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Thesis Approval Page

West Nile virus seroreversion and the influence of herd immunity on disease risk in a long-term study of free-ranging birds.

By

Eileen M. McKee

A Thesis Presented to the Graduate College of Governors State University

In Partial Fulfillment of the Requirements

For the Degree Master of Science, Environmental Biology

Approved:

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ABBREVIATIONS

AIC = Akaike Information Criterion
AICc = corrected AIC
c' = contact pattern
CDC = Centers for Disease Control and Prevention
CMI = cell-mediated immunity
CNS = central nervous system
CxFV = Culex flavivirus
D = duration of infectiousness
DF = Dengue fever
DPI = days post-inoculation
EEE = Eastern equine encephalitis
ELISA = enzyme-linked immune-sorbent assay
FMDV = foot-and-mouth disease virus
GMT = geometric mean antibody titer
HIA = hemagglutination inhibition assay
IR = mosquito infection rate
JEV = Japanese encephalitis virus
LACV = La Crosse virus
MAb = monoclonal antibody
MIR = minimum infection rate
PCR = polymerase chain reaction
PFU = plaque forming unit
PRNT = plaque reduction neutralization test
R_0 = basic reproduction number
R_e = effective reproduction number
S_0 = susceptible fraction of population
S_h = herd immunity threshold
SIR = epidemiological model (susceptible, infectious, recovered/removed)
SLE = St. Louis encephalitis
µMT = immune-deficient mice
USUV = Usutu virus
WEE = Western equine encephalitis
W_i = AIC parameter weight
WNV = West Nile virus
β = transmission probability

West Nile virus seroreversion and the influence of herd immunity on disease risk in a long-term study of free-ranging birds.

Abstract

The transmission and amplification of West Nile virus (WNV) is driven by the number of susceptible avian hosts. The quantity of susceptible hosts is determined by 1) recruitment of juvenile birds during the breeding season, 2) adult birds previously unexposed to WNV, and 3) adult birds with waning antibody titers. Although rarely tested, avian herd immunity and seroreversion could have a direct impact on *Culex* infection rate, a proxy for the reproductive number of WNV. In this study, we utilized data from a seven year study investigating WNV transmission ecology in suburban Chicago, Illinois. We tested the hypothesis that herd immunity in the avian community has an inverse relationship with WNV infection in *Culex* species mosquitoes. This longitudinal study, with repeated blood samples from individual free-ranging birds, revealed a rate of antibody decay such that most birds have undetectable titers after two years. Additionally, the magnitude of the antibody response, measured by the geometric mean antibody titer, was inversely associated with WNV infection in *Culex* species mosquitoes suggesting that herd immunity depends on a highly protective antibody titer. These results illustrate the need to understand the dynamics of the host immune response in relation to WNV transmission. Utilizing a unique, long-term data-base, this study contributes to understanding the mechanisms of WNV amplification in the urban environment.

Key Words: West Nile virus, avian seroprevalence, antibody persistence, avian seroreversion, herd immunity, epidemiological modeling

LITERATURE REVIEW

Background: The demand for biological resources needed to sustain the growth of human population and consumption has resulted in increased overlap of human, domestic animal, and wildlife habitats with unprecedented negative impacts on biodiversity and ecosystem stability (Maillard & Gonzalez 2006). The increased duration and intensity of human to human and human to animal contact has resulted in increased transmission of previously identified zoonoses as well as increased risk of contact with emerging pathogens, representing a significant threat to global health (Maillard & Gonzalez 2006; Jones *et al.* 2008). Loss of biodiversity may also amplify the risk of pathogen transmission. Studies have shown that increased species diversity can reduce vector-borne disease risk *via* the "dilution effect," whereby incompetent reservoir hosts dilute transmission among vectors and highly compatible reservoir hosts (Ezenwa *et al.* 2006; Keesing *et al.* 2006). Besides the public health concern, novel pathogens can cause catastrophic impacts on immunologically naïve wildlife species, a cause of extinction that may be secondary only to habitat loss and invasive species (Daszak *et al.* 2000). Exponential population growth, globalization and climate change will likely result in increased zoonotic epidemics, calling for a better understanding of host and vector populations as well as an increased ability to forecast pathogen persistence and amplification events (LaDeau *et al.* 2011).

Arboviruses: In the past 20 years, there has been a dramatic resurgence of globally epidemic "arboviruses", or arthropod-borne viruses, thought to be controlled or relatively unimportant to public health (Gubler 2002). Arboviruses

are maintained in nature through complex cycles involving interactions among the virus, hematophagous arthropod vectors, and vertebrate "reservoir" hosts under the appropriate environmental conditions (Monath 1993). The changing epidemiology of arboviruses is complex but involves the regional and global expansion of viruses and vectors, taking advantage of susceptible vectors and hosts (Gubler 2002). Of more than 500 arboviruses recognized worldwide, only a small subset of arboviral human pathogens has been isolated in North America and includes the La Crosse virus (LACV), St. Louis encephalitis (SLE), Eastern and Western equine encephalitis (EEE, WEE) and Dengue fever (DF) (Calisher 1994). However, these arboviruses continue to be a major cause of meningitis, encephalitis, or myelitis ("neuroinvasive disease") in North America. West Nile virus (WNV) is a mosquito-borne arbovirus (genus *Flavivirus*, family *Flaviviridae*) belonging to the Japanese encephalitis virus (JEV) antigenic complex along with closely related flaviviruses that also cause human disease: JEV in Asia, SLE in the Americas and Murray Valley encephalitis in Australia (De Madrid & Porterfield 1974; Gubler *et al.* 2007). WNV is now the dominant cause of encephalitis in the United States following its abrupt emergence in New York in 1999 (Riemann *et al.* 2008; Davis *et al.* 2008).

West Nile virus: First isolated in 1937 from the West Nile district of Uganda, WNV has expanded its native range of Africa, Asia and Europe and spread rapidly throughout its introduced range in North America, Canada, Mexico, Latin America and the Caribbean as far south as Argentina (Amore *et al.* 2010). WNV was first identified in the western hemisphere in New York City in

1999 and attributed as the cause of fatal disease in humans, horses and birds (Steele *et al.* 2000). Genetic analysis indicated its identity as a lineage I virus associated with those viruses implicated in major epidemics in Africa, India, the Middle East and Europe and closely related to the virus isolated in the Israeli epidemic of 1998 (Jia *et al.* 1999; Malkinson *et al.* 2002). Prior to the late 1990s, the presentation of WNV clinical illness in humans was primarily fever, arthralgia and rash with sporadic cases of encephalitis, and no apparent negative effects identified in wildlife (Peterson *et al.* 2004; Gubler 2007). Although the majority of viremic patients develop asymptomatic infection, approximately 20% of WNV-infected individuals develop symptoms ranging from West Nile fever to WNV-induced neuroinvasive disease, with the elderly and immunosuppressed most susceptible to severe forms of infection (Kim *et al.* 2008). The NY99 strain introduced into the United States appears to be more neurovirulent with over 35,350 cases of recorded human illnesses and 1,421 deaths reported by the Centers for Disease Control and Prevention (CDC) since 1999 and significant, unprecedented wildlife losses (Marra *et al.* 2004; Gubler *et al.* 2007; CDC 2012).

Following the identification of WNV positive birds in 2000 and 2001, Illinois led the nation in 2002 with the highest number of WNV human cases and 66 deaths (Ruiz *et al.* 2004), becoming "the largest arboviral meningoencephalitis epidemic documented in the western hemisphere" (CDC 2002). Illinois was second to California in 2005 with human cases and sixth in the nation in 2006 and has continued to produce WNV human illness every year from 2002 to 2012 (Ruiz *et al.* 2004; Illinois Department of Public Health 2012). Large numbers of

human WNV cases have been reported in urban areas of the United States (Komar *et al.* 2001; Allan *et al.* 2008). Most human cases from Illinois have occurred in highly clustered urban areas located in suburban Chicago with a considerably higher WNV case rate associated with moderate vegetative cover and population density (Ruiz *et al.* 2004; Ruiz *et al.* 2007).

WNV transmission cycle and viremia: The principal transmission cycle for WNV in natural settings appears to involve ornithophilic *Culex* spp. mosquitoes as vectors and birds as the reservoir host (Gubler *et al.* 2007). After ingestion of an infected vertebrate blood meal by the mosquito vector, WNV begins to replicate in the midgut by the second day, followed by rapid viral expansion to adjacent muscle and fat cells and finally the salivary glands by two weeks (Davis *et al.* 2008). The vector can then infect a susceptible host where replication of the virus (i.e., amplification) may reach levels sufficient for vector infection if the host is competent. A competent host is effectively able to become infected, survive to replicate the virus to sufficient levels, and transmit it to subsequent individuals (Gubler *et al.* 2007; Figure 1). The presence of arboviral particles in the blood of infected birds (i.e., viremia) remains at levels sufficient to re-infect vectors for 1-7 days and occasionally longer (Stamm 1966; Komar *et al.* 2003), with viremic titers greater than 10^5 PFU/mL considered infectious for *Culex pipiens* and *Culex quinquefasciatus*, two important WNV enzootic vectors in the United States (Komar *et al.* 2001). While other vertebrate hosts can become infected with WNV, they are considered “dead-end" hosts because their infective period is short, reducing the likelihood of a biting arthropod becoming

infected, or they do not typically develop viral titers high enough to infect mosquitoes (Marra *et al.* 2004; Weaver & Barrett 2004). Successful transmission of WNV is dependent on many complex factors including vector and host competency, availability of susceptible hosts, interactions between the host and vector communities, and environmental conditions such as temperature and rainfall (Marra *et al.* 2004).

WNV vectors: While WNV has been identified in 43 mosquito species, species vary in their competence. *Culex* mosquitoes, with species most active well after dusk and throughout the night, are identified as the main amplification vectors (Marra *et al.* 2004; Suom *et al.* 2010). *Culex* spp. have been found to breed effectively in artificial containers and storm drain systems (Allan *et al.* 2009) such as the catch basins built in the Chicago Lake Plain region where a cluster of WNV cases have occurred (Ruiz *et al*. 2004). Previously, increased abundances of *Culex* spp. have been recovered from catch basins in the Cook County area of Chicago (Geery & Holub 1989) and likely provided ideal breeding conditions during the WNV outbreak of 2002 (Ruiz *et al*. 2004).

By utilizing PCR (polymerase chain reaction) and DNA sequencing to identify mosquito feeding patterns on hosts, the ornithophilic mosquito *Culex pipiens* has been implicated as the epizootic vector in the Chicago area (Hamer *et al.* 2009). This species feeds on a large number of bird species (25 species), the most common being American robin (*Turdus migratorius*), house sparrow (*Passer domesticus*), mourning dove (*Zenaida macroura*) and northern cardinal (*Cardinalis cardinalis*; Hamer *et al.* 2008; Hamer *et al.* 2009). At a fine spatial

scale within the suburban Chicago study site, Hamer *et al.* (2011) found that the American robin and house sparrow produced 95.8% of the infectious *Culex pipiens* mosquitoes. Chaves *et al.* (2010*a*) suggested that rather than mosquito host preference, mosquito species in a community may rely primarily on host availability in a given landscape. Later studies in the area found that landscape heterogeneity, which was a predictor of increased vector biodiversity in the Chicago area, reduced the abundance of mosquitoes, in particular *Culex pipiens* (Chaves *et al.* 2011). However, *Culex pipiens* was the most common species identified during mosquito collections at this site for 2005, 2006 and 2007 (Hamer *et al.* 2009).

Mammalian hosts including humans were identified from additional *Culex pipiens* blood meals during the host feeding study in the Chicago study, suggesting that *Culex pipiens* may also serve as the epidemic or "bridge" vector responsible for transmission of WNV to humans (Hamer *et al.* 2008; Hamer *et al.* 2009). In a study of *Culex pipiens* in northeastern United States, Fonesca *et al.* (2004) found that a large proportion of individuals were hybrids of bird-biter and human-biter forms including *Culex molestus*, previously described as an anthropophagic species. Huang *et al.* (2009) identified genetic heterogeneity in the *Culex pipiens* population in the Chicago study site area, with the probability of genetic ancestry from *Culex pipiens f. molestus.* Such genetic heterogeneity could potentially predispose mosquitoes to feed more readily on mammals including humans.

Abiotic factors - temperature: WNV transmission activity has been well defined to the summer and early fall months in temperate areas when mosquito populations are at their highest, with peak viral activity usually recorded during periods of highest temperature and rainfall (Hayes 1989; Marra *et al.* 2004; Reisen *et al.* 2006*a*). Reisen & Brault (2007) examined the pattern of human disease in temperate North America in relation to temperature and found that WNV dispersed into new areas during years with above-normal temperatures, amplified during the following summers with above or normal temperatures and decreased or delayed virus activity with cooler summers. In the Chicago area, increased cumulative temperature was a significant predictor of increased mosquito infection and human cases from 2004 to 2008, potentially due to increases in vector growth rates, viral replication and oviposition (i.e., egg laying) activity (Ruiz *et al.* 2010). Hamer *et al.* (2008) suggested that the association between high environmental temperatures and increased rates of WNV dissemination among *Culex* spp. could be a result of shorter breeding cycles in mosquitoes and decreased incubation periods. Ruiz *et al.* (2010) found that air temperature in the Chicago study area influenced the magnitude and timing of increased *Culex* Minimum Infection Rate (MIR) within a season, with higher temperatures preceding an increase in mosquito infection. Lastly, Bertolotti *et al.* (2008) found that viral genetic diversity was higher in mosquitoes from residential sites in the Chicago study site where previous studies have shown a higher daytime temperature than in the adjacent rural areas. These data suggest that fine-scale temperature fluctuations and anthropogenic factors related to

urbanization could have direct effects on viral mutation and replication, or that temperature has an indirect effect on host-vector-virus interaction.

Abiotic factors - precipitation: In areas where *Culex nigripalpus* functions as the main vector, early seasonal droughts followed by rainfall have preceded WNV and SLE amplification episodes (Ruiz *et al.* 2010). In other areas of the United States, drought conditions have been associated with urban WNV as well as SLE outbreaks (Epstein & Defilippo 2002). Drought conditions may increase host and vector contact rates at water sources (Hamer *et al.* 2008) and favor breeding conditions in the organically rich water that forms in city storm drains and catch basins where decreased rain-flow prevents flushing of *Culex* larvae (Loss *et al.* 2009*a*). Chaves & Kitron (2010*b*) found that a marked change in ammonium ion levels following rainfall events reduced abundance of microbes, a mosquito larvae food source (Walker *et al.* 1991). Although the relationship of MIR to precipitation patterns in the Chicago area appears to be complex and variable, dry years often result in higher MIR. Additionally, there was an increased association of high MIR and/or human WNV cases when tandem conditions of high temperature and low rainfall occurred (Ruiz *et al.* 2010). In the same study area, Chaves *et al.* (2011) found similar patterns of increased entomological risk of disease transmission with highly variable rainfall regimes and relatively dry years. Overall, increased WNV transmission in the Chicago area has coincided with high temperatures and drought (Hamer *et al.* 2008).

WNV virulence in avian hosts: Epizootics of WNV have inflicted substantial morbidity and mortality on bird populations in the United States, with

tens of thousands of individual birds across North America testing positive for WNV. Infection with WNV was responsible for a particularly marked decline in American crows (*Corvus brachyrhynchos*; LaDeau *et al.* 2007). For example, crow abundance in Queens, NY declined by 90% (Hochachka *et al.* 2004). However, the largest negative impact on crow populations by state was found in Illinois (Foppa *et al.* 2011). Additionally, the authors noted a substantial regional decline in American robin and house sparrow populations due to WNV infection. While viremia and immune response to arboviruses are highly variable among susceptible avian hosts (Kuno 2001; Komar *et al.* 2003), the emergence of high avian mortality associated with WNV infection in the United States and Israel suggests the evolution of a more bird-virulent strain (Petersen & Roehrig 2001). Brault *et al.* (2004) found that crows experimentally infected with the North American NY99 strain, which has a $\geq$ 99.7% nucleotide identity to the Israeli 1998 strain, experienced 100% mortality compared to birds infected with Old World strains from Kenya (KEN) and Australia (Kinjun), supporting the genetic alteration of the virus. In a parallel evaluation, Langevin *et al.* (2005) examined WNV strain virulence in house sparrows and found that the NY99 and KEN strain (which differs in nucleotide identity from the NY99 by 3.4%) elicited significantly higher mortality and viremia compared to the Australian (KUN) strain, a genetically distant Lineage I strain. The authors attributed the difference in response to the immunologic naiveté of American crows to WNV compared to the house sparrow, a recently introduced species from Europe which may have been genetically selected for host resistance to historically circulating WNV

strains. Therefore, the combination of a more virulent strain and lack of previous exposure to WNV may have contributed to the extensive mortality of North American birds and could continue to pose a threat to naïve, rare and endangered avian species. In contrast, Peterson *et al.* (2004) suggested that the "dilution effect" might dampen the risk of an epizootic in tropical areas due to increased biodiversity compared with temperate regions.

WNV pathogenesis and histopathology: Buckley *et al.* (2006) demonstrated the replication of WNV in birds by the presence of non-structural protein (NS1), an RNA-binding protein required for viral replication and antibody recognition. Viremia, in the experimentally infected bird, has been shown to occur within 24 to 48 hours (Komar *et al.* 2003; Brault *et al.* 2004) followed by serum protective antibodies in those birds that survive infection (Styer *et al.* 2006; Stamm 1966). Birds that succumb usually die four to eight days post-infection (Komar *et al.* 2003). Following viremia and after spread of WNV to visceral organs such as the kidney and spleen, WNV spreads to the brain and spinal cord within three to five days in rodent models (Mehlhop *et al.* 2005). WNV was detected in the brain tissue of 88% of birds necropsied during the 1999 outbreak in New York. Brain lesions in 93% of these birds were marked by severe hemorrhage and there were multiple cases of meningitis (Steele *et al.* 2000). Peripheral blood monocytes and tissue macrophages appeared to be significant targets of the virus with extensive multiple organ changes (Steele *et al.* 2000). Necropsies of naturally infected American crows revealed that WNV antigen was widely disseminated in numerous organs with macrophages playing a key role in

the pathogenesis (Wunschmann *et al.* 2004). In immune-competent rodent models, WNV is largely cleared from the serum and peripheral organs by the end of the first week with the development of central nervous system infection in a subset of individuals exhibiting pathology similar to humans, and clearance of the virus from all tissues compartments within two to three weeks in surviving animals (Samuel & Diamond 2006). However, arboviral persistence for up to ten months has been detected at high levels in multiple organs of infected birds, with recrudescence or reactivation of the virus in infected birds hypothesized as a mechanism for local viral persistence (Reeves *et al.* 1958; Reeves 1990; Reisen *et al.* 2000; Gruwell *et al.* 2000; Reisen *et al.* 2003*a*; Reisen *et al.* 2006*b*; Reisen & Brault 2007; Kwan *et al.* 2012).

Avian immunity: Avian immunity, most commonly studied in poultry, is a complex system involving multiple cell types and soluble factors (i.e., antibodies) that must act together to provide an effective response to pathogenic challenge and can generally be compared to mammal immune mechanisms despite fundamental structural differences (Gerlach 1997). As in mammals, the immune system in birds is composed of the innate system with physical and chemical barriers, blood proteins such as complement and phagocytic cells as well as the adaptive system which includes maternal antibodies and humoral and cell-mediated immunity. In birds, the thymus and bursa of Fabricius are responsible for the antibody titer detected after an infection or immunization (Johnson 2005). The thymus is the primary site of T lymphocyte maturation and a less clearly defined role in the antibody response (Thorbecke *et al.* 1968; King & McClelland

1984). King & McClelland (1984) suggested that the thymus, rather than atrophying with age as in poultry, may re-enlarge after the annual breeding cycle in wild birds. The bursa of Fabricius, unique to birds, is the primary site of antibody producing B lymphocytes and the first site of immunoglobulin isotype IgM synthesis preceding the formation of IgG type antibodies (also called IgY in birds; Thorbecke *et al.* 1968). B cells migrate in young birds to secondary sites such as the spleen, which along with the bursa and bursa-dependent secondary lymphoid tissues, are responsible for the synthesis of circulating antibodies against invading microorganisms (King & McClelland 1984; Kollias 1986). The humoral immune response may take five to ten days to respond to a novel antigen but re-exposure of an antigen to a primed host results in a quicker second reaction (two to three days for an anamnestic response) as well as higher antibody titers (Gerlach 1997). Antibody titers reflect the degree to which blood serum can be diluted and still show protection toward a specific antigen (Zinkernagel 2003).

WNV antibodies: Natural (IgM) antibodies, an important link between the innate and adaptive immune response, are produced in the absence of antigenic stimulation and are present in serum long before the adaptive immune response is activated (Ochsenbein *et al.* 1999). Although their role is often debated, they may function in early resistance against viral infections by activating the classical complement pathway and clearing virus from the blood (Ochsenbein & Zinkernagel 2000). Acquired antibodies, however, appear to play an important role against WNV infection by preventing viral replication and limiting viral dissemination (Diamond *et al.* 2003*a*). Neutralizing antibodies interfere with

viral receptor binding, while the majority of virus-specific antibodies, which lack neutralizing capabilities, activate the complement system, promote cellular cytotoxicity and augment phagocytosis (Hangartner *et al.* 2006). Diamond *et al.* (2003*a*) detected WNV specific IgM antibodies in wild type mice (*Mus musculus*) four days after WNV infection concurrent with detection of high-grade viremia in immune-deficient mice (μMT). Despite the neutralizing capabilities of these IgM antibodies, they could not eradicate infection in the μMT mice treated with serum from the 4-day post infection wild type mice. All μMT mice in this experiment eventually succumbed to the virus. Only immune serum containing both anti-WNV IgM and IgG prevented morbidity and mortality in the μMT mice suggesting that the triggering of IgG by IgM was necessary to eradicate infection.

Immune IgG is believed to protect against WNV not only by direct neutralization of receptor binding but also by other mechanisms such as complement-mediated viral lysis or antibody dependent cytotoxicity (Diamond *et al.* 2003*b*). In further studies, the protective function against WNV of both natural IgM, without specific stimulation, and induced IgM were examined. Mice deficient in the production of secreted IgM but capable of secreting other immunoglobulin isotypes uniformly succumbed to WNV infection (Diamond *et al.* 2003*b*). Because natural IgM enhances phagocytosis, activates complement and primes the adaptive immune response, the authors suggested that a depressed specific IgG response contributed to lethal infection. Furthermore, low (≤ 1:25) anti-WNV induced IgM titers at day 4 post-infection resulted in mortality suggesting the importance of a strong anti-WNV IgM early in the course of

infection. Finally, Samuel & Diamond (2006) found a correlation between high-titer antibodies and protection against challenge in subsequent WNV vaccine studies.

Other immune components: The complement system is an important part of the innate immune system involved in pathogen recognition and clearance and has been demonstrated to control WNV infection in rodent studies by inducing a protective antibody response (Mehlhop *et al.* 2005). Further, WNV disseminated earlier into the CNS system of rodents deficient in certain complement proteins, and this caused deficits in both B and T cell responses that led to increased mortality (Mehlhop & Diamond 2006). Rodent studies have also shown the crucial role of T cells in clearing WNV from CNS tissues (Stewart *et al.* 2011). Finally, results of a study examining the role of cell-mediated immunity (CMI) in the absence of a humoral response against Newcastle virus in domestic chickens (*Gallus gallus domesticus*) found that CMI by itself was not protective against viral challenge (Reynolds & Maraqa 2000). The authors suggested that although CMI should not be discounted in limiting viral replication, antibodies were key components to protective immunity. All aspects of the immune system may be required in a host's defense against a pathogen. Responses may vary among individuals and populations and may be pathogen dependent (Matson *et al.* 2006*a*, Hawley & Altizer 2011). A full discussion of this topic, however, is beyond the scope of this paper, as the immune system is "arguably one of the most complex phenomena in biology" (Martinez *et al.* 2003).

Arboviral antibody response in birds: Arboviral antibody production, or seroconversion, can be detected around day 4 in birds (Komar *et al.* 2003). McLean *et al.* (1983) found that high antibody titers (equal to or greater than 1:320) in house sparrows experimentally inoculated with SLE were temporally related to inoculation, occurring within six weeks of viremia. However, the absence of a detectable antibody does not necessarily imply a lack of exposure (Anderson & May 1991) because individuals or species could produce short-lived antibodies or be refractory to infection (i.e., do not become infected or produce antibodies; Reisen *et al.* 2003*a*). For example, necropsy results of a bird 234 days post-inoculation with WEE indicated a viremia with a lack of detectable antibodies (Reeves 1990).

Seroconversion to WNV has been shown to occur within eight days in naïve domestic chickens, a sentinel species due to its relative resistance to WNV disease (Langevin *et al.* 2001; Fair *et al.* 2011). Experimental WNV infection in rock doves (or domestic pigeons; *Columba livia domestica*) resulted in an antibody response that rose early, fell, and then rose again between 3-7 weeks post inoculation (Komar *et al.* 2001). The ability to stimulate seroconversion in WNV vaccine studies in birds has resulted in variable results. Nusbaum *et al.* (2003) suggested that the failure of a killed equine vaccine to initiate a protective immune response in Chilean flamingos (*Phoenicopterus chilensis*) and red-tailed hawks (*Buteo jamaicensis*) could have been due to the inactivation of the virus. However, a killed equine vaccine used in a high dosage booster regimen resulted in seroconversion in multiple raptor species (Johnson 2005). Okeson *et al.* (2007)

suggested that variation among species in immune response to pathogenic challenge may be primarily due to underlying genetic and immune-competence differences after two of five bird species seroconverted to an inactivated whole WNV virus vaccine. Reisen *et al.* (2003*a*, 2003*b*) also found variable antibody response in birds experimentally inoculated with WEE and SLE viruses. It is not clear, however, the extent to which serum antibody levels predict generalized protection of an individual bird against a pathogen, although birds with high titers are better protected than those with low titers against certain infections (Gerlach 1997; Kuno 2001). Morbid eastern screech owls (*Megascops asio*) experimentally infected with WNV maintained a high level of viremia for 5-6 days before death despite initiating an antibody response (Nemeth *et al.* 2006). However, the authors found that those individuals that survived infection had a fourfold greater antibody titer than the morbid owls.

WNV antibody detection: WNV antibodies in birds can be detected serologically by plaque reduction neutralization test (PRNT), hemagglutination inhibition assay (HIA), and enzyme-linked immune-sorbent assay (ELISA) (Weingartl *et al.* 2003; Blitvich *et al.* 2003). However, PRNT is not suited for early diagnosis and can be expensive and laborious, and HIA can cross react to other closely related *flaviviruses* such as SLE (Ebel *et al.* 2002; Blitvich *et al.* 2003). Although direct ELISAs are sensitive and inexpensive, they are not suitable for screening from a wide variety of bird species due to the need for species specific secondary (reporter) antibodies (Martinez *et al.* 2003; Blitvich *et al.* 2003). An epitope-blocking ELISA was developed which measures the ability

of antibodies present in sera to block the binding of a monoclonal antibody (MAb) to a WNV-specific epitope on the NS1 protein in various species of birds and mammals (Ebel *et al.* 2002; Blitvich *et al.* 2003; Loroño-Pino *et al.* 2009). For North American bird species, MAb 6B6C-1 detects *flavivirus* cross-reactive antibodies in migratory and resident avian populations and MAb 3.1112G has been shown to differentiate between WNV and SLE (Blitvich *et al.* 2003). This assay has been shown to be comparable to PRNTs in early detection and superior in sensitivity for the examination of serum samples collected at 14 days post-infection (Blitvich *et al.* 2003). Nonetheless, disparities within and between assays can complicate the analysis of serologic data. For instance, McLean *et al.* (1983) found that antibody titers rose more rapidly with HIA than with PRNT and PRNT antibodies persisted longer, although the geometric mean antibody titers (GMT) were similar after two years. Reisen *et al.* (1992) found similar results and, though most studies examining antibody profiles utilize confirmatory PRNTs, the authors found that seroconversions to SLE detected by HIA were frequently undetectable by PRNT. Similarly, Reisen *et al.* (2003*a*) reported that enzyme immunoassays (EIAs) accurately detected SLE and WEE antibodies in infected birds, while PRNT appeared to be less sensitive for detection in infected birds during the first four weeks post-inoculation. Additionally, although EIA remained positive after four months, PRNT antibodies were no longer detectable. In further studies, PRNT antibodies to SLE decayed rapidly while EIA antibodies showed long-term persistence (Reisen *et al.* 2003*c*).

Antibody persistence: In the absence of circulating infection or antigen re-exposure, antibody titers have been shown to decrease gradually with natural infection, as well as after immunization (Zinkernagel 2003). The mechanisms responsible for immune persistence, however, are not clearly understood. Increased precursor B and T cells as well as memory B-cells produced by long-lived plasma cells have been shown to circulate in the absence of antigen (Zinkernagel *et al.* 1996; Crotty & Ahmed 2004). In antigen-dependent immunity, B cell differentiation into plasma cells is driven by persisting antigen from chronic infections or periodic low-level re-infections in the environment followed by the production of antibodies by memory B cells (Zinkernagel *et al.* 1996; Zinkernagel & Hengartner 2006; Amanna *et al.* 2007). However, in a study of human antibody persistence, antibody decay varied by antigen, with little correlation between peripheral B-cells and antibody titers suggesting that memory B-cells and antibody producing plasma cells may play different roles in the maintenance of protective immunity (Amanna *et al.* 2007).

While the need for antigenic re-exposure to obtain serologic memory continues to be debated (Kuno *et al.* 2001), Zinkernagel & Hengartner (2006) suggested that elevated antibody titers were a reflection of increased immunity or resistance to re-infection. The half-lives of detectable arboviral antibody responses in birds appear to be shorter than those of mammals (Stamm 1966; Kuno 2001). Avian antibody levels peak after several weeks, plateau for several months, and then decline below levels considered positive (Stamm 1966; Kuno 2001). The primary antibody response upon initial exposure to an antigen is first

dominated by IgM before switching to IgG (Duffy *et al.* 2000), and effective influenza vaccines are based on the induction of strain-specific IgG neutralizing antibodies (Tumpey *et al.* 2004). Griffiths & McClain (1985), however, found that IgG in chickens experimentally inoculated with EEE peaked within a few days and disappeared within 30 days, whereas the IgM immunoglobulin secondary response was relatively long-lasting. Similarly, Calisher *et al.* (1986) found that IgM antibodies to EEE persisted in chickens for more than 8 months. However, detectable IgG antibodies in the same study persisted through the 90 day study. The authors suggested that the use of a live virus in their study attributed to the longer lasting IgG antibodies. Reisen *et al.* (1992; 2001; 2003*b*; 2004*a*) observed a protective antibody response to WEE or SLE challenge in experimental inoculation studies up to 52 weeks even with immunosuppression, with a more rapid antibody decay observed with SLE antibodies. Chickens experimentally infected with WNV developed neutralizing antibodies as early as 7 days post-inoculation (dpi) with increasing titers by day 28 dpi (Langevin *et al.* 2001). Neutralizing antibodies to WNV appear to be long lasting (up to 36 months) in laboratory (Reisen *et al.* 2006*b*; Nemeth *et al.* 2009*a*) and flight cage studies (Wheeler & Reisen 2009). However, the temporal dynamics of the immune response in natural populations is still relatively unknown. Main *et al.* (1988) observed variable neutralizing antibody durations by species in a long-term recapture study of 17 passerine species. They were analyzing antibodies to an aviruluent strain of WEE (Highlands J virus) as well as EEE. Meister *et al.* (2008) observed that after natural infection with Usutu Virus (USUV) family

Flaviviridae, anti-USUV antibody titers in owl species dropped markedly within six months suggesting that *flavivirus* antibody titers were generally not very robust or long lasting. In WNV studies, Gibbs *et al.* (2005) observed antibody persistence over 15 months in naturally infected Rock pigeons (*Columba livia*). Additionally, naturally induced WNV neutralizing antibodies were observed in raptors throughout a four year study with secondary rises in titers presumably due to re-exposure and no clinical signs of disease, suggestive of a persistent protective response (Nemeth *et al.* 2008). Finally, Kwan *et al.* (2012) performed a long-term recapture study on wild house finches and house sparrows and found that several birds remained positive for WNV antibodies throughout the study, with one individual house finch consistently positive for five years. In contrast to captive studies, overall antibody persistence appeared to wane, perhaps as a function of age.

*Factors affecting immunity***:** Pathogen resistance in wild birds may be a costly, but necessary, energy investment influenced by factors such as physiology, environmental stressors, and seasonality (Staszewski *et al.* 2007; Hawley & Altizer 2011). For example, poor nutrition and parasitic or pathogenic infections can reduce CMI and cause bursal depletion and splenic lymphoid necrosis, thereby negatively impacting antibody production (Schmidt 1997; Glick *et al.* 1981; 1983). Additionally, stressful events cause the adrenal release of the primary steroid corticosterone. While initial release of corticosterone may allow for a more robust immune response to challenge in some transient stress-related situations (Hawley *et al.* 2006; Rubenstein *et al.* 2008; Martin 2009), studies

reflect that in general, chronically increased corticosterone results in immunosuppression by reducing the ability of the macrophages to facilitate antibody production (Cockrem & Silverin 2002; Johnson 2005; Matson *et al.* 2006*b*; Millet *et al.* 2007; Koutsos & Klasing 2008).

Elevated circulating levels of testosterone can also cause bursal depletion and elevation of corticosterone resulting in immunosuppression (Schmidt 1997). For example, Duffy *et al.* (2000) found that testosterone concentrations within the high normal physiological range and elevated corticosterone levels suppressed humoral and cell-mediated immunity in European starlings (*Sturnus vulgaris*). Additionally, Mougeot *et al.* (2005) found that testosterone implanted red grouse (*Lagopus lagopus scotica*) were significantly more susceptible to parasite infection. Finally, other studies have demonstrated the immunosupressive effects of elevated testosterone in house finches and house sparrows (Deviche & Cortez 2005; Evans *et al.* 2000).

Reproductive efforts and gender have also been reported to effect immune function. Deerenberg *et al.* (1997) quantified antibody production to sheep red blood cells in zebra finches (*Taeniopygia guttata*) and found that all non-breeding birds produced antibodies but only 47% of breeding birds produced antibodies. Additionally, the antibody response was negatively correlated to brood size. Parejo & Silva (2009) reported a weaker acquired immunity in males than females and Marshall *et al.* (2006) found a statistically significant sex-based difference in WNV antibody seroprevalence with females more likely to be seropositive.

However, other studies have reported no difference in gender based antibody response (Ringia *et al.* 2004; Godsey *et al.* 2005).

Reisen *et al.* (2003*c*) found that experimentally induced immunosuppression in house finches enhanced and lengthened WEE and SLE viremias and altered antibody response, though not in a consistent manner. Owen & Moore (2006; 2008) examined the immune function of several thrush species and found a significant decrease in cell-mediated immune response in migrating individuals compared to conspecifics outside the migratory season, with a detectable immunosuppression associated solely with the onset of migratory restlessness in a captive study. The authors suggested that immunosuppression could be an adaptive trade-off for the energetic costs of migration, resulting in increased susceptibility to disease transmission or in recrudescence (re-activation) of a latent infection. However, Buehler *et al.* (2008), in a study of immunity in captive versus free-living red knots (*Calidris canutus*), suggested that birds could use different immune strategies, such as non-specific immunity, in differing environments, investing more in defenses when disease risk is higher.

Finally, stress-related immunosuppression has been hypothesized as the cause of viral recrudescence of latent infections (Reeves *et al.* 1958; Reeves 1990; Reisen *et al.* 2001; Reisen *et al.* 2003*a*, Reisen & Brault 2007; Wheeler & Reisen 2009) since some arboviruses can remain infectious in particular organs or tissues despite high neutralizing antibody titers in serum (Kuno & Chang 2005). For instance, infectious WNV has been demonstrated to persist in rodents for up to four months with viral RNA detectable in tissues for up to six months despite the

presence of viral specific immune cells (Stewart *et al.* 2011). Nemeth *et al.* (2009*b*) found infectious WNV in house sparrows through 43 days and viral RNA through 65 days. However, detection of encephalitis relapses in birds has thus far been unsuccessful (Reeves 1990; Reisen *et al.* 2001; Reisen *et al.* 2003*b*; Reisen *et al.* 2003*c*; Reisen *et al.* 2004*a*; Reisen *et al*. 2004*b*; Gibbs *et al.* 2005; Nemeth *et al.* 2009*b*). Owen *et al.* (2010) found no effects of either testosterone or artificially induced migratory behavior on infectious status of previously viremic gray catbirds (*Dumetella carolinensis)* infected with WNV. Although, the authors did not measure antibody titers in the study, they suggested that low antibody titers could affect the ability for reactivation to a latent infection or re-infection.

Host age: Scott and Edman (1991) postulated that the immune status of young birds could affect arboviral transmission and amplification. Therefore, the waning of maternal antibodies to WNV in nestling and hatch-year birds could potentially provide a large population of susceptible hosts contributing to amplification. Meister *et al.* (2008) found that passively acquired maternal antibodies to USUV were detectable for up to two months whereas Buckley *et al.* (2006) found a decline within three weeks in sentinel hatchling chickens with WNV antibodies. Gibbs *et al.* (2005) found passively transferred WNV antibodies in rock doves only up to 30 days after hatching and Nemeth *et al.* (2008) found that maternal neutralizing antibodies to WNV appeared to be relatively short-lived in house sparrows, a passerine implicated as an important WNV reservoir host. Loss *et al.* (2009*b*) studied the role of nestling birds on amplification of WNV in the Chicago study site and found that nestling passerines

were not important for transmission. However, among hatch-year birds in the same area, there was a significant positive correlation between *Culex* WNV infection and subsequent seropositive birds, suggesting that hatch-year birds facilitate rapid WNV amplification by contributing to the susceptible population after waning of maternal antibodies (Hamer *et al.* 2008).

Age may affect other immune components as well. Results of a study by Palacios *et al.* (2009) examining natural antibodies and complement-mediated cell lysis in free-living tree swallows (*Tachycineta bicolor*) indicated that these innate immune components were not fully developed in fledglings. The authors suggested that humoral responses to T-cell dependent antigens developed later than T-cell independent antigens supporting the poor development of the adaptive immune system in young birds. Additionally, Parejo & Silva (2009) found higher levels of natural antibodies and complement in adult wild kestrels than in young birds, with adult birds less prone to parasitism. Tumpey *et al.* (2004) found that serum antibody responses after vaccination against avian influenza were also age dependent, with older turkeys reacting with a stronger and more consistent response after a single inoculation and younger turkeys requiring a second dose to elicit a comparable response. Santaella *et al.* (2005) found a higher WNV seroprevalence in adult white-tailed deer (*Odocoileus virginianus*) compared to yearlings. Gibbs *et al.* (2006) suggested that higher mortality in hatch-year birds could have contributed to the higher WNV antibody prevalence in adult birds compared to hatch-year birds in a Georgia study. However, others have found no significant difference between adult and hatch-year WNV seroprevalence (Garvin

et al. 2004; Ringia *et al.* 2004). In human studies of antibody persistence, antibody decay occurred in older individuals exposed to infection early in life (Anderson & May 1991). Similarly, Staszewski *et al.* (2007) discussed the waning of antibody persistence in older reindeer (*Rangifer tarandus tarandus*) in a study examining specific antibodies. WNV antibody decay in human patients however, appeared to be more pronounced in those younger than 65 years of age (Roehrig *et al.* 2003). Finally, Hukkanen *et al.* (2006) observed high individual variation in antibody persistence to WNV in pig-tailed macaques (*Macaca nemestrina*). Kwan *et al.* (2012) suggested that antibody decay in long-lived individuals may have contributed to the overall waning of WNV persistence observed in a California study. Therefore, antibody persistence may have age-dependent influence.

Avian WNV competence: WNV infection has been detected in at least 300 native and exotic bird species in North America; however, only a small number of species are likely to be WNV competent hosts, with species of the order Passeriformes representing the most competent reservoirs for WNV transmission (Marra *et al.* 2004; Peterson *et al.* 2004; Kilpatrick *et al.* 2006). Avian reservoir competence for WNV was determined by experimentally infecting 25 avian species in the New York area, the site of the 1999 emergence outbreak (Komar *et al.* 2003). The five most competent species for WNV were passerines: blue jay (*Cyanocitta cristata*), common grackle (*Quiscalus quiscula*), house finch, American crow and house sparrow. They sustained a viremic titer greater than 10^5 PFU/mL. These species had an average viremia that was greater in magnitude

and duration than species in other orders (Komar *et al.* 2003). It is not yet clear what mechanisms affect the susceptibility and competence variability among avian hosts to WNV, but they may include factors such as genetics, immune-competency and behavioral ecology (Marra *et al.* 2004).

Avian WNV seroprevalence and the "dilution effect": Seroconversion detection has been used for monitoring the spread of WNV as well as other arboviruses such as EEE and WEE (Weingartl *et al.* 2003). WNV seroprevalence, the number of individuals in a population with WNV antibodies, could also be used as a measure of host exposure and survival. Wilcox *et al.* (2007) suggested that bird species with lower WNV-associated mortality rates would have higher antibody prevalence such as the higher seroprevalence they found in fish crows (*Corvus ossifragus*) compared to American crows. In larger studies, northern cardinals and gray catbirds were among the highest seroprevalent species in a Georgia study (Gibbs *et al.* 2006). Cardinals also were the highest seroprevalent species in a Florida survey along with house sparrows (Godsey, Jr. *et al.* 2005). Across Illinois, Beveroth *et al.* (2006) found that although a large number of avian species were exposed to WNV from 2001 through 2004, American robins, house sparrows and northern cardinals, all competent hosts, were the most common seroprevalent species. Although mourning doves, another highly seroprevalent species in northern and central Illinois, are not considered highly competent due to their short viremia, Beveroth *et al.* (2006) suggested that the importance of this species in WNV transmission may be offset by its abundance. However, others have found that while host selection may be influenced by

abundance, certain species of birds appear to be overused (e.g., American robin) or underused (e.g., house sparrow) relative to their abundance, suggesting that other factors may be involved in host selection (Kilpatrick *et al.* 2006; Hamer *et al.* 2009). Still, abundant species with low competency could be important to the enzootic cycle by dampening WNV transmission. Several studies have examined this "dilution effect" in relation to WNV transmission by examining host heterogeneity and WNV infection. These studies have found that high host diversity resulted in decreased disease risk, including a zoo-prophylaxis effect, or decreased human infection, as avian hosts diverted vector meals away from humans (Ezenwa *et al.* 2006; Swaddle & Calos 2008; Allan *et al.* 2009; Koening *et al.* 2010).

American robins and "super-spreader" species: Studies by Kilpatrick *et al.* (2006) suggest that transmission of WNV is dominated by heterogeneity with intense transmission in only a few key "super-spreader" species, such as the American robin, important not only for WNV transmission but bird population impacts. "Super-spreader" species contribute disproportionately to disease transmission and are characterized by higher infectiousness or more frequent contact with susceptible hosts (Hawley & Altizer 2011). American robins are preferentially fed upon by the mosquitoes important for WNV transmission in the eastern United States, giving rise to the majority of secondary infections (Kilpatrick *et al.* 2006). In the Chicago area, Hamer *et al.* (2009) examined avian amplification fractions, a measure of the avian species-specific contribution to WNV transmission, and found that 66% of the infectious *Culex pipiens*

mosquitoes became infected by feeding on only a few competent species of birds, including American robins (35%), blue jays (17%) and house finches (15%). Rather than host preference though, Suom *et al.* (2010) suggested that ecological, spatial and avian behavioral factors influence successful blood meals from preferred hosts. American robins are considered urban tolerant birds with large communal roosts which may attract large numbers of mosquitoes. Their low anti-mosquito behavior could allow for a full blood meal by mosquitoes (Hamer *et al.* 2009). Girard *et al.* (2011) examined innate immune response against bacterial pathogens and found that American robins had significantly lower bactericidal capacity than house sparrows or gray catbirds, consistent with the variability of WNV competence in these hosts.

House sparrows: Studies in the 1950's in the Egyptian Nile Delta found that of 420 domestic and wild birds sampled, the hooded crow (*Corvus cornix*) and house sparrow had the highest WNV antibody seroprevalence at 65% and 42%, respectively (Hayes 1989). At the end of the 1974 WNV epidemic in South Africa, a high rate of seroprevalence was again found in the house sparrow (50%), a species with extremely large roosts observed at the time (Hayes 1989). In several areas of the United States, the house sparrow serves as the major host species for SLE transmission (McLean & Bowen 1980). A high competency reservoir host for WNV, the house sparrow is abundant and ubiquitous in North America (Komar *et al.* 2001). The most abundant seropositive hatch-year birds in the Chicago study site in 2005 were house sparrows, northern cardinals, American robins, and gray catbirds (Hamer *et al.* 2008). However, Hamer *et al.* (2009)

found that house sparrows appeared to be underused in relation to their high abundance in urban settings. In a subsequent study, Hamer *et al.* (2011) examined vector host use and force of infection (defined as the rate at which susceptible hosts acquire infection or seroconvert) at a fine spatial scale in the Chicago area and found that American robins and house sparrows produced 95.8% of the infectious *Culex pipiens* mosquitoes. These results suggest the importance of incorporating factors such as vector-host contact and host competence when analyzing the influence of host community structure on WNV transmission (Anderson & May 1991).

SIR modeling and herd immunity: In order to control infectious disease, a full appreciation of parasite, host and environmental relationships is needed because small changes in any of these factors can have dramatic impacts upon the spread of disease (Smith 1970). Zoonotic disease spread is dependent on many factors with one of the most important drivers being the proportion of the host population which is susceptible to infection (Smith 1970; Earn 2008). Those individuals that recover from infection will typically develop immunity against re-infection, although the intensity and duration of antibody response may be highly variable, probably due to genetic factors (Anderson & May 1991). For those individuals infected, the duration of infection is typically short relative to the expected life span of a host (Anderson & May 1991). Very few data are available on the natural longevity of wild birds; however, the potential longevity of several avian species has been obtained from the records of captive individuals under optimal or near-optimal conditions. The natural longevity of American robins is

at least nine or ten years, although the species has a potential longevity of at least 13 years and perhaps as much as 20 years (Farner 1945). Similarly, longevity in house sparrows may be greater than 13 years in the wild with annual survival of adult individuals at 57% (Nemeth *et al.* 2009*b*). Long-lived individuals could, therefore, seroconvert and become immune for a couple of seasons and then serorevert dependent on the rate of antibody decay and resume the role of a susceptible host. However, it should be noted that the average natural longevity for wild birds is often only a fraction of the potential lifespan, with 1.9 years a mean longevity among wild passerine species (Farner 1945). Day and Stark (1999) suggested that the drop in hemagglutination inhibition (HI) antibody titers to SLE at the end of 1991 following the 1990 epidemic could have been due to the replacement of short-lived infected mourning doves by non-immunes. Therefore, the temporal dynamics of natural immunity is also of epidemiological importance as it modifies the number of susceptible individuals in a population.

Mathematical modeling is used to examine the spread of infectious diseases in a population while incorporating individual characteristics of behavior and biology (Mishra *et al.* 2011). Stochastic (random) models are utilized when chance fluctuations are important. Deterministic, or compartmental, models (Figure 2) are used to interpret epidemiological trends and predict the spread of infectious disease at a population level (Trottier & Philippe 2001; Mishra *et al.* 2011). Individuals in a deterministic model are categorized into various compartments based on stages of infection experienced during the life of an individual (Mishra *et al.* 2011). For example, the SIR model (Figure 3) has three

host categories: susceptible individuals (S), infectious individuals (I), and recovered (immune) individuals (R). The total host population size, therefore, is N = S + I + R (Earn 2008; Mishra *et al.* 2011). In this model, individuals are considered susceptible at birth but, importantly, recovered individuals can become susceptible as their protective immunity decays (Trottier & Philippe 2001). In the basic SIR model, we assume homogenous (random) contact where each host has equal probability of contact with another host, so age, social structure or behavior are ignored (Trottier & Philippe 2001; Mishra *et al.* 2011).

This simplistic model also considers birth and death rates as stable, although population growth or dampening could alter the dynamics of transmission (Trottier & Philippe 2001). Additionally, the more simplistic model does not assume varying infection risk experienced by individuals as they age (Fine 1993). For example, in this model an individual with maternal antibodies to WNV will not become susceptible until waning of this passive immunity (Fine 1993). More complex combinations of the compartmental states can be used or other parameters can be added based on the biology of the infectious disease, details of a particular model or availability of data (Trottier & Philippe 2001). For example, vector-borne diseases such as WNV depend on links between host and vector, so Wonham *et al.* (2004) extended the classic SIR model to an eight-compartment model for WNV. Their model adds mosquito populations, allows for cross-infection between birds and mosquitoes in one season, and identifies removed birds caused by mortality. The more simplistic models, however, can still allow for meaningful predictions (Trottier & Philippe 2001).

With an SIR model, we can track movement between the compartments by applying parameters or variables based on estimates from empirical data or literature review to the mathematical equations governing the model (Mishra *et al.* 2011). For instance, the product of transmission probability (β), contact pattern (c') and duration of infectiousness (D, where D=1/rate of recovery) will give us R_0, or basic reproduction number, defined as the average number of hosts that become infected from a single infectious host in a completely susceptible population (Wonham *et al.* 2004). If R_0 is greater than 1, the pathogen is able to invade the susceptible population and an epidemic builds up (Trottier & Philippe 2001). Control programs such as mosquito abatement could therefore effectively reduce any of these components and directly impact R_0 (Mishra *et al.* 2011). However, when individuals acquire immunity through natural antibody production or immunization, the population is no longer entirely susceptible (Garnett 2005), so it is more appropriate to consider the effective reproduction number R_e, a time-dependent quantity that accounts for the population's reduced susceptibility (Cintr´on-Arias *et al.* 2009). The product of R_0 and the fraction of the population that is still susceptible (S_0) give us the effective reproductive number R_e. R_e measures protection against disease transmission in a population, because the higher the proportion of the population that is immune, the lower R_e (Garnett 2005). Control of infectious disease spread could be achieved by increasing this proportion of immune individuals above a critical level known as the herd immunity threshold (S_h). The herd immunity threshold is the proportion of immune individuals which must be exceeded if disease incidence is to decline

(S_h = 1-1/R_0; Fine *et al.* 2011). The herd immunity threshold represents a density of susceptibles which if exceeded would result in explosive incidence of disease (Fine *et al.* 2011). Immunity to measles in older children reached an estimated 80% by1988 in England (Gay *et al.* 1995). However, after a resurgence of measles incidence occurred, the authors utilized mathematical modeling to identify a critical threshold in excess of 90%. Fine (1993) discussed how most models lack the heterogeneity of real populations, resulting in unrealistically low immunity thresholds. Van Boven *et al.* (2008) determined the approximate "herd immunity threshold" to Newcastle virus following vaccination by examining immune status based on high and low antibody titers ($S_h = R_{low} - 1/R_{low} - R_{high}$) and found that a high fraction of birds (>85%) with high antibody titers was necessary to provide sufficient herd immunity. Therefore, the variability of immune response among species, an assumption most likely omitted from even the most elaborate mathematical models (Fine 1993), could directly impact herd immunity in avian populations affected by WNV. Specifically, it is possible that herd immunity may not be apparent if a certain threshold in antibody titer or seroprevalence has not been met.

Some authors use the term "herd immunity" or "herd effect" to describe the reduced risk of infection among susceptible individuals conferred by the protective presence and proximity of immune individuals (Fine *et al.* 2011). That is, large numbers of immune individuals can essentially reduce the risk of infection in the susceptible population (Katriel & Stone 2010). Most often this term has been used in vaccination programs, such as for measles and smallpox,

where the reduced force of infection among vaccinated individuals simultaneously reduces the exposure of still susceptible hosts (Mishra *et al.* 2011). For example, after the introduction of vaccines against pneumococcal and *Haemophilus* infection, a reduction in disease incidents in populations too old for vaccination was observed as well as a reduction in total disease incidence (Fine *et al.* 2011). Staszewski *et al.* (2007) found that mean anti-*Borrelia* antibody levels of recaptured sea-birds birds varied among years with mean increases in antibody levels related to exposure to ticks in the previous year. Meister *et al.* (2008) discussed the significant decline of Usutu Virus associated mortality in Austrian wild birds in recent years, paralleled with the steady increase in seropositive endemic birds. This pattern suggests a rapid establishment of herd immunity despite continuing viral circulation. Beveroth *et al.* (2006) found temporal variation in WNV seroprevalence in Illinois birds from 2001 to 2004. Seroprevalence increased from 2002 to 2003, decreased in 2004, and was higher in adult birds. In this study the high seroprevalence in 2003 likely reduced the number of susceptible hosts because adult birds of many species have high site fidelity. In a study of WNV seroprevalence in the common coot (*Fulica atra*) over a three year period in Spain, high WNV seroprevalence in 2003-2004 was followed the next year by decreased WNV activity. The authors suggested that effective herd immunity reduced the number of available hosts and, therefore, WNV transmission intensity (Figuerola *et al.* 2007). Finally, Kwan *et al.* (2012) found that elevated avian seroprevalence levels in 2004 was associated with reduced WNV outbreaks in subsequent years during a long-term study in

California. However, the reduction of adult bird herd immunity, presumably by recruitment of juveniles, eventually led to increased WNV human cases in subsequent years.

Some authors have also suggested that herd immunity may develop by super-infection exclusion, in which a pre-existing viral infection prevents subsequent infection with the same or closely related virus. WNV and SLE, for example, have a close antigenic relationship and have been demonstrated to produce strong cross-reactive antibodies (Tesh *et al.* 2002). Tesh *et al.* (2002) observed that in experimental studies with hamsters (*Mesocricetus auratus)*, infection with one or more *flaviviruses*, including SLE, conferred protection against or modification of subsequent infections of WNV. Fang & Reisen (2006) found that house finches experimentally infected with SLE were protected from challenge SLE viremia and had lowered challenge WNV viremia titers. Alternately, previous inoculation with WNV resulted in immunity against both viruses (Fang & Reisen 2006). Finally, Nemeth *et al.* (2009*c*) inoculated WNV antibody positive red-winged blackbirds (*Agelaius phoeniceus*) with JEV and found that individuals produced either significantly higher anti-WNV antibody titers or antibodies indistinguishable between JEV and WNV. Therefore, amplification of WNV could be reduced by herd immunity from other antigenically related viruses. However, Kanesa-Thasan *et al.* (2002) found that JEV immunization in humans did not induce neutralizing antibodies in sufficient titers to protect against subsequent WNV infection. Additionally, Newman *et al.* (2011) found that in the Chicago study site, 40% of WNV positive *Culex*

mosquitoes were also infected with *Culex flavivirus* (*CxFV*), an insect-specific *flavivirus*, demonstrating that mosquitoes could be co-infected with two *flaviviruses*. Similarly, data from a study in Texas indicated that both SLE and WNV could coexist, with no indication of viral displacement (Lillibridge *et al.* 2004).

Finally, in totally naïve populations, there is a dramatic increase in force of infection, a function of the proportion of susceptibles initially present in the population (Katriel & Stone 2010). Additionally, loss of immunity could impact force of infection and thereby increase disease transmission by affecting the number of susceptible individuals. Nemeth *et al.* (2009*a*) discussed how the multiple broods of up to eight chicks per brood in house sparrows could impact the influx of naïve offspring by decreasing the proportion of immune individuals necessary to provide herd immunity. Woolhouse *et al.* (1996) measured individual log antibody titers over time and applied a best fit regression line to estimate the fraction of susceptible individuals at a given time to foot-and-mouth disease virus (FMDV). With this information as well as the measure of vaccine potency from vaccine trials, the authors were able to estimate the R_0 and suggest that although vaccination could be effective in reducing transmission once an epidemic had begun, the absence of an increased vaccine regimen or other control measures would result in almost all susceptible cattle becoming infected. Therefore, the decline of maternal WNV antibodies in hatch-year birds as well as the decay of acquired WNV antibodies in adult birds could impact the rate at

which susceptible hosts enter the population with a subsequent effect on herd immunity.

Objectives: This study will examine the influence of avian herd immunity on WNV activity. The objectives for this study are 1) to utilize serial blood sampling from wild birds to assess natural longevity of WNV antibodies and 2) to assess the influence of avian herd immunity on WNV infection in *Culex* spp. mosquitoes. The hypothesis to be tested is that herd immunity in the avian community will reduce the WNV infection rate in mosquitoes. Herd immunity will be measured as the adult bird seroprevalence and geometric mean antibody titer (GMT) entering the transmission season and WNV activity will be measured by WNV infection in *Culex* spp. mosquito pools.

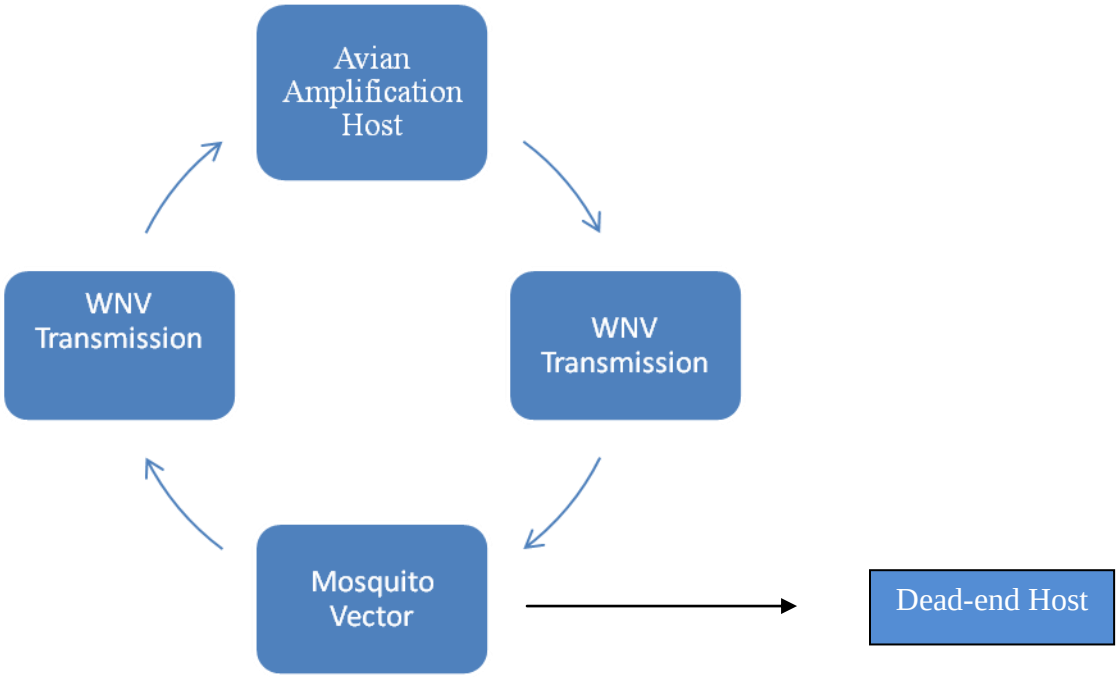

Figure 1. WNV Transmission Cycle

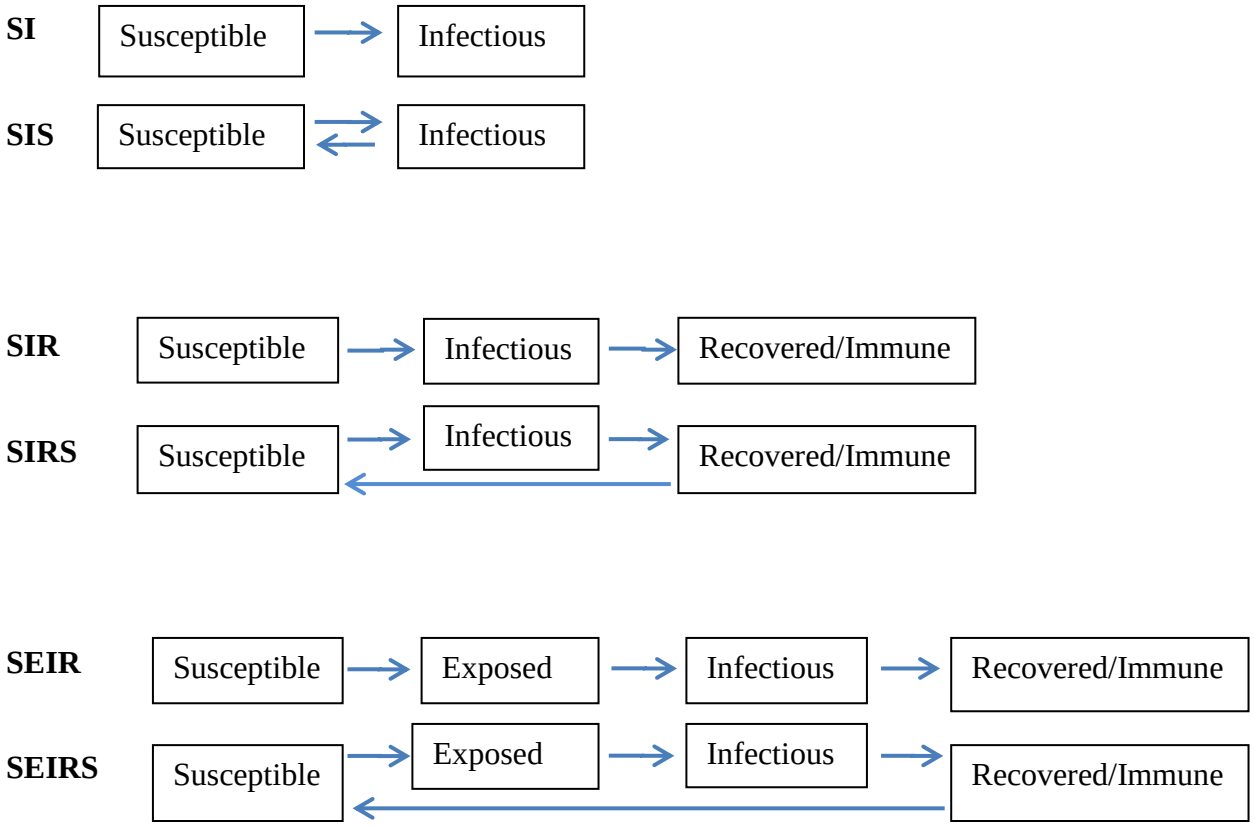

Figure 2. Common Models of Infectious Diseases
Source: Trottier & Philippe 200

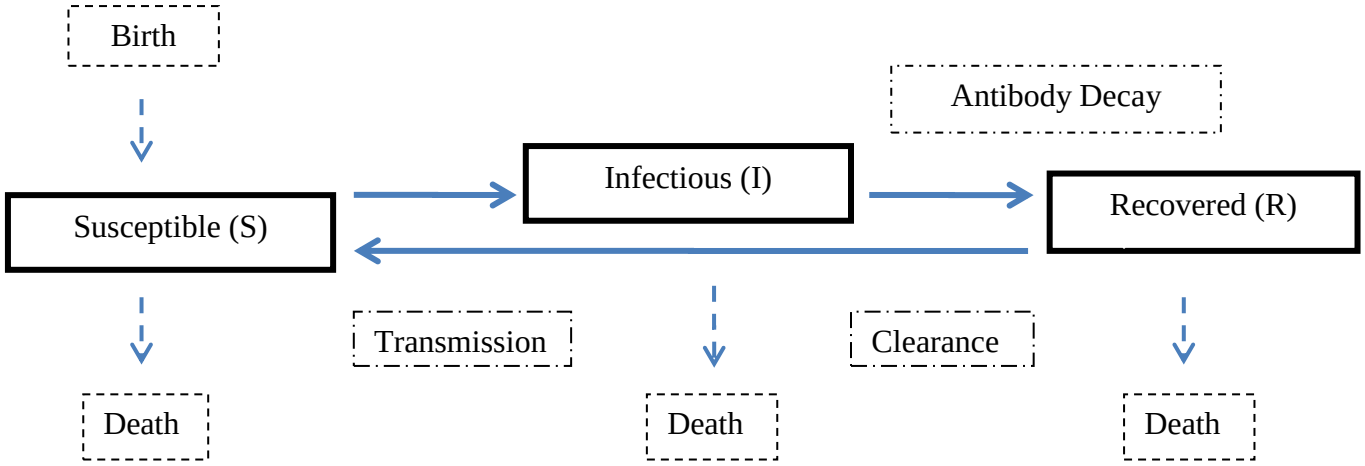

Figure 3. S-I-R model in which recovered individuals lose immunity

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West Nile virus seroreversion and the influence of herd immunity on *Culex* mosquito infection rate in a long-term study of free-ranging birds

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Abstract

The transmission and amplification of West Nile virus (WNV) is driven by the number of susceptible avian hosts. The quantity of susceptible hosts is determined by 1) recruitment of juvenile birds during the breeding season, 2) adult birds previously unexposed to WNV, and 3) adult birds with waning antibody titers. Although rarely tested, avian herd immunity and seroreversion could have a direct impact on *Culex* infection rate, a proxy for the reproductive number of WNV. In this study, we utilized data from a seven year study investigating WNV transmission ecology in suburban Chicago, Illinois. We tested the hypothesis that herd immunity in the avian community has an inverse relationship with WNV infection in *Culex* species mosquitoes. This longitudinal study, with repeated blood samples from individual free-ranging birds, revealed a rate of antibody decay such that most birds have undetectable titers after two years. Additionally, the magnitude of the antibody response, measured by the geometric mean antibody titer, was inversely associated with WNV infection in *Culex* species mosquitoes suggesting that herd immunity depends on the antibody titer magnitude. These results illustrate the need to understand the dynamics of the host immune response in relation to WNV transmission. Utilizing a unique,

long-term data-base, this study contributes to understanding the mechanisms of WNV amplification in the urban environment.

Introduction

Emerging infectious diseases, which are dominated by vector-borne and zoonotic pathogens, are a threat to global biodiversity and public health (Jones *et al*. 2008; Loyd-Smith *et al*. 2009). West Nile virus (WNV), a member of the Japanese encephalitis complex (JEV; genus *Flavivirus*) quickly spread across the United States with unprecedented mortality in humans, horses and birds (Lanciotti *et al*. 1999; Marra *et al*. 2004; Murray *et al*. 2010). Subsequent epidemics and epizootics have confirmed that WNV is now endemic to North America (Artsob *et al*. 2009; Lindsey *et al*. 2010) and is the only zoonotic *Flavivirus* to be identified on six continents (Komar & Clark 2006; Bosch *et al.* 2007). Successful transmission of WNV is dependent on many complex factors including the availability of susceptible hosts, host and vector competency and environmental conditions such as temperature and rainfall (Marra *et al.* 2004). In some years, cycles of accelerating mosquito-bird transmission, or amplification, can increase the risk of exposure to humans (Hamer *et al*. 2008*a*; Theophilides *et al*. 2006). WNV has become the dominant cause of encephalitis in the Unites States (Riemann *et al*. 2008; Davis *et al*. 2008) with 2012 exhibiting the highest number of human WNV cases since its initial detection in 1999 (CDC 2012).

Host susceptibility, proliferation of the pathogen within that host, and the duration of infectiousness of the host help determine the reservoir competency of a host for vector-borne pathogens (Richter *et al*. 2000; Komar *et al*. 2003). While

most mammalian hosts such as horses and humans can be infected and greatly impacted by the WNV, they are considered reproductive dead ends because they do not typically amplify the virus at sufficient concentrations to infect mosquitoes (Marra *et al*. 2004). High competency avian reservoir hosts for WNV exhibit a high and prolonged level of viral presence in the blood, or viremia (Komar *et al*. 2003). Although many North American bird species can develop an infectious viremia, a limited number of species, such as the American robin (*Turdus migratorius*) and house sparrow (*Passer domesticus*) are considered important in driving the transmission (Kilpatrick *et al*. 2006; Hamer *et al*. 2009; 2011; Simpson *et al*. 2011).

The humoral immune system is responsible for serum antibody titers detectable in avian hosts that survive WNV exposure or infection (Johnson 2005). Although the immune mechanisms of protection against WNV are not clearly understood, antibodies appear to be important for WNV clearance and protection against subsequent infection (Diamond *et al*. 2003*a*). In WNV vaccine studies, American crows *(Corvus brachyrhynchos)* with a vaccine induced antibody response were significantly more protected from mortality than those without anti-WNV antibodies (Bunning *et al*. 2007). Although recovered individuals typically develop immunity against re-infection (Anderson & May 1991) there appears to be considerable variation in avian immune response and antibody durations among individuals, populations, and pathogens (Main *et al*. 1988; Kuno 2001; Reisen *et al.* 2001; 2003*a*; 2004; Davison *et al*. 2008). Variation of serologic response to WNV may be primarily due to underlying genetic and

immune-competence differences (Johnson 2005; Hawley & Altizer 2011). Serologic response may be negatively influenced by factors such as physiology, environmental stressors, reproduction and seasonality (Millet *et al.* 2007; Okeson *et al*. 2007; Staszewski *et al*. 2007; Davison *et al*. 2008; Koutsos & Klasing 2008; Martin 2009). For example, poor nutrition and parasitic or pathogenic infections have been demonstrated to induce immunosuppression, reducing cell mediated immunity (CMI) and causing bursal depletion and splenic lymphoid necrosis, thereby negatively impacting antibody production (Schmidt 1997; Glick *et al.* 1981; 1983).

In a primary immune response immunoglobulin M (IgM) is produced earlier in the course of infection than IgG and persists for a relatively short time with subsequent IgG decay (Mayer 2006). For humoral immunity to be maintained, several mechanisms have been proposed including repeated exposure to the external antigen (Staszewski *et al*. 2007), antigen persistence within the host or antigen independent activation of memory B cells (Bernasconi *et al*. 2002). The persistence of antibodies to Hantavirus and to *Borrelia* has been observed for the expected life span of wild rodents (Mills *et al*. 1999; Hofmeister *et al*. 1999; Bunikis *et al*. 2004; Kuenzi *et al*. 2005). Henning *et al*. (2006) found declining antibody titers over a 3-year period in wild rabbits (*Oryctolagus cuniculus*) exposed to rabbit hemorrhagic disease virus. Persistence of antibodies to WNV has been examined in several species with longevity of 36 months in naturally re-exposed pig-tailed macaques (*Macaca nemestrina*) (Hukkanen *et al*. 2006); more than 3 years in pigs (*Sus scrofa*; Geevarghese *et al.* 1994); at least 15

months for horses (*Equus ferus caballus;* Ostlund *et al*. 2001); < 1 year in mesopredators (raccoons [*Procyon lotor*], opossums [*Didelphis virginiana*], and coyotes [*Canis latrans*]; Docherty *et al*. 2009) and ≥ 2 years in humans (Hayes 1989; Roehrig *et al*. 2003). Arboviral antibody production, or seroconversion, has been detected around day four in birds (Komar *et al.* 2003). Following primary infection, avian antibody levels peak for several weeks, plateau for several months, and then decline below levels considered positive (seroreversion; Stamm 1966; Kuno 2001; Ringia *et al*. 2004). The half-lives of detectable arboviral antibody responses in birds appear to be shorter than those of mammals (Stamm 1966; Kuno 2001). Reisen *et al*. (2000) found low neutralizing antibody titers to Western Equine Encephalitis (WEE) and St. Louis Encephalitis (SLE) viruses (both *Flaviviruses* in the JEV complex) in adult birds sampled during late-winter/early-spring suggestive of the rapid antibody decay seen in old infections. Komar *et al*. (2003) experimentally infected 25 species of birds with WNV and found that with the exception of two, all species that survived infection developed at least 70% neutralizing antibody activity with antibody persistence in rock doves (or domestic pigeons; *Columba livia domestica*) lasting the nine weeks of study. Further studies found antibody persistence for 15 months in naturally infected rock doves (Gibbs *et al*. 2005) and 12 months in captive fish crows (*Corvus ossifragus;* Wilcox *et al*. 2007). Finally, protective immunity against WNV challenge was observed for up to 36 months in 98.6% of experimentally inoculated and naturally infected house sparrows kept in free-flight cages (Nemeth *et al*. 2009). Although there was a ≥ 4-fold decrease in antibody titers

between 1 and 6 months in inoculated birds following peak infection, neutralizing antibody titers remained relatively constant in the subsequent 2 ½ years.

Results of captive and experimental inoculation studies, while providing valuable data, are not always reflective of avian antibody profiles in natural environments. For example, Smith *et al.* (1979) discussed the lack of SLE antibody persistence in adult birds from one season to the next. Additionally, Reisen *et al*. (1992) found that rock doves experimentally inoculated with SLE produced low-level transient seroconversions which contradicted the elevated seroprevalence rates observed during multiple field studies. Meister *et al*. (2008) observed that after natural infection with Usutu virus, another *Flavivirus* in the JEV complex, antibody titers in great grey owls (*Strix nebulosa*) and Tengmalm's owls (*Aegolius funereu*) dropped markedly within six months suggesting that anti-Usutu antibodies were generally not very robust or long lasting. Nemeth *et al.* (2008) observed naturally induced WNV neutralizing antibodies in raptors throughout a four year serial capture study with no clinical signs of disease, suggestive of a persistent protective response. Re-exposure events, however, could not be excluded as an explanation. Finally, Kwan *et al.* (2012) performed a long-term recapture study on wild house finches (*Carpodacus mexicanus*) and house sparrows and found that several birds remained positive for WNV antibodies throughout the study, with one individual house finch consistently positive for five years. However, there was the potential for re-exposure in these free-ranging individuals. More importantly, the overall antibody persistence of recaptured birds in the study waned over time in contrast to captive studies. The

proportion of the host population susceptible to infection is one of the most important drivers in the spread of a zoonotic disease (Smith 1970; Earn 2008). Therefore, the level of protective immunity attained in the host population that survives WNV infection could directly impact transmission of the virus by reducing the number of susceptible hosts (Beveroth *et al.* 2006). In particular, the competency and immune responses of "super-spreader" species, which reside primarily or exclusively in urban areas, could have implications for public health (Nemeth *et al*. 2009).

Another factor potentially influencing WNV amplification is the concept of "herd immunity" or "herd effect" in which the risk of acquiring infection among susceptible individuals in a population is reduced by the presence and proximity of immune individuals (Fine *et al*. 2011), including the protection of individuals whose immunity is waning (Stephens 2008). Additionally, as a significant proportion of hosts become immune, the pathogen's rate of transmission is constrained or reduced (Dobson 2009; Fine *et al*. 2011). So as avian hosts develop immunity to WNV, we may see decreased transmission of the virus. In a study of WNV seroprevalence in the common coot (*Fulica atra*) over a three year period in Spain, high WNV seroprevalence in 2003-2004 was followed the next year by decreased WNV activity (Figuerola *et al.* 2007). To examine the impact of herd immunity, Kwan *et al*. (2010*a*) calculated the wild bird seroconversion rate, which mirrored the temporal trend in cumulative seroprevalence, in a long-term WNV study in Los Angeles, California. The authors observed a consistent "protective effect" on sentinel chicken

seroconversions, a measure of tangential transmission. As herd immunity wanes with antibody decay and decreased viral activity, significant epizootic and epidemic events similar to novel exposure may occur, particularly if the appropriate triggers such as climate variables exist.

The "herd immunity threshold" is often used to determine the threshold proportion of immune individuals necessary to decrease incidence of infection (Mishra *et al.* 2011). Approximations from epidemiological data or vaccine research are often used to determine the parameters for these estimates (Katriel & Stone 2010). Van Boven *et al*. (2008) determined the approximate "herd immunity threshold" to Newcastle virus following vaccination by examining immune status based on high and low antibody titers and found that a high fraction of birds (>85%) with high antibody titers was necessary to provide sufficient herd immunity. Kwan *et al*. (2012) utilized WNV seroprevalence rates to estimate the herd immunity threshold in wild caught passerines in the California study and found that when levels exceeded 25%, human cases and positive dead birds decreased (Kwan *et al*. 2010*b*) as did sentinel chicken (*Gallus gallus domesticus*) seroconversions and mosquito infection rate (Kwan *et al*. 2012). In addition, the authors observed that as the proportion of seropositive birds waned to below 10% in subsequent years, positive dead birds and outbreaks of human cases were detected. Gibbs *et al*. (2006) found a relatively low level of WNV avian herd immunity in a four year capture study in Georgia. The authors suggested that this low proportion of immunity was insufficient to regulate WNV amplification. Therefore, it is possible that herd immunity may not be apparent if

a certain threshold of seroprevalence as well as antibody magnitude has not been met.

To determine the rate of seroreversion and to test the influence of herd immunity on WNV transmission, we utilized a seven year dataset studying the eco-epidemiology of West Nile virus in suburban Chicago, Illinois, a "hot spot" of WNV transmission (Hamer *et al*. 2008*b*; Ruiz *et al*. 2007). The objectives of this study were to assess WNV antibody persistence and the influence of avian herd immunity on WNV activity. We utilized serial blood sampling from wild birds to assess the natural longevity of WNV antibodies. We tested the hypothesis that herd immunity in the avian community would reduce the WNV infection rate in mosquitoes. Herd immunity was measured as the seroprevalence level and geometric mean antibody titer (GMT) entering the transmission season and WNV activity measured by WNV infection rate in *Culex* spp. mosquito pools.

Methods

Study site details: Mosquito and bird samples were collected over seven years (2005-2011) during the mosquito season, from mid-May to mid-October, in the metropolitan area of Chicago (Cook County; 87° 44' W, 41° 42' N) and consisted of multiple residential and "natural areas" sites representing a range of human population densities and land cover types as previously described (Hamer *et al*. 2008*b*; Loss *et al*. 2009). Temperature and precipitation data were obtained from the National Weather Service weather station for Midway Airport (2011) and were used to calculate cumulative degree weeks and cumulative precipitation as previously described (Ruiz *et al*. 2010).

Mosquito infection rate: Mosquitoes were collected using Centers for Disease Control and Prevention (CDC; Atlanta, GA) light traps and gravid traps (Hamer *et al*. 2008*b*). Mosquitoes were identified (Andreadis *et al*. 2005) and *Culex* spp. mosquito pools were tested for WNV using quantitative RT-PCR. Maximum likelihood estimates for infection rates (IR) were calculated by week and year using the pooled infection rate (Biggerstaff 2006) in the program Excel (Microsoft, Redmond, WA). In order to identify the time periods to utilize avian serology data for our statistical models, we considered a few weekly infection rate thresholds of 5, 10 and 29 per 1,000 mosquitoes. These thresholds identify the beginning and end of the enzootic transmission season in each year and allowed us to evaluate the seroprevalence and GMT of the appropriate population of birds.

Bird sampling: Wild birds were captured at each site on a rotational basis using 36-mm mesh nylon mist nets (Avinet, Inc., Dryden, NY). Birds were identified to species, weighed, measured, aged, sexed and banded with numbered U.S. Fish and Wildlife Service leg bands (U.S. Department of Interior Bird Banding Laboratory, Federal Bird Banding Permit #06507). All fieldwork was carried out under appropriate collecting permits with approvals from the Institutional Animal Care and Use Committee at Michigan State University (Animal Use Form 12/03-152-00 and UIUC Animal Use Protocol #03034). Blood samples were collected by jugular or brachial venipuncture, with blood volume collected not exceeding 1% of the bird's body weight or 0.2 mL. Collected blood was added to BA-1 diluent and stored on ice packs until serum was separated via centrifugation within five hours of collection. Supernatants

were transferred to 2.0-ml cryovials and stored at -20°C or -80°C. Epitope blocked enzyme-linked immunosorbent assay (b-ELISA) was used to detect WNV antibodies using 100 µl of serum at 1:20 dilution and a 1:4000 dilution of 6B6C-1 monoclonal antibody (MAb) (Hamer *et al*. 2008*b*). Two positive chicken serum controls and four negative controls were used for reference on each plate. Samples testing positive upon first screening were serially diluted up to 1:640 and re-tested to determine endpoint titers.

Serostatus of recaptured birds: Recapture data was used to determine the behavior of antibody titers in serially sampled wild birds. We utilized end-point titers to assess antibody decay for all recaptured individuals following an initial seropositive sample. The end-point titer calculations were adjusted to consider that different amounts of serum were obtained from different samples and then log-transformed. To estimate the rate of antibody waning, we plotted the end-point titers of these serially sampled birds by month following the initial seropositive record. We first included all serially sampled birds for a conservative estimate of antibody persistence, then produced a second estimate after removing birds with overt evidence of a natural re-exposure event. Birds were considered naturally re-exposed if the antibody titer increased more than 1:100 and if this increase occurred during a period of enzootic activity based on concurrent collections of *Culex* spp. mosquitoes.

Antibody titers of serially sampled birds were plotted by the number of months post-initial seropositive sample for both the conservative estimate and estimate with recaptures removed. We calculated the log transformed slope of

end-point titers following the initial seropositive sample for each bird and then averaged this slope to determine an overall rate of seroreversion. We determined a mean rate of seroreversion for house sparrows, northern cardinals (*Cardinalis cardinalis*), gray catbirds (*Dumetella carolinensis*), and other combined species. We utilized the mean corrected log transformed antibody titers for all juvenile house sparrows, northern cardinals, gray catbirds, and other combined species throughout the entire study as an initial value.

Herd immunity: Avian seroprevalence, defined as the percentage of WNV antibody positive birds in the given population, was determined for each year (cumulative and by site) using multiple criteria. The seroprevalence calculation was restricted to those species known to breed locally that have been implicated in the enzootic cycle (Hamer *et al*. 2011) and only first capture samples were used to avoid pseudo-replication. Seroprevalence is commonly calculated over the entire enzootic season (Gibbs *et al*. 2006; Kwan *et al*. 2012) which we have done for comparative purposes. However, Kilpatrick *et al*. (2007) discussed the shortcoming of seroprevalence studies that utilize data collected over the entire season, because the probability of having WNV antibodies differs with capture date. We attempted to address this issue by identifying the appropriate populations of birds with IR thresholds determining the enzootic season each year and excluding seroprevalence during the peak periods. Specifically, we utilized adult and juvenile bird data at the end of the WNV enzootic cycle in the previous year and adult bird data prior to WNV activity in the current year. We utilized the IR by week to determine the seasonal enzootic cycle and applied multiple

temporal IR thresholds to determine the "cut-off" weeks. The two most important avian amplification hosts in our study region, American robin and house sparrow (Hamer *et al*. 2011) were then analyzed individually for seroprevalence by year (cumulative and by site). Statistical models were applied to datasets with a minimum sample size (i.e., total number of serology results for a site within year) of 10 or 20. Results for the two sample size cut-offs were not different so only the cut-off of 10 is reported. All ELISA end-point titers utilized for the geometric mean antibody titer (GMT) calculations were corrected for serum variation among samples and calculated by year using the same criteria we used to calculate seroprevalence. GMT was calculated for seropositive samples with a minimum sample size of 5. Standard deviations and 95% confidence intervals for GMT were calculated on log transformed antibody titers (*ln* [y+1]) and back transformed.

Statistical methods: Linear mixed models were utilized to identify the influence of herd immunity on virus activity for each year. The response (dependent) variable was the *Culex* spp. IR and the predictor (independent) variable was the avian seroprevalence prior to virus activity in a given year. Cumulative degree weeks and cumulative precipitation, both of which are known predictors of WNV transmission intensity (Ruiz *et al*. 2010), and geometric mean antibody titers were included as covariates in the model. Site and year were included as random factors. Statistical analysis was performed using SAS PROC MEANS (1994) and R v2.11.1 statistical programming language. We used the Akaike Information Criterion (AIC) to determine the ranking of candidate models

for statistical analysis (Burnham & Anderson 2002). AIC values were corrected (AICc) for low sample size and increased complexity. AIC weights (w_i) were calculated to determine the relative importance of the parameters.

Results

Culex IR: The total number of *Culex* spp. mosquito pools (# individuals) tested in 2005-2011 and utilized by the statistical models in this study were 1,163 (21,196), 1,685 (24,332), 1,441 (13,200), 218 (3,823), 2,670 (39,891), 13,667 (270,426) and 1,954 (11,637) respectively. The highest yearly IR was observed in 2005 and 2006 (14.7 per 1,000 mosquitoes) followed by 2010 (11.0; Table 1). The lowest yearly IR was observed in 2011(0.5).

Antibody decay: We serially sampled 236 individual birds but only 41 had a seropositive sample followed by subsequent recaptures. At a minimum, we obtained a seropositive sample followed by one subsequent sample but some individuals had up to four serial samples. The average length of time between serial samples was 235 days, ranging between 13 days and 36 months. The species composition included northern cardinal (n=21), house sparrow (n=12), gray catbird (n=5), American robin (n=1), red-winged blackbird (*Agelaius phoeniceus*; n=1), and brown-headed cowbird (*Molothrus ater*; n=1). The mean WNV log transformed seroreversion rate for all species combined was -0.26 units per month (Figure 4). The mean antibody seroreversion rate for cardinals, house sparrows, gray catbirds, and other species combined was -0.19, -0.13, -0.32, and -2.18 units per month, respectively. This is a conservative estimate because the

calculations included individuals suspected of re-exposure to WNV. To plot the rate of seroreversion or antibody decay by species, we calculated a log transformed mean corrected antibody titer for all juvenile seropositive samples from 2005-2011 (all recaptures removed as well as those not involved in the enzootic cycle) by species to represent a starting point with house sparrows (n=62) having a mean corrected antibody titer of 5.16; cardinals (n=34) a titer of 5.10; gray catbirds (n=12) a titer of 4.95 and all other species combined (n=45) titer of 4.54.

Sample size decreased to 33 individuals when suspected re-exposures were removed. The mean seroreversion slope for all species combined after re-exposed samples were removed was -0.70 units per month (Figure 5). The seroreversion slope for house sparrows (n=9), cardinals (n=17), gray catbirds (n=4), and other species combined (n=3) was -0.51, -0.60, -0.44, and -2.18 units per month, respectively (Figure 5).

Herd immunity: During the seven year study, we obtained a total of 5,775 blood samples from 87 species of birds but only 4,716 samples were utilized in our seroprevalence calculations after those individuals not meeting our criteria were removed. Seroprevalence was highest during the first year of study, 2005, at 22% for all birds and American robins and 29% in house sparrows (weekly IR threshold of 29, Table 1, Figure 6). However, the GMT during 2005 was the lowest of the study at 1:35 (Table 2, Figure 6). In 2010, seroprevalence was the lowest of the study at 5% with an elevated GMT of 1:142. We observed an

elevated seroprevalence in conjunction with an elevated GMT in 2008, 2009 and 2011.

Herd immunity and Culex infection rate: Mixed models were developed to investigate the influence of avian herd immunity and weather on *Culex* infection rate. Statistical analysis was performed with SAS and R which produced similar results. A model selection procedure indicated that the data were best fit by a model (lowest AIC and highest weight) that included GMT as a covariate and site and year as random effects (Table 3). The second best model included GMT and temperature using degree week (DW) as covariates. The GMT covariate had a negative relationship with *Culex* infection rate with an $\alpha = 0.1$ ($\beta = -0.029$, S.E. = 0.016, $t_{27} = -1.84$, $p = 0.08$; Figure 7B). The covariates in the second model were not significant (GMT: $\beta = -0.025$, S.E. = 0.016, $t_{25} = -1.51$, $p = 0.14$; DW: $\beta = 0.194$, S.E. = 0.230, $t_{25} = 0.84$, $p = 0.41$). Temperature (DW) in our study was positively associated with *Culex* IR as expected, although not statistically significant when analyzed with GMT (Figure 7C, 8). We observed a weak positive association between seroprevalence and IR (Figure 7A) and a positive association between precipitation and IR (Figure 7D).

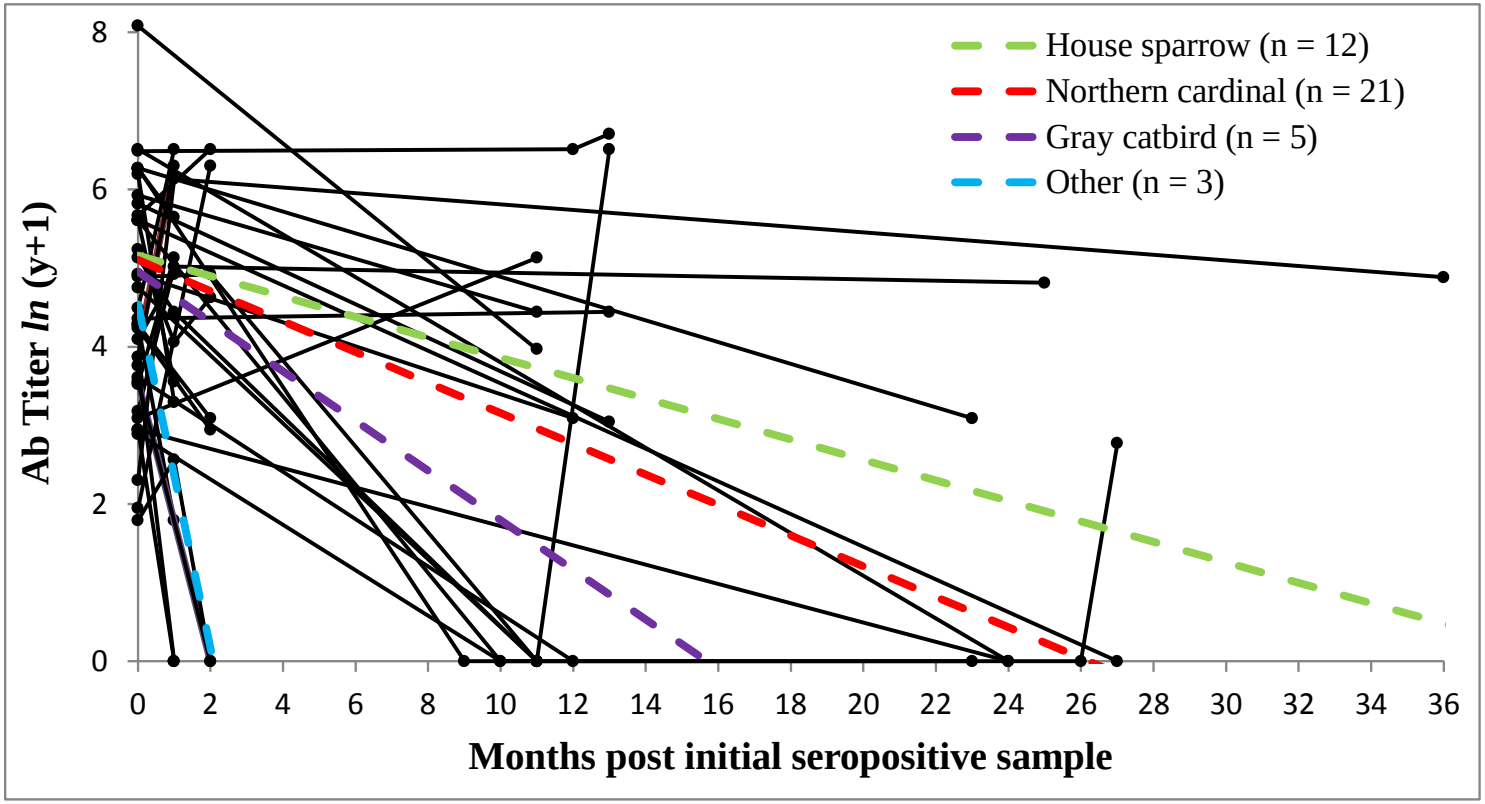

Figure 4. Log-transformed antibody titers for recaptured birds during months following initial seropositive sample. Mean seroreversion rates are provided for house sparrow, northern cardinal, gray catbird, and other avian species**.**

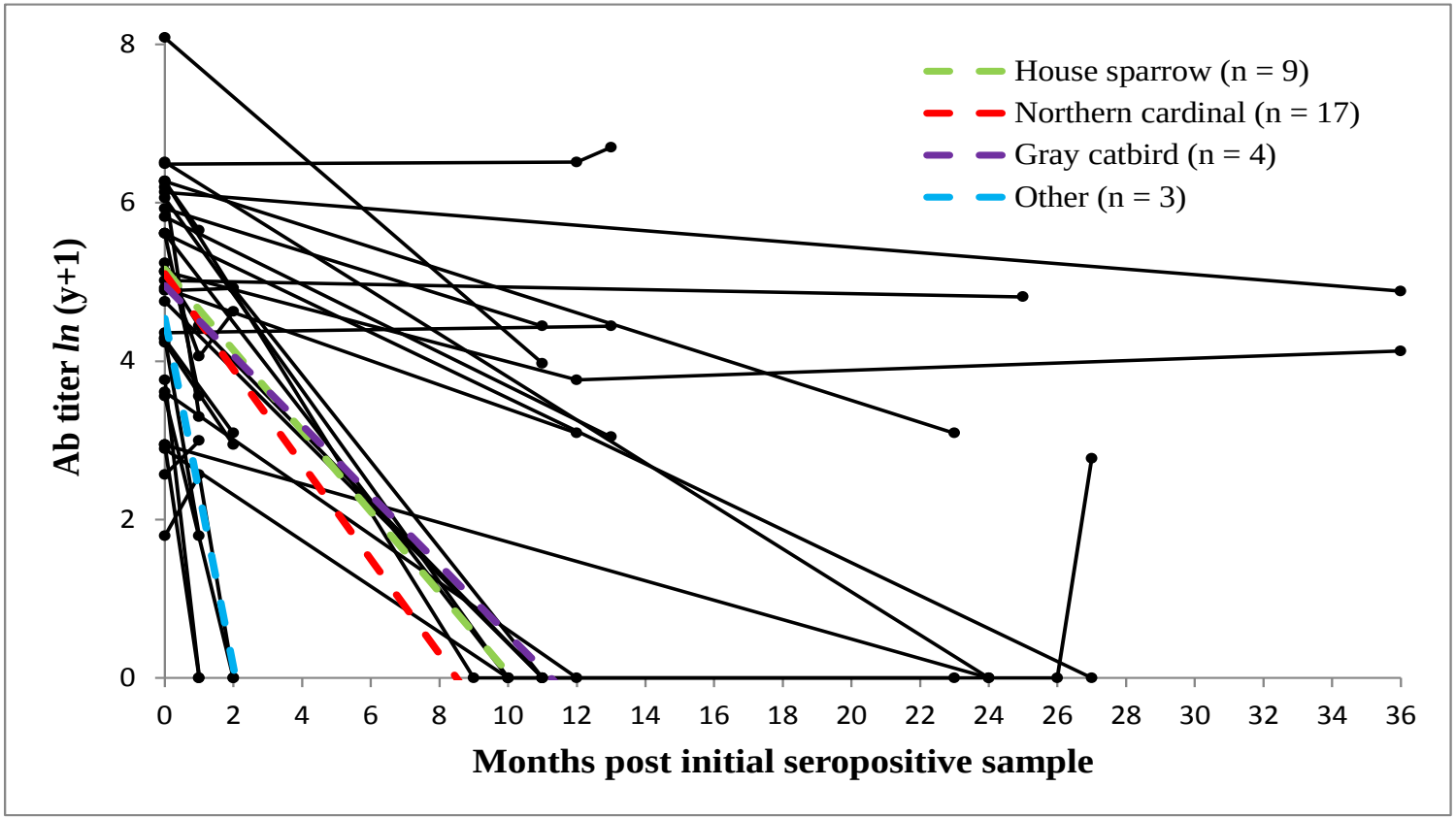

Figure 5. Log-transformed antibody titers for recaptured birds during months following initial seropositive sample. Individuals with evidence of re-exposures to WNV were removed. Mean seroreversion rates are provided for house sparrow, northern cardinal, gray catbird, and other avian species.

Table 1. Yearly *Culex* infection rate (IR; # positive per 1,000). Total avian seroprevalence (Sero.; %) by year utilizing temporal thresholds of seasonal enzootic activity defined by weekly mosquito infection rates. Total American robin (AMRO) and house sparrow (HOSP) seroprevalence for IR of 29. Total adult (AHY) and juvenile (HY) seroprevalence calculated by entire season. Statistical models conducted on data structured by site nested within year. N (sample size).

	Years						
Parameters	2005	2006	2007	2008	2009	2010	2011
IR	14.70	14.70	10.72	6.85	5.34	10.98	0.50
Sero.[a]	21.32	9.33	8	16.36	13.13	5.23	11.86
(N)	(475)	(525)	(375)	(220)	(297)	(172)	(132)
Sero.[b] (N)	21.32	11.15	9.94	16.36	13.13	5.23	11.86
	(475)	(646)	(513)	(220)	(297)	(172)	(132)
Sero.[c] (N)	21.80	12.82	10.28	13.82	13.13	6.67	11.86
	(536)	(741)	(603)	(275)	(297)	(195)	(132)
Sero.[d] (N)	22.48	4.00	6.74	11.30	7.73	5.99	5.57
	(863)	(1,112)	(1,135)	(345)	(608)	(336)	(269)
AMRO (N)	22.1	10.1	5.5	15.2	11.1	5.4	24.5
	(77)	(89)	(91)	(33)	(54)	(56)	(53)
HOSP (N)	28.8	12.6	6.5	15.6	17.3	8.5	14.6
	(73)	(309)	(201)	(135)	(110)	(59)	(48)
AHY Sero. (N)	23.46	4.60	10.93	16.49	11.76	7.25	11.19
	(405)	(653)	(540)	(194)	(340)	(145)	(134)
HY Sero. (N)	21.62	3.10	2.89	4.64	2.61	4.71	0
	(458)	(459)	(595)	(151)	(268)	(191)	(135)

[a] Calculated utilizing IR threshold of 5. [b] Calculated utilizing IR threshold of 10. [c] Calculated utilizing IR threshold of 29.
[d] Calculated for entire season.

Table 2. Yearly *Culex* infection rate (IR; # positive per 1,000). Total avian geometric mean antibody titer (GMT) utilizing temporal threshold of seasonal enzootic activity defined by weekly *Culex* infection rate of 29. Results of GMT[a] included in statistical calculation structured by site nested within year. CI (95% Confidence interval), N (sample size) and SD (standard deviation).

	Years						
Parameters	2005	2006	2007	2008	2009	2010	2011
IR	14.7	14.7	10.7	6.9	5.3	11.0	0.5
GMT[a] (CI) (N, SD)	35 (26,47) (75, 3.6)	180 (140, 230) (102, 1.3)	59 (42, 83) (68, 1.4)	114 (72, 181) (38, 1.4)	151 (103, 222) (41, 1.2)	142 (61, 332) (13, 1.4)	134 (83,215) (21, 1.0)
GMT[b] (N, SD)	69 (194, 1.5)	70 (45, 1.7)	60 (78, 1.1)	167 (39, 1.4)	134 (47, 1.1)	151 (23, 1.2)	116 (14, 1.1)
AHY GMT (N, SD)	29 (95, 1.3)	61 (31, 1.8)	81 (61, 1.2)	162 (32, 1.3)	127 (39, 1.1)	125 (14, 1.2)	116 (14, 1.1)
HY GMT (N, SD)	160 (98, 1.3)	97 (14, 1.3)	62 (17, 1.1)	191 (7, 1.5)	224 (8, 1.1)	204 (9, 1.1)	0

[a] Calculated utilizing IR threshold of 29.
[b] Calculated for entire season.

Table 3. Candidate models for predicting *Culex* infection rate with site and year included as random effects in the linear mixed effect model. Increased predictive accuracy determined by lowest AIC score (AICc corrected for sample size and complexity) and highest weight (w_i).

Model	AICc	ΔAICc	w_i
GMT+(1/site)+(1/year)	66.9	13.9	0.000795
GMT+DW+(1/site)+(1/year)	73.26	20.3	0.000795
Total10+GMT+DW+(1/site)+(1/year)	82.4	29.4	0.000000
Total10+GMT+Precip140+DW+(1/site)+(1/year)	89.1	36.1	0.000000
Total10+GMT+Precip140+DW+(1/site)+(1/year)	89.1	36.1	0.000000
Total10+GMT+(1/site)+(1/year)	93.1	40.1	0.000000
DW+(1/site)+(1/year)	113.7	60.7	0.000000
Precip2040+(1/site)+(1/year)	114.7	61.7	0.000000
Precip140+(1/site)+(1/year)	114.8	61.8	0.000000
Total10+(1/site)+(1/year)	115.1	62.1	0.000000
Precip140+DW+(1/site)+(1/year)	121.0	68.0	0.000000
Total10+DW+(1/site)+(1/year)	135.5	82.5	0.000000
Total10+Precip140+(1/site)+(1/year)	138.5	85.5	0.000000

GMT (geometric mean antibody titer).

Total 10 (seroprevalence for IR threshold of 10. Sample size of 10.

DW (degree week).

Precip 140 (rainfall for weeks 1-40 of the year).

Precip 2040 (rainfall during the WNV active season, weeks 20-40).

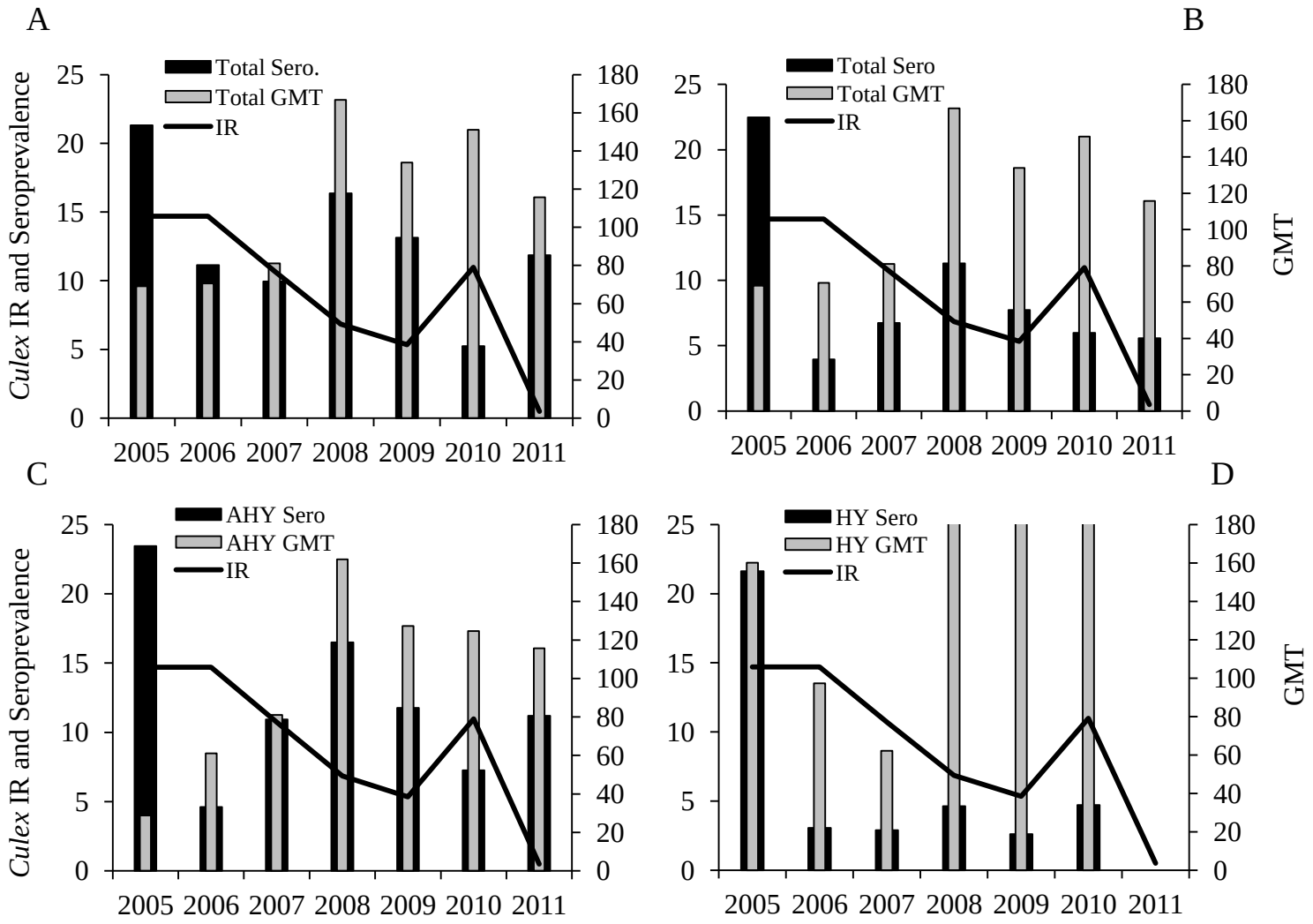

Figure 6. Yearly *Culex* infection rate (IR), avian seroprevalence (%), geometric mean antibody titer (GMT) for 2005-2011. A. Total seroprevalence and GMT calculated utilizing temporal threshold of seasonal enzootic activity defined by weekly *Culex* infection rate of 29. B. Total seroprevalence and GMT calculated for entire enzootic season. C. Adult (AHY) and D. Juvenile (HY) seroprevalence and GMT for entire enzootic season.

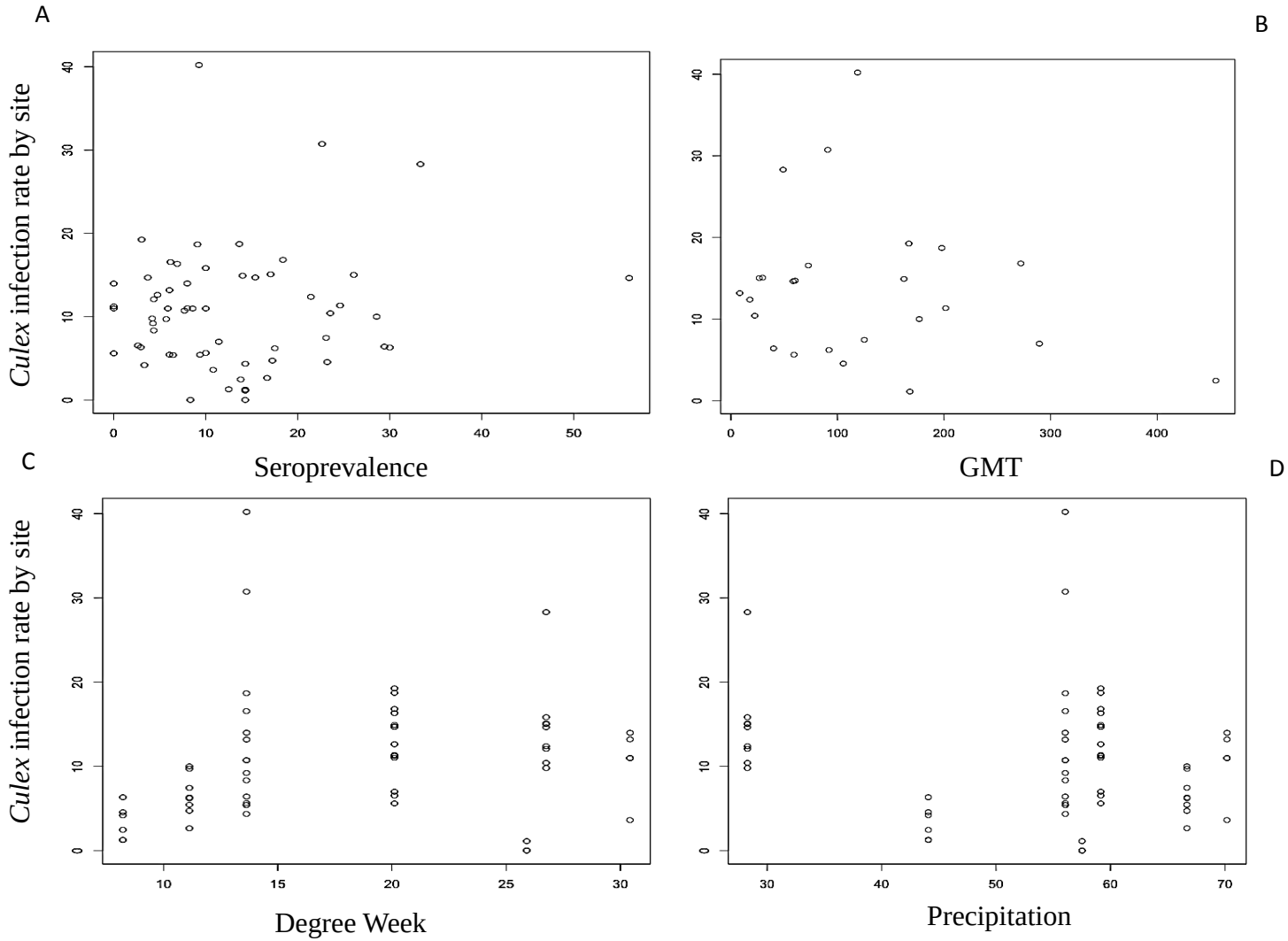

Figure 7. Diagnostic plots for models explaining variation in *Culex* infection rate (# positive per 1,000). All data are plotted by site and year. A. avian seroprevalence, B. geometric mean antibody titer (GMT), C. degree week and D. precipitation.

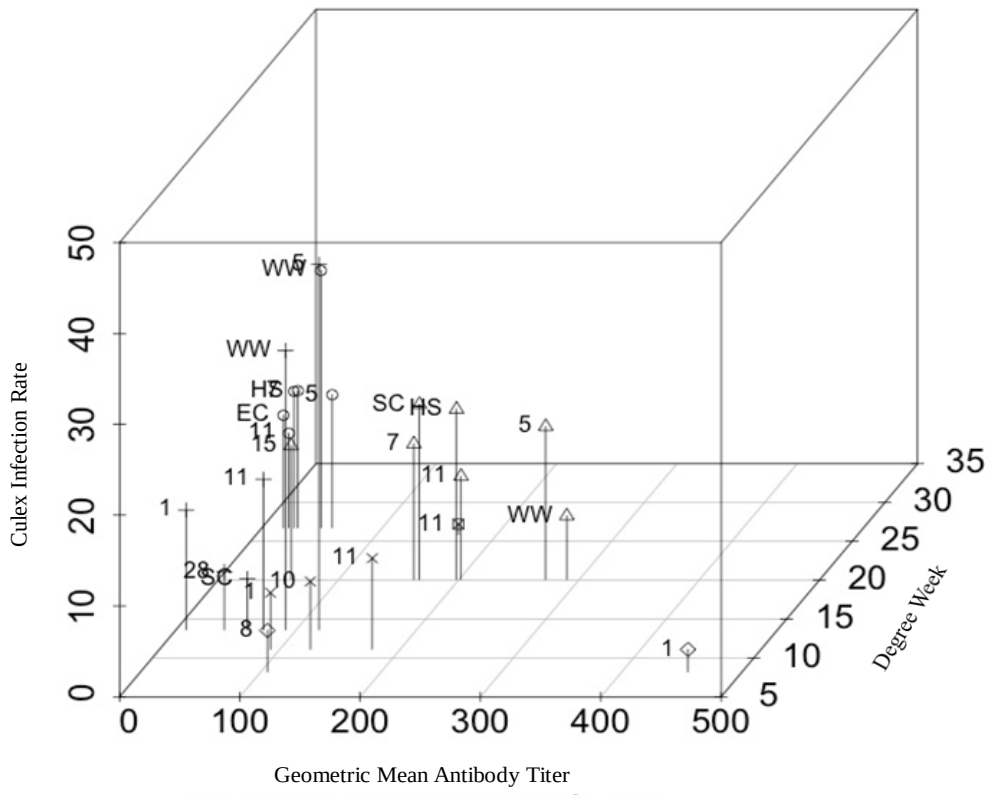

Figure 8. Geometric mean antibody titer plotted by *Culex* infection rate, and total avian seroprevalence by site and year. Symbols indicate year and labels indicate site.

Discussion

Antibody persistence: The temporal dynamics of natural immunity is of epidemiological importance as it modifies the number of susceptible individuals in a population. We report a measure of IgG antibodies produced after WNV infection in wild caught birds. Our quantified rate of antibody decay indicates that most birds will serorevert by the second transmission season following initial exposure to WNV. It should be noted that while we believe that by removing those individuals with overt signs of re-exposure we are presenting a more accurate reflection of antibody decay, our results represent a minimum loss of antibody titer over time because we probably still have some birds that were re-exposed included. Additionally, we cannot determine the time of initial exposure.

Following initial clearance of an antigen, memory B and T cells levels are reduced by apoptosis or removal to a resting state (Schijns *et al.* 2008). The production of antibodies following B-cell differentiation is driven by persisting antigen from chronic infections or periodic low-level re-infections (Zinkernagel *et al.* 1996; Zinkernagel & Hengartner 2006; Amanna *et al.* 2007). In the absence of persistent infection or antigen re-exposure, antibody titers gradually decrease, with the stimulation and duration of immune memory varying by antigen and species (Zinkernagel 2003; Amanna *et al.* 2007; Schijns *et al*. 2008). However, precursor B and T cells as well as memory B-cells can circulate in the absence of antigen (Zinkernagel *et al.* 1996; Crotty & Ahmed 2004). Therefore, the need for antigenic re-exposure to obtain serologic memory continues to be debated (Kuno 2001; Zinkernagel & Hengartner 2006).

Antibody persistence may also have age-dependent influence (Komar *et al.* 2012). Cichon *et al*. (2001) found a decline in anti-SRBC antibodies in older (5-6 years old) collared flycatchers (*Ficedula albicollis*). Similar results were found in barn swallows (*Hirundo rustica*) vaccinated for Newcastle Disease virus (Ardia & Schat 2008). Finally, Kwan *et al*. (2012) suggested that the overall waning of WNV antibodies observed in their recapture study may have been a factor of immune changes in especially long-lived individuals.

The mean seroreversion slope for house sparrows appears to correlate with long lasting protection (several seasons) in this species (Nemeth *et al*. 2009). However, long-lived individuals could potentially seroconvert and become immune for a couple of seasons and then serorevert dependent on the rate of antibody decay and resume the role of a susceptible host. Limited data are available on the natural longevity of wild birds; however, the potential longevity of several avian species has been obtained from the records of captive individuals under optimal or near-optimal conditions. The natural longevity of American robins is at least nine or ten years, although the species has a potential life span of at least 13 years and perhaps as much as 20 years (Farner 1945). Similarly, longevity in house sparrows may be greater than 13 years in the wild (Nemeth *et al.* 2009). However, it should be noted that the average natural life span for wild passerines is often only a fraction of the potential lifespan (Farner 1945). However, Kwan *et al*. 2012 found that acquired WNV immunity significantly increased the mean duration of life in avian hosts. Therefore, the rate of antibody decay in birds could essentially be utilized to determine a threshold of protection

in birds, if indeed, birds serorevert to a susceptible status. We were only able to use one American robin for the seroreversion analysis. Further examination of the temporal patterns of antibody response in natural populations should be further examined, particularly in "super-spreader" species.

Herd Immunity: Immunity to WNV in the avian community could be a limiting factor in the transmission cycle by influencing the number of susceptible hosts in a given transmission season, thereby reducing disease incidence. Our data suggests a significant association between avian antibody titer and WNV infection rate in mosquitoes. While increased temperature is typically associated with increased infection rates (Ruiz *et al*. 2010) for a variety of reasons (Hayes 1989; Marra *et al.* 2004; Reisen *et al.* 2006; Ruiz *et al*. 2010; Chaves *et al.* 2011), the summer of 2011 in the Chicago area was an exception to this pattern. While temperature is important, our results suggest that a combination of multiple factors may determine the magnitude of enzootic transmission. Specifically, avian hosts with a high magnitude of antibody titer may be of more importance in explaining variation in mosquito infection rate than abiotic variables such as temperature and precipitation.

Serologic surveys can be complicated by disparities between serologic assays. Plaque-reduction neutralization tests (PRNT) are considered the standard for arbovirus serologic testing (Ebel *et al*. 2002). However, the required use of live WNV has prevented widespread use of the PRNT as a diagnostic tool due to the associated zoonotic risk (Komar *et al*. 2009; Sotelo *et al*. 2011). PRNT may not detect old infections (Reisen *et al*. 2003*b*) and is less sensitive for early

detection compared to epitope-blocking ELISAs (b- ELISA) Sotelo *et al*. 2011. Results of b-ELISA correspond well with PRNT results and are sensitive to the detection of even very low titers of WNV antibodies (Blitvich *et al*. 2003; Gibbs *et al*. 2005). While ELISA assays do not directly measure neutralizing antibodies like PRNT, humoral immune responses to WNV comprise both neutralizing and non-neutralizing antibodies (Diamond *et al.* 2008), the latter of which can be detected by b-ELISAs (Blitvich *et al*. 2003). It should be noted, however, that neutralizing antibodies are generally considered to have a greater longevity than non-neutralizing antibodies (Blitvich et al. 2003; Sotelo *et al*. 2011).

While antibodies are a useful indicator of protective immunity (Hammarlund *et al.* 2003), other non-humoral mechanisms may mediate protection as well (Schijns *et al*. 2008). McLean *et al*. (1983) found that house finches experimentally inoculated with SLE were protected from viral challenge at 24 months even though neutralizing antibodies were no longer detectable. In WNV vaccination studies, American robins survived challenge infection and viremia was reduced to non-infectious levels, even though a detectable antibody response was not elicited (Kilpatrick *et al*. 2010). Therefore, focusing on seroprevalence as the sole measure of protective immunity may underestimate the actual prevalence of resistant hosts to WNV.

Seroprevalence can be used as a measure of host exposure and survival. However, the detection of antibodies as well as high antibody magnitude could be an indication of recent or current infection (McLean et al. 1983; Anderson & May 1991; Hawley & Altizer 2011). As such, regional serosurveys have been

performed as surveillance for evidence of WNV infection (Beveroth *et al.* 2006; Gibbs *et al*. 2006; Blitvich *et al*. 2009). McLean *et al.* (1983) found that high antibody titers (equal to or greater than 1:320) in house sparrows experimentally inoculated with SLE were temporally related to inoculation, occurring within six weeks of viremia. In WNV studies, experimentally infected eastern screech owls (*Megascops asio*) maintained a high level of viremia for 5-6 days before death despite initiating an antibody response (Nemeth *et al.* 2006). Additionally, fish crows (*Corvus ossifragus*) vaccinated against WNV elicited an antibody response which prevented death but failed to prevent an infectious viremia (Bunning *et al*. 2007).

Despite low seropositive sample size in some years for hatch-year birds, we observed an elevated GMT for juveniles in our study when calculated by season which may be a reflection of recent infection following the waning of maternal antibodies. Hamer *et al*. (2008*b*) found a significant positive correlation between *Culex* IR and subsequent seropositive juvenile birds in the Chicago study site suggesting that hatch-year birds facilitate rapid amplification of WNV by contributing to the proportion of susceptibles. To improve the accuracy of our seroprevalence and GMT calculations for data analysis we identified a specific population of individuals prior to enzootic activity by utilizing our *Culex* IR thresholds. Specifically, we only included hatch-year birds from the end of the previous season to reduce the inclusion of those individuals actively infected or involved in the enzootic transmission cycle. However, adult birds with low antibody titers due to antibody decay would still be included in our data set.

Interpretation of seroprevalence can be further complicated because a seronegative sample does not necessarily prove a lack of viral exposure (Anderson & May 1991; Kuno 2001). Individuals or species could produce short-lived antibodies or be refractory to infection (i.e., do not become infected or produce antibodies; Reisen *et al*. 2003*b*). Additionally, the extent to which detectable antibody levels predict generalized protection against subsequent infection is not clearly understood (Gerlach 1997; Kuno 2001; Nemeth *et al*. 2009). Diamond *et al.* (2003*b*) suggested that a depressed specific IgG response contributed to lethal WNV infection in rodent studies. In foot and mouth disease vaccine studies, Woolhouse *et al.* (1996) described a threshold antibody titer, below which cattle were susceptible to infection. Finally, undetectable or low antibody titers may leave a bird susceptible to either relapse of a latent WNV infection or re-infection (Reisen *et al*. 2001; Owen *et al*. 2010; Kwan *et al.* 2012). Therefore, the assumption that all seropositive individuals become dead-end hosts could be challenged (Kuno 2001, Kuno & Chang 2005).

Re-exposure of a primed host to an antigen should result in quicker reaction and higher antibody titers (Zinkernagel 2003). In general, elevated antibody titers are believed to be a reflection of increased immunity or resistance to re-infection (Gerlach 1997; Zinkernagel & Hengartner 2006; Nauta *et al*. 2009). In human vaccine studies, protection is positively correlated to antibody titers above a determined threshold (Nauta *et al*. 2009). The level of protection against WNV challenge, in rodents, generally correlated with high WNV antibody titers (Samuel & Diamond 2006; Shrestha *et al*. 2008). Ardia & Schat (2008)

demonstrated that in adult passerines such as barn swallows (*Hirundo rustica*) and tree swallows (*Tachycineta bicolor*), high levels of immune response correlated with higher survival rates. Finally, Nemeth *et al*. (2006) found that screech owls (*Megascops asio*) that survived WNV infection had a fourfold greater antibody titer than morbid owls.

The highest seroprevalence and lowest GMT in our study (juvenile and adult birds combined) were observed in 2005, which may have been the result of low WNV activity in the preceding two seasons (2003-2004; Ruiz *et al*. 2007). Reisen & Brault (2007) suggested that the transient nature of avian herd immunity could be due to antibody decay and population turnover. The influx of unexposed adult and juvenile birds in addition to adult birds with waning antibody titers may have contributed to a decline in the level of herd immunity in 2005 (Figure 6C). Similar results were observed with the more traditional measurement of seroprevalence over an entire season. However, as GMT increased in conjunction with seroprevalence in later years, *Culex* IR decreased. We observed a renewed viral amplification in 2010 despite elevated GMT which may have been related to the lower peak seroprevalence levels below 5%. Kwan *et al*. 2012 found that herd immunity levels < 10% in the adult avian population were followed by WNV outbreaks. Therefore, utilizing seroprevalence levels alone may not fully explain herd immunity in a population. Rather, seroprevalence in association with a high antibody titer may be more important to achieving a sufficient level of protection. We were unable to obtain a sufficient sample size of antibody positive house

sparrows and American robins for all years of our study, making interpretation of species-level herd immunity difficult.

Compartmental models are used to interpret epidemiological trends and predict the spread of infectious disease at a population level (Trottier & Philippe 2001; Mishra *et al.* 2011). The transition of an avian host from the removed compartment back to the recovered compartment due to seroreversion could have important consequences on the reproduction number R_0 but this factor has not been considered in epidemiological models of WNV. Additionally, Fine (1993) suggested that the variability of immune response among species was an assumption most likely omitted from even the most elaborate mathematical models. Therefore, our data could eventually be applied to a WNV model to better our understanding of pathogen persistence and amplification events. This study highlights the complexity of long-term immunologic responses in natural host populations. Environmental conditions play a key role in determining the timing and intensity of the WNV cycle so may be influenced by unpredictable changes in climate and in the environment. However, we found that WNV antibody titer in the avian community in Chicago was a more important driver of WNV transmission and amplification than climate variables.

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