the entire southeastern United States, are found in all physiographic regions and land-use types, are closely associated with humans, are easy to catch using mist nets, have a high seroprevalence rate, and develop a strong antibody response to WNV. The habitat flexibility and abundance of northern cardinals also makes them excellent candidates for indicators.

While rock doves and common ground doves fulfilled many of the requirements for an avian indicator, these species were poorly distributed across the landscape and may have more utility as indicators in very localized areas. This is illustrated by the extraordinarily high WNV seroprevalence in rock pigeons in urban centers in Georgia. Variation in seroprevalence within these urban areas however can be extreme; rock pigeons captured in Charleston, South Carolina, had no antibodies to WNV, while those captured in Savannah, Georgia averaged 25%

seroprevalence (Gibbs, unpublished data). In smaller cities around Georgia, seroprevalence rates in rock pigeons ranged from zero to 10%. This variation may be associated with very localized habitat conditions affecting vector abundance. Considering the human populations associated with these urban areas, the potential utility of this species in such settings warrants additional study.

The WNV reservoir competence of 25 North American avian species has been assessed in experimental studies (Komar et al. 2003), and based on the susceptibility to infection and the level and duration of viremia, blue jay, common grackle, house finch, American crow, and house sparrow had the highest reservoir competence. Mortality rates in these species were 75% (n=4) for blue jays, 33% (n=6) for common grackles, 100% (n=2) for house finches, 100% (n=8) for American crows, and 50% (n=6) for house sparrows. In the current study, members of each of these five species were found to be WNV antibody positive. Seroprevalence levels for the common grackle, house finch, and house sparrow were extremely low ($\leq$2.0%), and were moderate for the blue jay and American crow (4.3 and 9.7%). These prevalence estimates do not appear to reflect the trend in mortality rates observed by Komar et al. (2003).

The highest seroprevalence in the current study was observed in rock pigeons, which have been shown to circulate WNV viremia titers in excess of 10^5 plaque forming units/mL during natural infection (Allison et al. 2004), and are predominately refractory to experimentally induced disease (Komar et al. 2003). Reservoir competence has not been determined for northern cardinals, common ground doves, and many of the other bird species that were seropositive in this study. As with WNV-related mortality, host competency represents another area where supporting data are needed to fully understand the avian component of WNV epidemiology.

The increase in WNV antibody prevalence from 2001 (0.4%) to 2002 (5.2%) is consistent with the initial detection of WNV in Georgia during the summer of 2001 (CDC, 2005a). During that year, WNV was isolated from a moribund Cooper's hawk found in the Atlanta area in June (Figure 5.3). The second case, a dead American crow from Lowndes County (south Georgia), was found in July; WNV positive dead birds were consistently reported from this date until November (SCWDS, unpublished data). The low antibody prevalence (0.4%) observed in 2001 may partially reflect the timing of sampling, because 71% of the 1,762 birds serologically tested in that year were sampled prior to July. In addition, the distribution of WNV in Georgia during this initial year, as confirmed by virus isolation from dead birds, appears to have been restricted (Figure 5.3).

The detection of antibodies during 2000 (0.8%) probably resulted from combined or individual effects of limited virus transmission within the state prior to its initial detection in 2001 or the sampling of migrating birds that were infected in the northeastern United States where WNV was first introduced. Antibodies were detected in American crows, boat-tailed grackles, and rock pigeons sampled between June and August 2000. Based on the sample dates, these can be regarded as resident birds. Eight of the 12 WNV antibody positive birds from 2000 were sampled in counties found positive for WNV by dead bird surveillance in 2001. Positive ring billed gulls and American coots were sampled between November and December 2000 and, in this case, could represent seropositive migratory birds. In both cases, however, a very low prevalence of antibodies and the possibility of false positive results restrict interpretation of these data (Koller et al. 1968).

Logistic regression analyses consistently identified year, species, age, and land use as significant variables in determining antibody prevalence (Table 5.4). Year was included as a

significant variable in both the All Variables model, which included results from all species tested from 2002 to 2004, and for the northern cardinal model based on data collected during these same years. The inclusion of the year variable is not surprising considering that data were collected from the three years immediately following the initial detection of WNV in Georgia. However, the increase in antibody prevalence over the three years was relatively gradual, only increasing 2.1% and 4.8% annually. This slow rate of prevalence increase was not expected considering the introduction of WNV into a naïve avian community and four years of annual transmission. It is possible that these results accurately reflect a low level of endemic WNV transmission occurring in Georgia and this interpretation is supported by relatively few reported human cases (120) of WNV from 2001 to present (GDPH, 2005).

Species was a significant variable in the All Variables, 2002, 2003, and 2004 logistic regression models (Table 5.4). Antibodies to WNV were detected in 37 avian species and antibody prevalence varied greatly among species. Results of this study were similar to those seen in New York and California in that, of the species tested, rock pigeons and northern cardinals had the highest seroprevalence rates against WNV (Komar et al. 2001b; Reisen et al. 2004; Godsey et al. 2005). The reasons for the host prevalence differences observed between rock pigeons/northern cardinals and other avian species may be related to: 1) differences in host susceptibility and mortality; 2) species specific selection by vector species; 3) species specific utilization of land use types or habitats that enhance vector contact, or 4) species specific variation in immune response.

Individual species vary greatly with regard to their susceptibility to WNV induced disease. Mortality in experimental infections of both wild and domestic birds has ranged from 0 to 100%. Such differential mortality is important with regard to interpretation of serologic data

for various species. For example, because high mortality rates have been reported in both naturally (Eidson et al. 2001a; Eidson et al. 2001b) and experimentally-infected American crows (McLean et al. 2001; Komar et al. 2003), the 7.8% antibody prevalence observed in American crows in 2004 does not necessarily reflect a low infection rate. Relatively few species have been challenged with WNV under experimental conditions (Komar et al. 2003). Species-specific mortality rates for most North American avian species, data which would greatly facilitate the interpretation of serologic data, is therefore unknown. Without this information to support field serologic surveys, the roles of individual species in the epidemiology of WNV cannot be readily determined.

The variation in antibody prevalence observed among species may be influenced by species-related host selection by competent mosquito vectors, the juxtaposition of vectors and hosts through shared habitats, or both. *Culex* species have shown preference for particular avian species (Apperson et al. 2004; Lord and Day, 2000). *Culex pipiens* complex mosquitoes from Tennessee had a strong preference for northern mockingbirds, while *Culex pipiens* in New Jersey and New York had a preference for American robins and tufted titmice (Apperson et al. 2004). Specific host selection was also demonstrated in *Culex nigripalpus* by assessing that vector's tendency to feed on domestic chickens or northern bobwhite (*Colinus virginianus)*(Lord and Day, 2000). In that study, *Cx. nigripalpus* had a strong preference for bird-baited traps containing chickens. The link between the primary mosquito species involved in WNV transmission in the southeastern United States and avian species with high WNV seroprevalence has not yet been explored. Of primary concern in Georgia is *Culex quinquefasciatus,* which is distributed throughout the state and feeds on both mammals and birds (Darsie and Ward, 2005;

Loftin et al. 1997). Determination of which avian hosts this species prefers might help to explain in part seroprevalence differences among wild birds in Georgia.

It is unlikely that the variation in seroprevalence was influenced by differing immune responses. Detectable immune responses have been observed in all experimental infections of birds with WNV. In addition, antibodies to WNV in naturally infected rock pigeons have been shown to persist for greater than 15 months (Gibbs et al. 2005). The higher antibody prevalence in adult than hatch year birds, as well as rise in seroprevalence among adult birds over the three years, also suggest long-term WNV antibody persistence in avian species.

Antibodies were detected in hatch year birds in 2002, 2003, and 2004, indicating that WNV transmission occurred during each year. Although antibody prevalence in hatch year birds increased each year, overall prevalence was low and never exceeded 3.8% for hatch year birds of all species combined or northern cardinals. Increases in antibody prevalence were observed annually in hatch year birds but never exceeded 1% between years. Whether this slight increase may reflect higher rates of maternal antibody transfer or an increased rate of infection cannot be determined. Maternal antibodies to WNV have been shown to persist for up to 33 days in rock pigeons (Gibbs et al. 2005); this information is unknown for other North American species. The low seroprevalence which was observed in hatch year birds might also reflect mortality experienced by hatch year birds.

The inclusion of land use in all logistic regression models except the 2004 model was most likely due to the fact that, by 2004, the distribution of WNV had become complete across the landscape. The inclusion of land use as a significant variable may also have been affected by species distribution. Land use types utilized by the birds in this study varied greatly according to behavioral characteristics and available food sources (e.g. rock pigeons preferred urban areas,

Canada geese preferred wetland environments). Land use was not a significant variable in the logistic regression model for the northern cardinal where the potential confounding effects of species was controlled (Table 5.4). In contrast, land use was included in the rock pigeon model (Table 5.4); it is important to note that this was the only model that also included county as a significant variable. These can both be explained by the localized nature of seropositive results for this species as almost all positive rock pigeons were sampled from a single site in Fulton County (Allison et al. 2004).

Physiographic region was identified as an important variable only in the northern cardinal logistic regression model (Table 5.4) and was most likely a result of the widespread distribution of northern cardinals in comparison to other species. The patchy distribution of other avian species most likely decreased the fit of the physiographic region variable and thus prevented inclusion in the models. The physiographic region differences observed in all birds in 2002 may have resulted from WNV distribution differences associated with virus introduction. As shown in Fig. 5.3a, seroprevalence was highest in the coastal plain and piedmont regions for 2002. This mirrors the clustering of virus isolation positive dead bird cases seen shortly after the initial identification of WNV in the state of Georgia (Fig. 5.3c), suggesting that there were at least two sites of initial virus introduction into the state. The physiographic region variable was not significant in subsequent years (2003, 2004) as WNV became established throughout the state. Differences were detected between physiographic regions in 2003 and 2004 for northern cardinals, with seroprevalence lowest in the coastal area. The reason for this is not understood but could be related to variation in natural vector composition or mosquito control efforts. Northern cardinals were often sampled in suburban settings and many of the coastal counties of Georgia have very active mosquito abatement programs.

In the logistic regression analyses, the mountain physiographic regions (Southern Blue Ridge and Southern Ridge and Valley) were combined. Because the geologic characteristics of these areas are not uniform, additional comparisons were made. The Southern Blue Ridge forms the southern limit of the Great Smoky Group and is characterized by rugged mountains ranging in elevation of 3500-4700 feet, gently rolling valleys up to 2000 feet below the adjacent summits, and steep slopes to the lower elevations in the south (Clark and Zisa, 1976). The Southern Ridge and Valley is characterized by elevations of 700-1600 feet and wide valleys from 50 to 700 feet below mountain ridges (Clark and Zisa, 1976). The seroprevalence in birds captured in the Southern Blue Ridge (2.6%) was significantly lower than in birds tested from the Southern Ridge and Valley (7.1%) ($p \leq 0.01$). This may reflect lesser vector abundance and diminished diversity due to colder temperatures associated with the higher elevations within the Southern Blue Ridge physiographic region.

Serologic surveillance of wild bird species for WNV does not lend itself to prediction of short-term human risk. The strength of dead bird surveillance and mosquito surveillance strategies has been repeatedly proven as an effective strategy for detecting areas of WNV amplification prior to the onset of human cases (Montashari et al. 2003; Guptill et al. 2003; Andreadis et al. 2004). The identification of a serologic avian indicator does, however, provide a potential tool for understanding long-term patterns associated with the establishment of WNV throughout the United States. Such data can also be used to support or supplement other surveillance strategies. Results from this study clearly show a state-wide distribution of WNV in Georgia and annual transmission. Results also demonstrate that risk of infection is associated with land use, but it is important to emphasize that the observed differences in prevalence were minor. We believe that these minor differences relate to the biology of the predominant WNV

vector species (*Cx. quinquefasciatus*) in Georgia, which has a statewide distribution and often is dependent on breeding sites associated with human activity. Such human activity can be directly associated with or in close proximity to any land use type.

Finally, results demonstrate that despite four years of WNV transmission in Georgia, a relatively low proportion of the avian population has immunity to the virus. This immunity to WNV among the avian community in Georgia is not a limiting factor to WNV transmission and considering the slow rate of increase observed in antibody prevalence over these four years, it is possible that antibody prevalence will never reach a level high enough to effectively regulate WNV amplification. This lack of immunity suggests that the enzootic WNV cycle will persist in Georgia and will likely be regulated primarily by vector population dynamics, particularly *C. quinquefasciatus,* which has accounted for the majority of positive mosquito pools statewide (SCWDS, unpublished data). At present, it appears that these mosquito populations are sufficient for annual transmission to birds.

This pattern is not only suggested by the results of this study, by also by a consistent but low incidence of human WNV cases in the state (CDC, 2005a). Further evidence for this proposed pattern is provided by SLEV, which is also vectored by *Cx. quinquefasciatus* (Mitchell et al. 1980). Considering the few birds that tested positive for SLEV antibodies in this study and the few human cases of SLEV that have been historically reported in Georgia (CDC, 2005b), the vector in Georgia appears insufficient to sustain an SLEV epidemic.

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Table 5.1. Antibody prevalence against WNV of selected peridomestic avian species tested by plaque reduction neutralization test (PRNT)

	2000			2001			2002			2003			2004			total		
Species	**n**	**No. pos***	**% pos†**	**n**	**No. pos**	**% pos**	**n**	**No. pos**	**% pos**	**n**	**No. pos**	**% pos**	**n**	**No. pos**	**% pos**	**n**	**No. pos**	**% pos**
Rock pigeon (*Columba livia*)	127	2	1.4	190	0	0	183	59	32.2	179	46	25.7	168	48	28.6	847	155	18.0
Northern Cardinal (*Cardinalis cardinalis*)	30	0	0	152	1	0.6	523	45	8.6	976	146	15.0	1319	251	19.0	3000	443	14.8
Common ground dove (*Columbina passerina*)	0	0	0	5	0	0	2	1	50.0	16	7	43.8	38	7	18.4	61	15	24.6
Grey catbird (*Dumetella carolinensis*)	37	0	0	3	0	0	32	2	6.3	73	8	11.0	119	15	12.6	264	25	9.5
Northern Mockingbird (*Mimus polyglottos*)	5	0	0	14	0	0	85	4	4.7	118	16	13.6	107	12	11.2	329	32	9.7
Brown thrasher (*Toxostoma rufum)*	3	0	0	8	0	0	77	3	3.9	126	4	3.2	113	5	4.4	327	12	3.7
House finch (*Carpodacus mexicanus*)	10	0	0	42	0	0	236	2	0.9	237	5	2.1	454	12	2.6	979	19	1.9
House sparrow (*Passer domesticus*)	0	0	0	15	1	5.0	203	1	0.5	716	14	2.0	123	2	1.6	1057	18	1.7
Tufted titmouse (*Baeolophus bicolor*)	5	0	0	18	0	0	128	1	0.8	149	1	0.7	189	1	0.5	489	3	0.6
Canada goose (*Branta Canadensis*)	789	0	0	782	0	0	486	1	0.2	201	2	1.0	351	5	1.4	2609	8	0.3
All species tested (n=83)‡	1450	12	0.8	1762	7	0.4	2703	140	5.2	4009	292	7.3	4153	418	10.1	14077	869	6.2

* No. pos = number positive

† % pos = percent positive

‡ Note that the number positive for the 10 selected species may not match the number positive for all species since many species with low WNV seroprevalence were not included in the table.

Table 5.2. Land use as determined by site observation for selected peridomestic avian species.

	2002				2003				2004			
Species	**Ag**	**US**	**F**	**W**	**Ag**	**US**	**F**	**W**	**Ag**	**US**	**F**	**W**
Rock pigeon (*Columba livia*)	1/18* (5.6)†	58/164 (35.4)	0/0	0/0	0/10	46/167 (27.5)	0/1	0/0	0/0	48/152 (31.6)	0/2	0/14
Northern cardinal (*Cardinalis cardinalis*)	16/116 (13.8)	13/185 (7.0)	15/151 (9.9)	1/70 (1.4)	36/203 (17.7)	66/409 (16.1)	44/358 (12.3)	0/5	53/319 (16.6)	129/616 (20.9)	68/375 (18.1)	1/9 (11.1)
Common ground dove (*Columbina passerina***)**	1/2 (50.0)	0/0	0/0	0/0	0/1	4/4 (100.0)	3/10 (30.0)	0/1	2/12 (16.7)	3/15 (20.0)	2/11 (18.2)	0/0
Grey catbird (*Dumetella carolinensis*)	0/12	1/9 (11.1)	1/8 (12.5)	0/3	0/5	2/23 (8.7)	2/2 (100)	0/0	5/51 (9.8)	9/53 (17.0)	1/14 (7.1)	0/0
Northern Mockingbird (*Mimus polyglottos*)	3/51 (5.9)	1/17 (5.9)	0/11	0/5	11/73 (15.1)	3/32 (9.4)	2/11 (18.2)	0/1	3/44 (6.8)	5/43 (11.6)	4/19 (21.1)	0/0
Brown thrasher (*Toxostoma rufum)*	0/26	2/25 (8.0)	1/21 (4.8)	0/4	3/62 (4.8)	0/33	1/29 (3.4)	0/1	3/44 (6.8)	1/34 (2.9)	1/34 (2.9)	0/0
House finch (*Carpodacus mexicanus*)	0/108	2/94 (2.1)	0/32	0/1	0/38	4/107 (3.7)	1/91 (1.1)	0/0	1/55 (1.8)	8/257 (3.1)	3/142 (2.1)	0/0
House sparrow (*Passer domesticus*)	1/182 (0.5)	0/5	0/15	0/0	12/608 (2.0)	2/98 (2.0)	0/8	0/2	1/67 (1.5)	1/54 (1.9)	0/1	0/0
Tufted titmouse (*Baeolophus bicolor*)	0/10	0/59	1/48 (2.1)	0/10	0/26	1/56 (1.7)	0/63	0/3	0/32	0/93	1/63 (1.6)	0/0
Canada goose (*Branta Canadensis*)	0/62	0/361	0/30	1/32 (3.1)	0/31	1/118 (0.8)	0/23	1/28 (3.6)	0/0	2/89 (2.2)	3/109 (2.8)	1/154 (0.6)

* Number of birds positive for WNV antibodies by plaque reduction neutralization test/ number of birds tested in land use type

† Seroprevalence in land use type

Ag- agriculture, US- urban suburban, F- forested, W- wetland

Table 5.3. Comparison of northern cardinals (*Cardinalis cardinalis*) with all avian species tested as indicators in the physiographic regions of Georgia

	NOCA pos	NOCA tested	NOCA prev (%)	95% confidence interval (%)	Allsp pos	Allsp tested	Allsp prev (%)	95% confidence interval (%)
2002								
C	4	114	**3.5**	0.96 – 8.74	9	291	**3.1**	1.42 – 5.79
CP	33	240	**13.8**	9.66 – 18.77	60	946	**6.3**	4.87 – 8.08
M	3	47	**6.4**	1.34 – 17.54	3	301	**1.0**	0.21 – 2.88
P	5	121	**4.1**	1.36 – 9.38	78	1165	**6.7**	5.33 – 8.29
2003								
C	11	113	**9.7**	4.96 – 16.75	18	304	**5.9**	3.55 – 9.20
CP	67	447	**15.0**	11.81 – 18.64	122	1709	**7.1**	5.97 – 8.47
M	26	153	**17.0**	11.41 – 23.90	43	591	**7.3**	5.32 – 9.68
P	43	263	**16.3**	12.09 – 21.38	109	1405	**7.8**	6.41 – 9.28
2004								
C	15	109	**13.8**	07.91 – 21.68	32	260	**12.3**	8.57 – 16.93
CP	112	677	**16.5**	13.82 – 19.56	161	1668	**9.7**	8.28 – 11.17
M	26	123	**21.1**	14.30 – 29.42	43	458	**9.4**	6.88 – 12.44
P	98	409	**24.0**	19.90 – 28.40	184	1767	**10.4**	9.03 – 11.93
02,03,04								
C	30	336	**8.9**	6.11 – 12.50	59	855	**6.9**	5.29 – 8.81
CP	212	1364	**15.5**	13.66 – 17.58	343	4323	**7.9**	7.15 – 8.78
M	55	323	**17.0**	13.09 – 21.58	89	1350	**6.6**	5.33 – 8.05
P	146	793	**18.4**	15.77 – 21.29	371	4337	**8.6**	7.74 – 9.43

NOCA = Northern cardinal, Pos = positive, Prev = prevalence, Allsp = All species, C=coastal, CP=coastal plain, M=mountain, P=piedmont

Table 5.4. Age comparison of birds sampled for West Nile virus antibodies, 2002-2004

	All species		Northern cardinal (*Cardinalis cardinalis*)	
Year	**Hatch year**	**Adult**	**Hatch year**	**Adult**
2002	9/584 (1.5)*	131/2122 (6.2)	3/159 (1.9)	42/364 (11.5)
2003	38/1333 (2.9)	258/2674 (9.6)	11/373 (2.9)	136/603 (22.6)
2004	46/1279 (3.6)	373/2874 (13.0)	25/653 (3.8)	228/666 (34.2)

*Number positive/total in age group, (%)

Table 5.5. Logistic regression models for determining the importance of variables on wild bird West Nile virus seropositivity.

Model	Variable	Coefficient	Chi square	P value
All variables	Intercept	-772.7	62.8483	<0.0001
	Year	0.3831	62.0195	<0.0001
	Age	1.3961	151.8588	<0.0001
	Land use	0.1826	19.2263	<0.0001
	Species	0.0309	210.9856	<0.0001
2002	Intercept	-6.5575	201.4639	<0.0001
	Age	1.3787	15.4466	<0.0001
	Land use	0.2344	5.8733	0.0154
	Species	0.0468	82.7853	<0.0001
2003	Intercept	-5.0063	365.1339	<0.0001
	Age	1.2304	46.2230	<0.0001
	Land use	0.2875	0.0691	<0.0001
	Species	0.0232	40.5223	<0.0001
2004	Intercept	-4.5070	484.5349	<0.0001
	Age	1.4958	87.5473	<0.0001
	Species	0.0291	87.9973	<0.0001
Northern cardinal (*Cardinalis cardinalis*)	Intercept	-1259.9	63.0196	<0.0001
	Year	0.6269	62.6369	<0.0001
	Age	2.4697	194.6457	<0.0001
	region	0.2025	9.0379	0.0026
Rock pigeon (*Columba livia*)	Intercept	-6.8477	12.4473	0.0004
	Age	1.1010	5.9414	0.0148
	Land use	1.2641	4.5185	0.0335
	County	0.0220	5.8546	0.0155

Figure 5.1. Geographic distribution by county of wild avian species tested for antibodies to West Nile virus by plaque reduction neutralization test (PRNT) in 2000 – 2004.

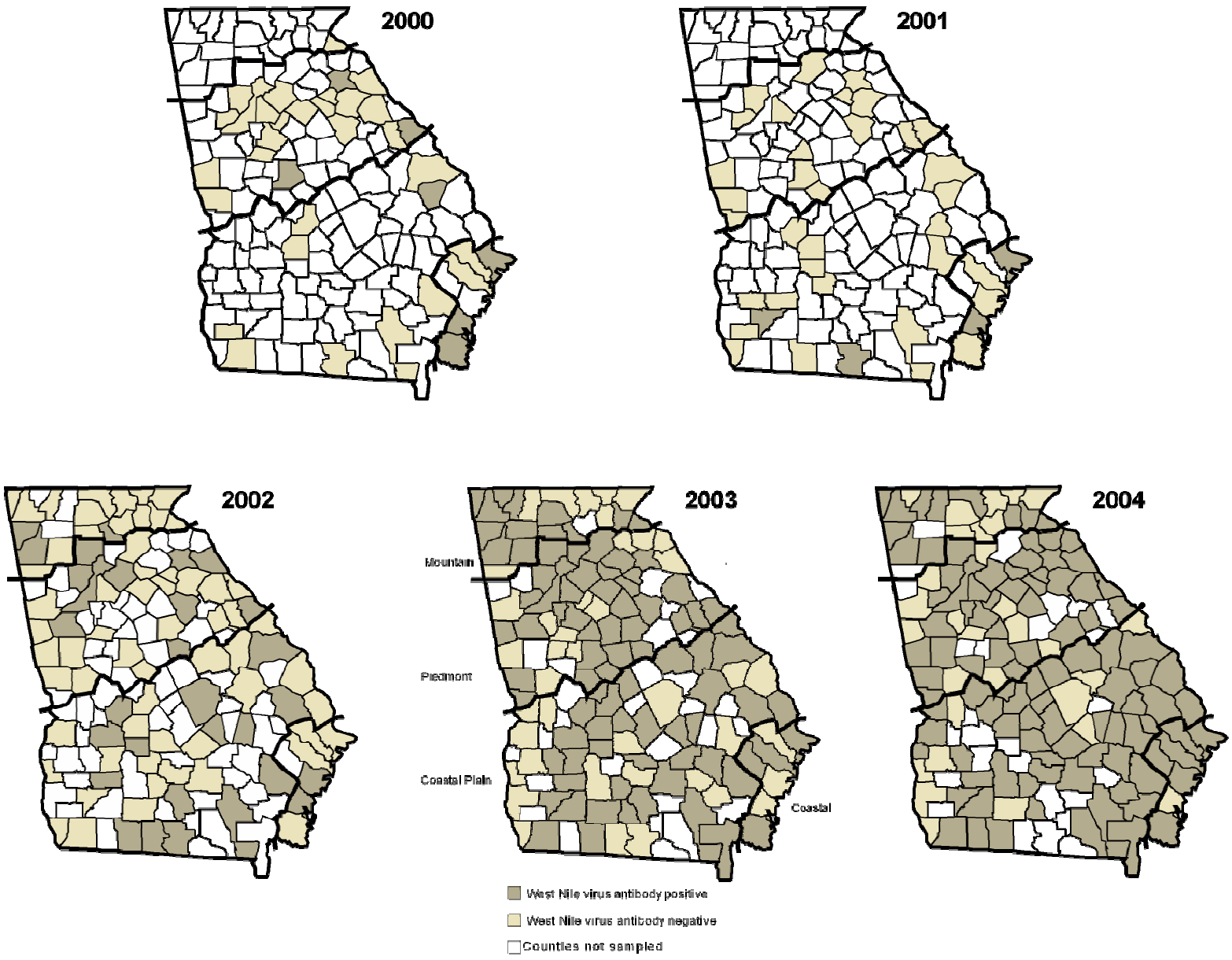

2000
2001
2002
2003
2004
Mountain
Piedmont
Coastal Plain
Coastal
West Nile virus antibody positive
West Nile virus antibody negative
Counties not sampled

Figure 5.2. West Nile virus antibody titers as determined by plaque reduction neutralization test of wild avian species in Georgia for 2002-2004.

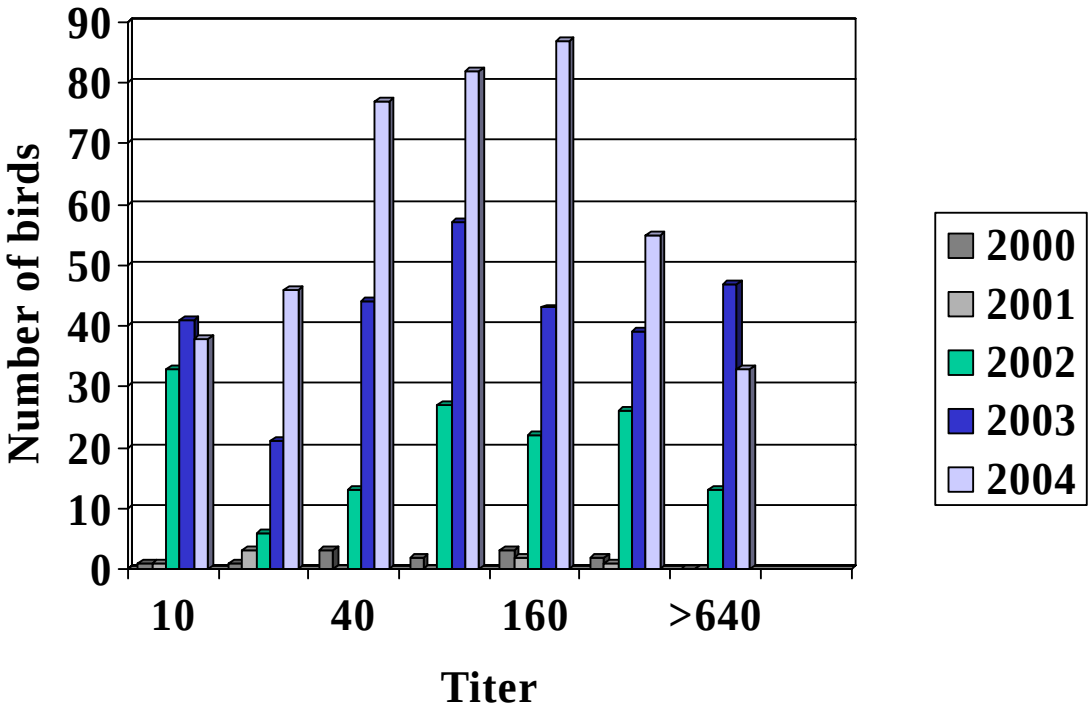
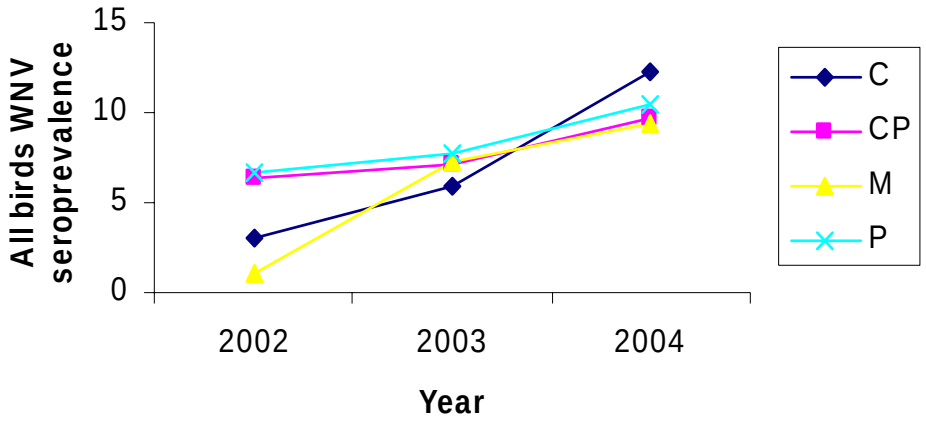
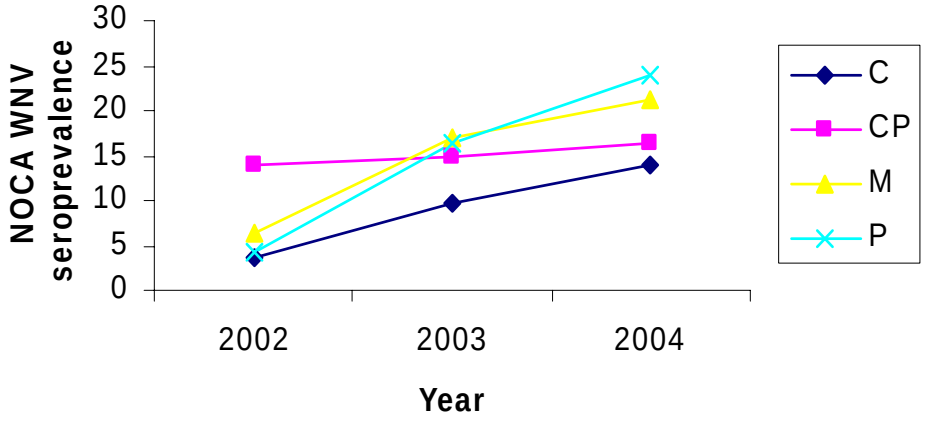


Figure 5.3. West Nile virus (WNV) seroprevalence of all avian species and northern cardinals (*Cardinalis cardinalis*) tested in four physiographic regions of Georgia for 2002-2004 compared to the distribution of West Nile virus isolation positive dead birds in 2001: Coastal (C), Coastal plain (CP), Mountain (M), and Piedmont (P); A: all birds tested for WNV, B: northern cardinals (*Cyanocitta cristata*), C: WNV positive dead bird distribution in 2001

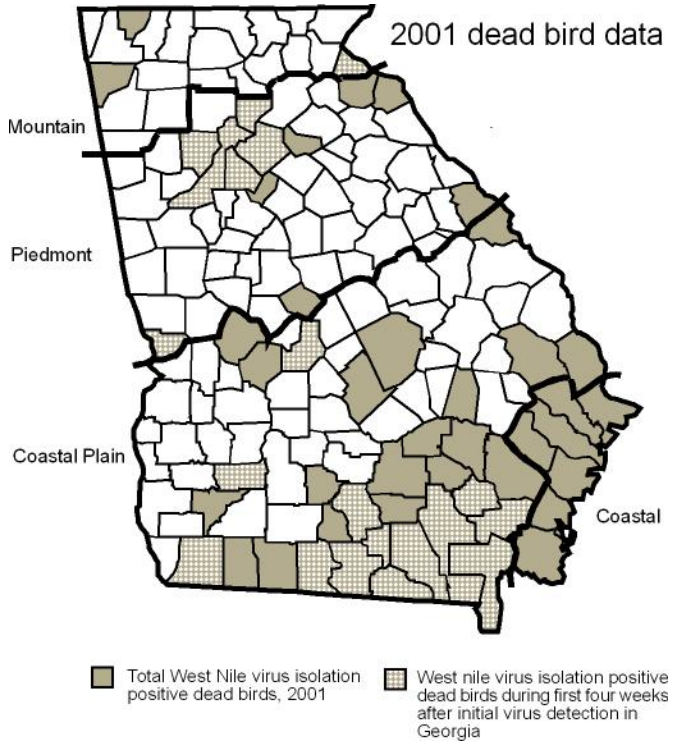
a.


All birds WNV seroprevalence
15
10
5
0
2002
2003
2004
Year
C
CP
M
P

b.


NOCA WNV seroprevalence
30
25
20
15
10
5
0
2002
2003
2004
Year
C
CP
M
P

c.


2001 dead bird data
Mountain
Piedmont
Coastal Plain
Coastal
Total West Nile virus isolation positive dead birds, 2001
West nile virus isolation positive dead birds during first four weeks after initial virus detection in Georgia

CHAPTER 6

Factors affecting the geographic distribution of West Nile virus in Georgia, USA: 2002-2004[1]

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[1]Gibbs SEJ, Wimberly MC, Madden M, Masour J, Yabsley MJ, Stallknecht DE. To be submitted to *Vector-Borne and Zoonotic Diseases*.

Abstract

The distribution of West Nile virus (WNV) is dependent on the occurrence of both susceptible avian reservoir hosts and competent mosquito vectors. Both factors can be influenced by geographic variables such as land use/landcover, elevation, human population density, physiographic region, and temperature. The current study uses geographic information systems (GIS) and logistic regression analyses to model the distribution of WNV in the state of Georgia based on a wild bird indicator system, and to identify human and environmental predictor variables that are important in the determination of WNV distribution. A database for Georgia was constructed that included 1) location points of all the avian samples tested for WNV, 2) local land use classifications including temperature, physiographic divisions, land use/landcover, and elevation, 3) human demographic data from the U.S. Census, and 4) statistics summarizing land cover, elevation, and climate within a 1 km radius landscape around each sample point. Logistic regression analysis was carried out using the serostatus of avian collection sites as the dependent variable. Temperature, housing density, urban/suburban land use, and mountain physiographic region were important variables in predicting the distribution of WNV in the state of Georgia. While weak, the positive correlation between WNV antibody positive sites and the urban/suburban environment was consistent throughout the study period. The risks associated with WNV endemicity appear to be increased in urban/suburban areas and decreased in the mountainous region of the state. This information may be used in addressing regional public health needs and mosquito control programs.

INTRODUCTION

West Nile virus (WNV) (*Flaviridae, Flavivirus*) is a vector borne pathogen of global importance. The geographic range of this virus has expanded since its discovery in Uganda in 1937 (Smithburn et al. 1940) and now includes Africa, Asia, Europe, North America, Central America and the Caribbean (McIntosh et al.1968; Hubalek and Halzouka 1999; Malkinson and Banet 2002a; Steele et al. 2000; OIE 2004; Cruz et al. 2005; Quirin et al. 2004). The distribution of WNV is dependent on the occurrence of both susceptible avian reservoir hosts and competent mosquito vectors, and both can be influenced by geographic variables such as land use/landcover, elevation, human population density, physiographic region, and temperature.

The potential influence of environmental and social factors on WNV transmission has been of great interest since the discovery of the virus. Taylor et al. (1956) noted differences in WNV seroprevalence in humans and hooded crows (*Corvus corone sardonius*) from study zones along the Nile River Delta. While climatic and geologic features in these areas were not markedly different, there were significant differences in human population density and land use patterns. The increased mosquito breeding habitat and improved farmland created by irrigation in the Nile River Delta was also cited as one of the most important man-made modifications to the environment influencing the transmission of WNV (Hayes 1989).

In a more recent study, spatial analysis of WNV case distribution in the New York City area in 1999 revealed that vegetation abundance was significantly and positively associated with human WNV cases (Brownstein et al. 2002). This association was used to predict areas of greatest human risk for WNV infection; the model constructed in the study showed that the less populated suburban regions were at greatest risk. A study in the Chicago area found that risk factors associated with clusters of human cases and dead birds included vegetation, age, income,

race, distance to reported WNV positive dead birds, age of housing, mosquito control activities, and geological factors (Ruiz et al. 2004). Mosquito abatement activities accounted for approximately 53% of the variation between clusters in this study. In Florida, spatial and temporal differences in periods of drought and rain were associated with variability in human WNV cases and infection of sentinel chickens (Shaman et al. 2005). The authors suggested that close proximity of birds and mosquito vectors during times of drought are responsible for increased virus transmission.

Geographic information systems (GIS) have also been applied to the analysis of surveillance data associated with dead bird submissions. Dead bird data was shown to be an effective indicator of WNV amplification and could be used to predict potential areas of high human risk at least 13 days prior to the onset of human illness in those areas (Theophilides et al. 2003). Another study applied a spatial scan statistic to detect small-area clustering of dead birds (Mostashari et al. 2003). This information was used to predict areas of active virus transmission and served as a basis on which to target mosquito surveillance activities. In addition, a retrospective study was conducted of dead crow report data from Chicago in 2002 (Watson et al. 2004). Spatial analysis of this data showed that human cases were three times more likely to occur inside areas of high early-season crow deaths.

Environmental conditions affecting both avian reservoir hosts and the mosquito vector populations may regulate WNV amplification. Identifying such factors will not only aid in understanding WNV epidemiology, but also will serve in predicting and possibly reducing the risk of WNV infection. In a recent study (Gibbs, *companion paper*), wild birds were used as fine scale indicators of WNV transmission over the physiographic and land use variation present in Georgia. The current study builds on this work by examining these data using GIS and logistic

regression analyses to predict the distribution of WNV in the state of Georgia based on a wild bird indicator system, and to identify human and environmental predictor variables that are important in the determination of WNV distribution.

MATERIALS AND METHODS

West Nile virus database construction

The serologic database used for the current study was derived from previous work in which avian serum samples (n = 10,865) from 70 species of birds captured throughout Georgia at approximately 200 sampling locations during the summers of 2002 - 2004 were tested by plaque reduction neutralization test (PRNT) for antibodies to WNV and St. Louis encephalitis virus (SLE) (Gibbs, *companion paper*). Antibodies to WNV were detected in 850 (7.8%) of these samples.

A database for Georgia was constructed that included 1) location points of all the avian samples tested for WNV, 2) local land use classifications including temperature, physiographic divisions, land use/landcover, and elevation, 3) human demographic data from the U.S. Census, and 4) statistics summarizing land cover, elevation, and climate within a 1 km radius landscape around each sample point. The GIS software packages used to develop the database, extract summary statistics and create map displays were the Environmental Systems Research Institute (ESRI) ArcView 3.3 and ArcGIS 9 (ESRI, Redlands, CA, USA). Data layers for each environmental factor were created as ArcView shapefiles in the Universal Transverse Mercator (UTM) grid coordinate system tied to the North American Datum (NAD) of 1983. The database comprised three seasons (May-October of 2002, 2003, and 2004) of avian samples collected within the state of Georgia and tested for antibodies to WNV by PRNT.

Coordinates of the sample points were recorded in the field with hand-held Garmin Global Positioning System (GPS) (Garmin International Inc., Olathe, KS, USA) units in the UTM coordinate system. The sampled data points were arbitrarily distributed based on sampling opportunities and located in the two UTM Zones (16 and 17) that cover the state of Georgia. Buffers of 1 km around each sampled data point were created with the ArcGIS proximity function in Arc Toolbox. This circular area was then used to clip out a 1-km radius area from each environmental factor dataset corresponding to each sample data point.

If one or more birds at a collection site were positive for WNV antibodies, that site was classified as positive. Site status was used in the analysis rather than WNV antibody status of individual birds in an effort to avoid bias introduced by species differences in prevalence and geographic distribution (Gibbs, *companion paper*).

Logistic regression

Explanatory variables Explanatory variables used in logistic regression modeling included land use/lancover, physiographic region, elevation, minimum and maximum temperatures for January and February, and 2000 census data for the state of Georgia which included human population per acre and per hectare, and housing density per acre and per hectare.

A landscape-level land use/landcover of Georgia dataset was created by the University of Georgia, Institute of Ecology Natural Resources Spatial Analysis Laboratory and obtained from the Georgia GIS Clearinghouse (http://www.gis.state.ga.us). The Landsat Landcover (18 class) dataset, dated 1998, was produced from Landsat Thematic Mapper (TM) satellite imagery of 30-m spatial resolution. Based on the requirements of this project, we reduced the number of classes to five: urban-suburban, forest, agriculture, wetland, and other. This dataset was

converted to an ArcView grid with a cell size of 30 x 30 meters. A second dataset of local land use characteristics for each sampling site was created based on field observations recorded during avian sample collection.

The physiographic divisions dataset was derived from the U.S. Geological Survey (USGS) “Physiographic Provinces” ESRI Export File (e00), dated 1992 and based on a 1:7,000,000-scale map. These data were obtained from the Georgia GIS Clearinghouse. Physiographic divisions are areas having similar topography, rock types, geology and geomorphic history as defined by USGS. The original data in the Lambert Conic Conformal coordinate system were reprojected to the UTM coordinate system, NAD 83. We used four major physiographic divisions: mountains, piedmont, coastal plain, and coastal. These data were also converted to an ArcView grid with a cell size of 30 x 30 meters.

The source for the elevation dataset was the USGS 1:24,000 scale National Elevation Dataset (NED), dated 1999. Data were obtained from the Georgia GIS Clearinghouse. The file format was ERDAS Imagine (.img) in a geographical projection (latitude and longitude). The data were converted to an ArcView grid file and reprojected to the UTM coordinate system, NAD83.

The source for temperature datasets was monthly mean maximum and minimum temperature for January and February acquired from the National Climatic Data Center (NCDC) http://www.ncdc.noaa.gov/oa/ncdc.html. Data from the weather stations in nine Georgia climatic divisions were combined with data from stations in eleven adjacent divisions of the neighboring states (totaling approximately 300 stations) in order to insure an accurate and complete spatial interpolation. A Kriging interpolation method was employed in ArcView to create a continuous surface of temperature data for the study area. The resulting triangulated irregular networks

(TINs) of temperatures covering the state of Georgia was created for January and February of 2002, 2003, and 2004. The TINs were subsequently converted to GRID format for spatial analysis in ArcView. Grids for temperature were created with a cell size of 100 x 100 m. Four grids were created for each year - January mean minimum temperature, January mean maximum temperature, February mean minimum temperature and February mean maximum temperature.

Housing density was computed using U.S. Census data from 2000. Block-level data for the state of Georgia was queried from the CensusCD 2000/Short Form Blocks dataset (GeoLytics, Inc., East Brunswick NJ, 08816). Census blocks are the smallest spatial units at which census data is released, and their sizes vary depending on population density. They can be as small as a city block in urban areas, or as large as 400 acres in rural areas. The total count of housing units within each census block was normalized by the acreage of each block to generate housing density.

An overlay analysis function in ArcView, clip, was used to extract the grid data cells of environmental factors within each 1-km radius buffer area surrounding the sample points. The ArcView Map Calculator function was then used to extract and summarize the average values of environmental factors within a 1-km buffer area surrounding each sample data point. Mean maximum and minimum temperature for January and February, physiographic divisions, land use/landcover, and elevation were thus summarized. Data for temperature and elevation were summarized as a weighted mean for each buffer area, while land use/landcover and physiographic divisions were calculated as the percent area covered by each class in the buffer area. Housing density was obtained from the census block within which each sample point fell. Categorical variables, including physiographic province and local land use, were each coded as a set of (0, 1) dummy variables corresponding to each physiographic or land use class.

Logistic regression models. Logistic regression analysis was carried out using S-Plus 6.1 (Insightful Corporation, Seattle, WA 98109) using the serostatus of avian collection sites as the dependent variable. A forward stepwise procedure with a $p < 0.05$ was used to determine which environmental variables maximized the fit of the statistical model based on our data. Accuracy (percent of testing sites correctly classified), sensitivity (percent of positive testing sites correctly classified), and specificity (percent of negative testing sites correctly classified) were computed for the model. Also, the area under the receiver operating characteristic curve (AUC ROC) and the max rescaled R^2 were calculated as indices of the fit of the model (Fielding and Bell, 1997; Nagelkerke 1991).

RESULTS

Avian samples were collected at sites within 151 counties of the state of Georgia (Fig 6.1). The sampling sites were distributed throughout all land use types and physiographic regions present in the state. West Nile virus antibody prevalence data for the individual avian samples on which the site data were based are shown in Table 6.1.

For the 2002 data, four variables were significantly related to WNV serostatus (Table 6.2). The probability of WNV being present in an area increased with field-observed urban-suburban land use and minimum January temperature. A unimodal response to the natural logarithm of housing density was seen in the dependent variable (serostatus), and the second-degree polynomial term was included in the final model. The probability of a site being WNV positive was highest at densities of approximately 1 housing unit per 10 acres, and was lower both in more rural areas and in more heavily populated areas. The 2003 model included only the field observed urban-suburban land use variable, which was positively associated with WNV

presence. In the 2004 model, the probability of WNV positive sites was lower in the mountains than in other physiographic regions. As with the 2002 model, the second-degree polynomial term for the natural logarithm of housing density indicated that the probability of a site containing a WNV positive bird was highest at intermediate housing densities of approximately 1 housing unit per 10 acres.

The accuracy, sensitivity, specificity, maximum rescaled R^2, and area under the receiver operating characteristic curve (AUC ROC) of the 2002 model were much greater than in the 2003 and 2004 models (Table 6.3).

The serologic status of collection sites that were sampled in more than one year are listed in Table 6.4. Approximately half of the sites which were initially negative changed status to positive upon re-sampling. Few sites initially found positive changed to a negative status.

DISCUSSION

The results of this study illustrate the widespread distribution of WNV in the state of Georgia in just three years after introduction. Antibodies against WNV were found for each sample year in both adult and hatch year birds, indicating that the virus was able to over-winter and become endemic in the state (Gibbs, *companion paper*). This finding is also supported by dead bird and mosquito surveillance in Georgia (SCWDS, unpublished data). As demonstrated by the poor accuracy of the logistic regression models in the last two years of the study, environmental and demographic variables became less important in determining the distribution of the virus as time progressed. This is consistent with logistic regression findings based on data

from individual avian samples in which the local land use variable was less important in the 2003 than 2002 model, and was not included at all in the 2004 model (Gibbs, *companion paper*).

The thorough coverage of WNV across the Georgia landscape was most likely facilitated by the presence of several competent vectors. *Culex quinquefasciatus*, the primary vector in the state, as well as *Cx. nigripalpus, Cx. restauns,* and *Cx. salinarius*, each include Georgia in their distributions (Darsie and Ward, 2005). The different behavioral characteristics of these mosquitoes, including host and habitat preference, allow for transmission in a diversity of environments. *Cx. quinquefasciatus* may be found in abundance in human modified habitats such as residential areas (Reisen, 1992). Such modifications include creation of mosquito habitat in flower pots, used tires, flooded basements, sewage treatment areas, and water-catchment basins in housing developments. There are few areas within the state of Georgia that are not heavily impacted by human activities.

Sites testing positive for WNV antibodies in 2002 may represent the areas to which WNV was first introduced in 2001. Serologic data from 2002 revealed two loose foci of positive sites, one in the coastal plain and one in the metro Atlanta area. A similar spatial distribution was also observed in dead bird surveillance data from 2001 (Gibbs, *companion paper*). In the following transmission seasons, the range of the virus might have then extended through local bird movements rather than being reintroduced by migratory species. The effects of the land use and physiographic region on site serologic status during 2002 may have been enhanced due to a more localized distribution associated with initial introduction.

The landscape-level land use/landcover variables (based on land cover data within a 1-km buffer area surrounding the sample point) were discarded in all three of the models. The local variable for urban-suburban land use variable (based on field observation), however, was

included in both the 2002 and 2003 models. This suggests that there is a very localized association between the presence of WNV and human land use within the immediate sampling location. The practice of capturing birds around feeders may have influenced this association. This may also reflect the importance of microhabitat rather than broader habitat patterns within the 1- km radius area surrounding the capture site in determining avian WNV exposure.

The overall fit of the 2004 model was better than the 2003 model, but neither predicted the presence of WNV as accurately as the 2002 model. Housing density was a strong predictor of WNV presence in 2002, with higher numbers of positive sites situated in areas with intermediate housing densities. While not as strongly correlated with WNV positive sites as it was in 2002, the housing density was also included in the model for 2004. Together with the inclusion of urban-suburban land use data (based on field observation) in 2003, this information supports the contention that human activities in the urban/suburban landscape provide reservoir host and vector habitats suitable for efficient WNV transmission.

While weak, the positive correlation between WNV antibody positive sites and the urban/suburban environment was consistent throughout the study period. The human impact on disease ecology has been studied intensively as humans continue to expand and modify their environment. In an assessment of emerging pathogens of wildlife in North America between 1998 and 2000, the majority of outbreaks were linked to human activities (Dobson and Foufopoulos, 2001). As demonstrated by a number of arboviruses, disease emergence is most often related to human activities that increase disease vector habitats or change the density of nonhuman vertebrates involved in virus amplification (birds in the case of WNV) (Shope 1997; Mackenzie et al. 2004; Anonymous, 1994). Urbanization and deforestation have been linked to emergence of arboviruses such as Rift Valley fever, SLE, and dengue (Wilson 1994). Despite an

abundance of review literature on the topic, minimal data are available to confirm the impact of human activities on disease epidemiology (Kuiken et al. 2003). The current study provides some of these data, as well as insight into the potential of a human altered environment, specifically the urban/suburban environment, to support vector borne disease. Human activities not only support mosquito populations, but also provide food, nesting, and roosting habitat for both native and introduced birds.

The importance of minimum January temperature in the 2002 model reflects the dependence of WNV transmission on mosquito vectors. Areas experiencing extended periods of freezing temperatures during the winter are less likely to support year-round WNV transmission than sites at the southern limits of the state. The inclusion of mountain physiographic region in the 2004 model also emphasizes the importance of elevation, temperature, and physiographic region in WNV epidemiology. These three variables are interrelated in Georgia, and in combination appear to limit the transmission of WNV. Reasons for this limitation probably relate to lower temperatures in mountainous regions, consequent decreases in mosquito abundance, and differences in avian species composition.

The changing serologic status of re-sampled sites in this study agrees with antibody prevalence data obtained from individual birds in this dataset (Gibbs, *companion paper*); antibody prevalence for all species increased from 5.2% to 7.3% to 10.1% in 2002, 2003, and 2004, respectively. The results were consistent between years, reflecting the increasing geographic distribution of WNV during the study. Approximately 30% of the sites initially negative converted to positive sites, however, only 8.7% went from positive to negative. This is surprising considering the low prevalence and small sample size at some sites.

As demonstrated by this work, WNV is now distributed throughout the state of Georgia and poses a health risk to humans, livestock, and wildlife in all physiographic regions and land use types; the challenge we experienced in developing a model with high sensitivity and specificity for the data set reflects this broad distribution. The data were grouped according to sampling site rather than by individual serum sample in an effort to decrease bias potentially introduced by differences in susceptibility and antibody formation in avian species. This modeling based on site status will also work in future studies; however, a targeted approach towards sites with good avian indicators (such as the northern cardinal) would be a better approach. Seroprevalence data from individual samples could then be included in the model, making it more accurate in defining the influence environmental and demographic factors have on WNV distribution. The importance of these factors will most likely change as the environment becomes increasingly impacted by human population expansion.

The trends observed both in this study and work conducted on WNV antibody prevalence in avian species (Gibbs, *companion paper*) suggest that the virus will continue to circulate in the environment at endemic levels. The risks associated with endemicity appear to be increased in urban/suburban areas and decreased in the mountainous region of the state. This information may be used in addressing regional public health needs and mosquito control programs; priority should be placed on campaigns aimed at decreasing man-made mosquito habitats in urban/suburban areas.

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Table 6.1. West Nile virus antibody prevalence in avian samples by site.

	# locations	# positive	Site prevalence (%)	Average sample size (range)	Average antibody prevalence at pos. sites (%)
2002					
Land use					
Ag	63	13	20.6	15.3 (1-77)	11.9
F	41	8	19.5	10.2 (1-56)	25.8
U/S	63	15	23.8	14.9 (1-288)	23.4
W	11	1	9.0	15.2 (1-37)	11.1
Phys region					
C	16	2	12.5	14.7 (1-37)	18.8
CP	74	24	32.4	13.3 (1-56)	18.0
M	32	3	9.4	9.4 (1-74)	7.2
P	56	8	14.3	17.3 (1-288)	28.9
Total	**178**	**37**	**20.8**	**14.0 (1-288)**	**18.9**
2003					
Land use					
Ag	74	38	51.4	21.2 (2-72)	13.8
F	56	27	48.2	15.9 (1-65)	21.3
U/S	79	49	62.0	15.5 (1-137)	19.0
W	6	2	33.3	16.2(4-28)	3.6
Phys region					
C	21	9	42.9	14.4 (3-32)	14.7
CP	83	49	59.0	20.1 (1-53)	15.8
M	43	19	44.2	13.3 (2-65)	25.2
P	68	39	57.4	18.2 (1-137)	16.7
Total	**215**	**116**	**53.9**	**17.6 (1-137)**	**17.7**
2004					
Land use					
Ag	43	32	74.4	23.3 (2-47)	15.2
F	56	35	62.5	19.4 (2-83)	14.3
U/S	112	70	62.5	16.7 (1-139)	18.8
W	11	2	18.2	16.9 (5-41)	5.0
Phys region					
C	16	10	62.5	16.25 (4-30)	16.1
CP	91	64	70.3	18.3 (1-53)	17.0
M	29	13	44.8	15.8 (1-37)	17.0
P	86	51	60.0	20.5 (1-139)	16.5
Total	**222**	**139**	**62.6**	**18.7 (1-139)**	**16.6**

Ag – agriculture, F – forest, U/S – urban/suburban, W – wetland, C – coastal, CP – coastal plain, M – mountain, P – piedmont, Phys region – physiographic region

Table 6.2. Logistic regression models for predicting the distribution of West Nile virus in the state of Georgia based on a wild bird indicator system.

Year	Variable	Coefficient	Wald statistic	P
2002	Minimum January temperature	0.02	3.31	0.0009
	log(housing/hectare + 0.01)	-1.62	-3.28	0.0010
	$[\log(\text{housing/hectare} + 0.01)]^2$	-0.30	-3.11	0.0019
	Urban-suburban land use based on field observation	1.74	3.25	0.0012
2003	Urban-suburban land use based on field observation	0.61	2.11	0.0350
2004	Mountain physiographic region	-0.01	-2.72	0.0066
	log(housing/hectare + 0.01)	-0.21	-1.56	0.1190
	$[\log(\text{housing/hectare} + 0.01)]^2$	-0.09	-2.29	0.0223

Table 6.3. Comparison of three models constructed for predicting distribution of West Nile virus in the state of Georgia based on a wild bird indicator system.

Analysis*	2002	2003	2004
Accuracy (%)	83.1	55.6	64.9
Sensitivity (%)	76.9	64.6	65.8
Specificity (%)	83.6	50.4	58.6
Maximum rescaled R^2	0.239	0.028	0.072
AUC ROC†	0.751	0.57	0.622

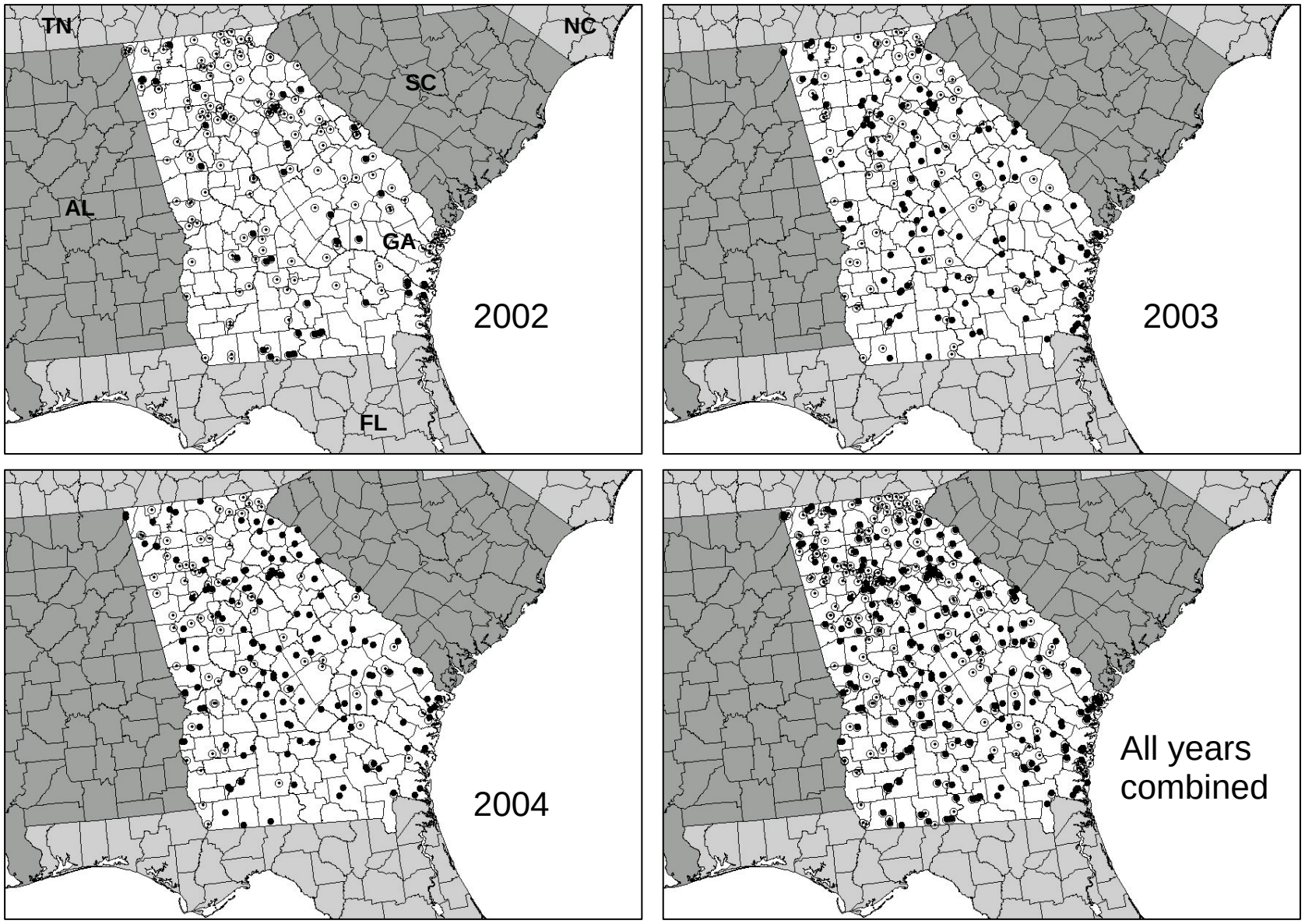
* A sampling site was considered positive if $\geq$ 1 bird tested positive for WNV antibodies by plaque reduction neutralization test (PRNT).

† Area under the receiver operating characteristic curve (AUC ROC)

Table 6.4. West Nile virus serologic status of re-sampled collection sites

Years	# sites re-sampled	Site status				
		Negative to negative	Positive to positive	Negative to positive	Positive to negative	Variable
2002-2003	64	21	13	22	8	-
2003-2004	81	14	43	11	8	-
2002-2004	35	10	7	17	1	-
2002-2003-2004	27	3	6	12	1	5

Fig. 6.1. Distribution of West Nile virus antibody positive and negative sites in the state of Georgia for 2002-2004



● Site with West Nile virus seropositive birds

⊙ Negative site

CHAPTER 7

CONCLUSIONS

West Nile virus (WNV) [*Flaviviridae, flavivirus*] has a transmission cycle between mosquitoes and birds, and causes encephalitis in humans and a variety of other animals (Smithburn, 1940). Prior to 1999, epidemics had only occurred in Africa, Asia, and Europe (McIntosh, 1968; Hubalék and Halouzka, 1999; Murgue, 2002). In 1999, WNV was introduced to the United States and over the next six years spread from North America through to Central America (Steele, 2000; Farafan-Ale, 2004; OIE, 2003). WNV is currently an emerging zoonosis in the Western Hemisphere and is having an important impact on public health, animal health, and ecosystem health. The combined objective of the three projects included in this dissertation was to define the role that peridomestic avian species play as amplifying hosts and sentinels of WNV in Georgia.

Chapter 3: WNV detection in Blue Jays

Detectability of WNV in blue jays varies by tissue and is dependent on the diagnostic test employed. Lung, brain, and heart tissues of naturally infected blue jays in this study contained the highest WNV titers of the eight tissues examined. For blue jays, dead bird surveillance efforts utilizing virus isolation can be limited to one or more of these tissues. Gross and microscopic pathology were the least effective procedures for WNV diagnosis in blue jays. WNV antigens were most often detected in kidney, heart, liver, and lung tissue by IHC, making them the most optimal tissues for use with this procedure. Due to variability in staining between

tissues, the authors recommend using at least three of the optimal tissues when performing surveillance to increase sensitivity. Nested RT-PCR proved to be the most sensitive diagnostic test applied in this study, and did not detect viral RNA in any of the tissues from five WNV negative blue jays. The high WNV titers present in these blue jay tissues reinforces the need for personal protection when handling the birds and performing post-mortem exams. The high titers also illustrate the potential for blue jay tissues to be a source of WNV infection if ingested by scavenger species.

Immunohistochemistry, virus isolation, and RT-nPCR are all useful techniques in WNV surveillance. We have shown, however, that when employing these techniques, tissue selection is critical for immunohistochemistry (heart, kidney, liver, and lung were best) and virus isolation (based on tissue titer, brain, heart, and lung were best). With RT-nPCR however, it seems possible to detect viral RNA in a variety of tissues of positive birds, allowing the investigator to choose the tissue based on ease of collection.

Chapter 4: Antibody persistence to WNV in pigeons

This study showed that the minimum duration of antibody persistence to WNV in rock pigeons is 15 months, there is little long-term variation in antibody titers, and there is no serological evidence of viral recrudescence. Based on these findings, the avian population immunity to WNV can be expected to increase as WNV establishes itself in North America. The persistence of antibodies to WNV in an avian species for over a year complicates interpretation of multi-year studies involving serologic surveillance of wild bird populations. Since the antibody titers in this study remained at high levels, pigeons may maintain neutralizing antibody titers to WNV for several years. Seroprevalence of WNV in avian populations may therefore

increase while transmission of the virus in an area remains stable over time. Because species variation in the persistence of antibodies to WNV may exist, antibody persistence in other avian species should be evaluated.

The results in this study proved to be highly test-dependent and serologic results should be interpreted with this in mind. The HAI test was not as effective as the PRNT or ELISA in this study. To our knowledge, this is the first report detailing the persistence of avian maternal antibodies to the North American strain of WNV. The role of nestlings in WNV amplification cycles may be reduced by maternal antibody persistence. In the case of pigeons, the additional opportunity for transfer of passive immunity from not only the hen, but also the cock, increases the proportion of squabs with resistance to WNV infection.

Chapter 5: WNV antibodies in avian species of Georgia

Logistic regression analyses consistently identified year, species, age, and land use as significant variables in determining antibody prevalence. The inclusion of the year variable is not surprising considering that data were collected from the three years immediately following the initial detection of WNV in Georgia. The slow rate of prevalence increase was not expected considering the introduction of WNV into a naïve avian community and four years of annual transmission. The reasons for the host species prevalence differences observed between rock pigeons/northern cardinals and other avian species may be related to: 1) differences in host susceptibility and mortality; 2) species specific selection by vector species; 3) species specific utilization of land use types or habitats that enhance vector contact, or 4) species specific variation in immune response. Antibodies were detected in hatch year birds in 2002, 2003, and 2004, indicating that WNV transmission occurred during each year. The inclusion of land use in all logistic regression models except the 2004 model was most likely due to the fact that, by

2004, the distribution of WNV had become complete across the landscape. Physiographic region was identified as an important variable only in the northern cardinal logistic regression model and was most likely a result of the widespread distribution of northern cardinals in comparison to other species.

Northern cardinals met all of the criteria for an avian indicator. They are distributed throughout the entire southeastern United States, are found in all physiographic regions and land-use types, are closely associated with humans, are easy to catch using mist nets, have a high seroprevalence rate, and develop a strong antibody response to WNV. The habitat flexibility and abundance of northern cardinals also makes them excellent candidates for indicators. While rock pigeons and common ground doves fulfilled many of the requirements for an avian indicator, these species were poorly distributed across the landscape and may have more utility as indicators in very localized areas.

Chapter 6: Factors affecting the geographic distribution of West Nile virus in Georgia

The results of this study illustrate the widespread distribution of WNV in the state of Georgia just three years after introduction. Antibodies against WNV were found for each sample year in both adult and hatch year birds, indicating that the virus was able to over-winter and become endemic in the state. As demonstrated by the poor accuracy of the logistic regression models in the last two years of the study, environmental and sociological variables became less important in determining the distribution of the virus as time progressed. Data from individual avian samples are consistent with logistic regression findings based on sampling site.

The thorough coverage of WNV across the Georgia landscape was most likely facilitated by the presence of several competent vectors. The different behavioral characteristics of the

mosquito vectors, including host and habitat preference, allow for transmission in a diversity of environments. *Cx*. *quinquefasciatus* is found in close association with humans and human modified habitats, and there are few areas within the state of Georgia that are not heavily impacted by human activities.

Sites testing positive for WNV antibodies in 2002 may represent the areas to which WNV was first introduced in 2001. Serologic data from 2002 revealed two loose foci of positive sites, one in the coastal plain and one in the metro Atlanta area. While weak, the positive correlation between WNV antibody positive sites and the urban/suburban environment was consistent throughout the study period. As demonstrated by a number of arboviruses, disease emergence is most often related to human activities that increase disease vector habitats or change the density of nonhuman vertebrates involved in virus amplification (birds in the case of WNV) (Shope, 1997; Mackenzie, 2004; Anonymous, 1994). The current study provides some data on the impact of human activities on disease epidemiology, as well as insight into the potential of a human altered environment, specifically the urban/suburban environment, to support vector borne disease.

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