DISSERTATION

AVIAN IMMUNITY TO WEST NILE VIRUS

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY NICOLE M. NEMETH, ENTITLED AVIAN IMMUNITY TO WEST NIILE VIRUS, BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

AVIAN IMMUNITY TO WEST NILE VIRUS

As West Nile virus (WNV) becomes endemic throughout much of North America, it continues to have detrimental effects on countless birds of various taxonomic groups. However, many birds survive infection, mounting an effective immune response. This dissertation focuses on the avian immune response to WNV, including naturally and experimentally-induced antibody duration and passive transfer of immunity. In addition, persistent WNV infection is a potential factor in altering pathogenesis if immunity were to wane.

The duration and protection provided by anti-WNV antibodies was documented in house sparrows (*Passer domesticus*) and raptors for 3-4 years. Antibody levels were relatively stable over time, and protected against viremia in the former and recurrence of clinical disease in the latter.

Passive transfer of WNV immunity from hen to eggs and chicks was characterized in domestic chickens (*Gallus gallus domesticus*). Eggs from both seropositive and seronegative hens were either sacrificed to test for WNV antibody in yolks or chicks artificially inoculated to examine viremic and serologic responses. Concurrently, age-associated differences in response to WNV infection were documented. The passive transfer experiment was repeated in house sparrows to explore this phenomenon in a passerine species; passive transfer was less prevalent in sparrow versus chicken chicks, was of shorter duration, and was less protective.

Persistent WNV shedding, viremia, and tissue infection was examined in house sparrows, with juveniles sampled more intensively on a shorter time scale (30-65 days) and adults sampled at 1, 6, 12, 18, and 24 months post-infection. Infectious WNV was isolated from an oral swab, spleen, and kidney of several individuals at 30 DPI, but not from sera after 6 DPI or swabs after 15 DPI. However, WNV was detected in an oral swab by RT-PCR at 44 DPI and was in multiple tissues from most sparrows at 30 DPI, and from kidney and spleen of two individuals at 65 DPI. These findings suggest that WNV infection in tissues may persist beyond the acute stage of infection, while implications for natural transmission and avian health remain unknown.

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CHAPTER 1

LITERATURE REVIEW

History of West Nile virus

West Nile virus (WNV; family Flaviviridae, genus *Flavivirus*) has been described as a virus whose range includes temperate, subtropical, and tropical thermal zones, existing in a range of habitats, including coastal plains, river deltas, forests, semi-arid habitats, and highland plateaus (Peiris and Amerasinghe 1994). The past and present geographical distribution of WNV reflects this description. WNV is an emerging arthropod-borne virus that has been recognized as an important encephalitis-causing pathogen of humans, but in its emergence has also become an important disease-causing pathogen of avian wildlife (Hayes 2001, Marra et al. 2004).

West Nile isolates thus far have been classified into two lineages; lineage 1 isolates are from central and North Africa, Europe, Israel, India, Asia, and North America whereas lineage 2 isolates are from central and southern Africa and Madagascar (Petersen and Roehrig 2001, Burt et al. 2002). Lineage 1 viruses have led to clinical human encephalitis, whereas lineage 2 viruses are generally maintained in enzootic foci in Africa and have not been documented to cause clinical disease in humans (Petersen and Roehrig 2001). Further, different WNV genotypes have led to varied levels of neurovirulence in a murine model. North American WNV isolates led to neuroinvasive disease in mice that

were not age-dependent, and differences in neuroinvasive phenotype were not associated with the source of the isolate (e.g., mosquito, bird, or mammal) (Beasley et al. 2002).

The first documented isolation of a neurotropic virus, later named West Nile virus, came from a native woman with febrile illness in the West Nile province of Uganda in 1937 (Smithburn et al. 1940). Subsequently, human populations in Uganda, Kenya, Belgian Congo, and the Sudan tested seropositive (Smithburn and Jacobs 1942, Smithburn 1952, Hurlbut et al. 1956). While the extent of the potential effects of WNV in humans was unknown at that time, various WNV-like viruses were isolated from human sera in Egypt, and were later determined to be WNV. These strains were pathogenic in mice, hamsters, rhesus monkeys, and chick embryos (Melnick et al. 1951). Experimental inoculation of human cancer patients with WNV was characterized by detectable viremia in most patients, as well as slight increases in body temperature, anorexia, and malaise; clinical signs attributable to mild encephalitis were noted in several of the inoculated patients (Southam and Moore 1952). In the vicinity of Cairo, a number of humans, mostly children, were seropositive and virus positive for WNV but lacked specific clinical signs attributable to WNV infection (Melnick et al. 1951). Soon after, clinical illness was described in a WNV-infected child, and consisted of rapid onset of fever, gastro-intestinal abnormalities, malaise, rash, lymphadenopathy, but no neurological signs, with subsequent improvement within one week. Two adults inoculated in the laboratory experienced fever, muscle fatigue, headache, and gastro-intestinal abnormalities; both improved over a slightly longer course than the child. WNV-associated encephalitis and death were considered rare in these relatively early cases (Hurlbut et al. 1956).

Early studies of the ecology of WNV suggested that in addition to being susceptible to viral infection, humans may also serve as competent or 'active' hosts of the virus (Southam and Moore 1952, Taylor et al. 1956). It was noted that human populations with high WNV seroprevalence were in close proximity to fruiting and other trees that attracted a variety of bird species, including house sparrows (*Passer domesticus*), palm doves (*Streptopelia senegalensis*), hooded crows (*Corvus corone sardonius*), buff-backed herons (*Bubulcus ibis ibis*; also known as cattle egrets), rock pigeons (*Columba livia*), and kestrels (*Falco tinnunculus*). Humans also lived in close proximity to domesticated birds and mammals, including cows, sheep, goats, donkeys, camels, chickens, ducks, pigeons, and geese (Taylor et al. 1956).

While studies were taking place in Egypt, WNV activity in Israel began to reveal additional information about the virus (Bernkopf et al. 1953, Goldblum et al. 1954). The first WNV outbreak in Israel was believed to have taken place in 1941 (Malkinson and Banet 2002). However, the first isolation of WNV in Israel did not occur until 1951, and resulted from a WNV outbreak in humans within an agricultural settlement. The outbreak was characterized by illness, especially in infants and young children, including fever headache, back and limb pain, anorexia, and vomiting (Bernkopf et al. 1953). Additional human cases in Israel included clinical manifestations such as malaise, fever, weakness, drowsiness, headaches, and pain in the chest and back (Goldblum et al. 1954). West Nile virus activity continued in Israel, documented by seroconversions of sentinel chickens in 1965-66 (Nir et al. 1969). However, subsequent WNV activity in Israel remained undetected until a human outbreak in 1980 (Katz et al. 1989), with another more recent outbreak in 2000, and a bird-virulent strain detected in 1997-99 (Weinberger et al. 2001).

In South Africa, a number of WNV isolates were obtained in the early-mid 1960s, and the mosquito *Culex univittatus* was implicated as the primary vector, feeding mostly on birds and thereby implicating them as the major vertebrate reservoir host (McIntosh et al. 1967). Adverse effects of WNV on humans, birds, and horses (e.g., neurological manifestations) thus far were rare, and this pattern continued into the 1990s (Malkinson and Banet 2002). However, there were some exceptions. Several WNV outbreaks in France in the 1960s involved fatal neurologic disease in humans and horses (Panthier et al. 1966, and Panthier 1968 cited in Gubler 2007). Outbreaks in 1962 led to isolation of WNV from *Cx. modestus* mosquitoes and humans in France in 1964 (Hannoun et al. 1964 cited in Murgue et al. 2001).

While reports of WNV cases were relatively rare from the mid 1970s to mid 1990s, from 1996 to 2000, human and equine outbreaks of fatal encephalitis occurred in Romania, Morocco, Tunisia, Italy, Russia, Israel, and France (Zeller and Schuffenecker 2004). Initial isolations of WNV in Central Europe were from mosquitoes (*Aedes cantans*) in west Slovakia in 1972 (Labuda et al. 1974) and later from pooled *Ae. vexans*, *Ae. cinereus*, and *Cx. pipiens* in the Czech Republic in 1997 (Hubálek et al. 1998). Detection of WNV transmission appeared to be increasing, with an epidemic of human cases (including fatalities) in Algeria in 1994 and equine cases in Morocco in 1996 (Le Guenno et al. 1996, El Harrack et al. 1997 and Tber et al. 1996 cited in Murgue et al. 2002). A noteworthy WNV epidemic in Romania in 1996 (the first in 20th century Europe) led to a human case fatality rate of 4.3% (n = 393) (Tsai et al. 1998, Savage et al. 1999, Malkinson and Banet 2002). During this outbreak, the first WNV isolate from Romania was recovered from a pool of *Cx. pipiens* (Tsai et al. 1998). Sporadic human

infections in the southeast part of Romania followed the 1996 outbreak (Cernescu et al. 2000). In addition, phylogenetic analyses of a Romanian mosquito isolate suggested that the virus was introduced from sub-Saharan Africa to northern Africa, and into southern Europe (Savage et al. 1999). Continued transmission was observed in this region through 1997-98 with a human outbreak in Tunisia (Triki et al. 2001 cited in Murgue et al. 2002), significant deaths of farmed geese in Israel, and clinical disease in horses in Italy (Cantille et al. 2000, Autorino et al. 2002, Gubler 2007).

In the Volgograd region of Russia, a WNV isolate from a human during a 1999 outbreak was most closely related to isolates obtained from Kenya and Senegal, while an isolate from a mosquito during this same outbreak was most closely related to isolates from Egypt and Romania (Platonov et al. 2001). Of 318 human patients tested during the 1999 outbreak in Russia, 58% demonstrated evidence of WNV infection, with 40 (12.6%) fatalities. This outbreak was characterized by abnormally high rates of human death and illness, and while viral genomic factors may have contributed to the high rates of morbidity and mortality among humans, climatic factors favoring mosquito vectors may have also led to increased transmission levels during this time (Lvov et al. 2000).

West Nile virus is still considered a threat in Europe (Tsai 1997), as well as other parts of the historical range. Recent outbreaks occurred in southern France in 2000 and 2004, evidenced by serologic testing and in some cases, disease in humans, horses, and/or birds (Murgue et al. 2001, Durand et al. 2002, Murgue et al. 2002, Jourdain et al. 2007b). During the latter outbreak, WNV isolates from a healthy, live house sparrow and common magpie (*Pica pica*) were sequenced, and showed greater identity with lineage 1 West Nile viruses isolated from Europe, the Mediterranean, and Kenya versus those

isolated from Israel and North America (Jourdain et al. 2007b). In 2003, WNV reemerged in Morocco, causing an equine outbreak, though no human or bird cases were reported. During this outbreak, sequencing of an isolate from a horse brain revealed a strain that was closely related to the European/Mediterranean/Kenyan cluster of lineage 1 WNV, similar to the recent isolates from France (Schuffenecker et al. 2005).

The rate and incidence of WNV outbreaks is unpredictable, with a currently widespread geographic distribution within the endemic range of the virus (Zeller and Schuffenecker 2004). As with the recent emergence of WNV in the Western Hemisphere, zoonotic pathogens have become increasingly mobile and widespread, in part due to global human travel, and trade and commerce, including the sale and shipping of both domestic and wild-caught animals (Kuiken et al. 2003, Karesh et al. 2005, Marano et al. 2007). Unprecedented fatalities in humans and birds in the past ten years also suggest greater virulence of more recently isolated WNV strains, traits which have allowed it to exploit its host and facilitated its spread (Gubler 2007).

Birds as reservoirs and dispersers of West Nile virus

In the 1950s, birds were implicated as potential reservoir hosts of WNV following serosurveys in the Nile Delta and experimental infection studies in a variety of avian species indigenous to the region. The initial isolation of WNV from birds occurred from the Nile Delta region of Egypt, and was from the blood, spleen and/or brain of a rock dove, and hooded crows (Work et al. 1953). Species examined experimentally included the house sparrow, hooded crow, buff-backed heron, palm dove, and kestrel; researchers observed species differences in viremia profiles, and therefore ability to serve as reservoir hosts (Work et al. 1955). Shortly thereafter, additional studies revealed relatively high

seroprevalence rates in house sparrows and hooded crows in Egypt (Taylor et al. 1956). The hypothesis that birds acted as reservoirs of WNV in nature was gaining strength.

Isolation of WNV from birds and mosquitoes was relatively rare during early investigations, but when isolations were achieved, they provided additional insight into transmission and movement of the virus. From 1965-67, virus isolates were obtained in Israel from *Cx. univittatus* mosquitoes, and from a wagtail (*Motacilla alba*) and turtledove (*Streptopelia turtur*) (Nir et al. 1972). Isolates from *Cx. univittatus* complex mosquitoes in Kenya in 1998 proved phylogenetically similar (demonstrating a "sister relationship") to WNV isolated from *Cx. pipiens* in Romania in 1996 (Miller et al. 2000). WNV was isolated from white storks and a lappet-faced vulture in Israel in 1998 and in 1999-2000, from various birds at zoos or in urban settings, including white-eyed gull, feral pigeons, collared dove, and rosella (Malkinson and Banet 2002).

Data from experimental infection studies in Africa, Europe, the Middle East and Russia lent further evidence to the role of birds are reservoirs and dispersers of WNV. A range of species were deemed susceptible to Egyptian strains of WNV via controlled infections, including mice, hamsters, rhesus monkeys, and chick embryos (Melnick et al. 1951). The majority of house sparrows (12/16) and all hooded crows (13/13) originating from the Nile Delta region proved highly susceptible to relatively high viremia titers and mortality rates following experimental mosquito-inoculation with the Ar-248 strain of WNV isolated from a pool of *Cx. antennatus* in Egypt. In contrast, none of seven kestrels, none of five buff-backed herons, and none of four palm doves succumbed to infection and had relatively low viremia levels (Work et al. 1955). In Russia, experimental inoculations of birds indicated that some species of birds were highly

susceptible to viremia (detectable for 4 to 5 days) but not to clinical disease or death [i.e., pheasant (*Phasianus colchicus*), gray heron (*Ardea cinerea*), crow, rook (*Corvus frugilegus*), pintail (*Anas acuta*), pochard (*Aythya ferina*), and pigeon] (Chunichin and Leonova 1985 cited in Lvov 1994). In Israel, domestic geese were experimentally inoculated with a WNV strain isolated from a clinically ill goose within that country. Eight of nine geese had detectable viremia between 1-7 days post-inoculation (DPI), and peak viremia titers were observed from 2-4 DPI (Malkinson and Banet 2002).

Birds have been suspected in the movement and spread of WNV since early in the documented history of the virus. Bird migration has been considered a major mechanism of intra- and intercontinental dissemination of WNV (Malkinson et al. 2002, Peterson et al. 2003). Prior to the 1999 spread of WNV to North America, migratory birds had been implicated in the spread of WNV between Europe and Africa (Tsai 1997, Miller et al. 2000). For example, migrating birds were believed to have played a potential role in the spread and subsequent outbreaks of WNV in the Camargue region of France, possibly carrying the virus while migrating from Africa or from Eastern Europe (Jourdain et al. 2007c). It is possible that migratory birds are responsible for periodic outbreaks of WNV or reintroductions in various regions of Europe, such as the Rhone Delta of France, the Volga Delta of Russia (Hayes 2001). However, the rapid spread of WNV and the pattern of spread from east to west across the continent of North America fail to support migratory birds as the major route of intercontinental spread of the virus (Rappole and Hubálek 2003).

The introduction of West Nile virus to the Western Hemisphere

West Nile virus was first documented in the Western Hemisphere in 1999. The virus arrived to New York City, accompanied by cases of human encephalitis and the death of numerous free-ranging American crows (*Corvus brachyrrhynchos*) and other bird species (both native and non-native to North America) (Lanciotti et al. 1999). Rapid spread of WNV occurred along the eastern seaboard of the United States within the first year of its arrival (Marfin et al. 2001). The spread continued, and by 2003 WNV had reached the west coast of the United States (Petersen and Hayes 2004, Reisen et al. 2004). The spread of WNV across the United States has been associated with numerous epizootics in horses and birds, as well as epidemics in humans, as the epicenters varied with each subsequent transmission season (McLean 2006, Gubler 2007).

The distribution of WNV has undergone relatively recent and rapid expansion both north and south of United States borders, with evidence of circulation in Canada since 2001 (Lindsay et al. 2003, Pepperell et al. 2003, Shuai et al. 2006), Mexico since 2002 (Estrada-Franco et al. 2003), and El Salvador since 2003 (Komar and Clark 2006). West Nile virus continued its spread to the Caribbean Islands of Cuba, Puerto Rico, Bahamas, Cayman Islands, Jamaica, Hispaniola, and Guadeloupe (Campbell et al. 2002, Dupuis et al. 2003, Quirin et al. 2004, Komar and Clark 2006, Pupo et al. 2006, Gubler 2007, Barrera et al. 2008). More recently, WNV activity (virus isolations and/or serologic) has been evident in birds and horses in South America, including Colombia, Trinidad, Venezuela, and Argentina (Morales et al. 2006, Bosch et al. 2007, Gubler 2007, Diaz et al. 2008).

The means for the arrival of WNV to North America remain unclear (Gubler 2007). Possible explanations for the unexpected arrival of WNV to New York City include via a viremic human, viremic bird, stowaway mosquito, or intentional introduction of the virus (Calisher 2000). Some experts believe that migratory birds were critical for the long-distance spread and mosquitoes for enzootic transmission (Peiris and Amerasinghe 1994, Peterson et al. 2003, Rappole and Hubálek 2003), and models suggest that spread may have been due to dispersing resident birds (Rappole et al. 2006). While the possible explanations for the arrival and spread of WNV in the New World remain unproven, it is likely that the post-arrival spread was facilitated by a broad range of vertebrate host and vector species; these include at least 300 bird species, 30 non-avian vertebrate species, and 61 mosquito species. The impacts of WNV on North America have also been due to the emergence of a more virulent strain of the virus with an increased potential to cause epidemics. While the exact mechanism(s) for the unprecedented expansion of this virus will never be entirely clear, it appears that WNV has become established and will continue to persist in the Western Hemisphere (Gubler 2007).

West Nile virus isolates obtained during the New York City outbreak in 1999 most closely resemble (> 99% homology) a strain originally isolated from a goose carcass in Israel in 1998 (Jia et al. 1999, Lanciotti et al. 2002). Further, a WNV isolate from the brain of a Chilean flamingo (*Phoenicopterus chilensis*) that died at a zoo in New York in 1999 was genetically characterized for comparison to existing WNV isolates. These analyses demonstrated the greatest similarity between a NY99 WNV strain and isolates from Egypt (WNV-EGY-Eg101) and Israel (WNV-Israel-98), also supporting a

Middle East origin of WNV NY99 (belonging to lineage 1; i.e., viruses circulating in western Africa, the Middle East, eastern Europe, Australia, and the United States) (Jia et al. 1999). When American crows were inoculated with either Old or New World WNV strains, an isolate obtained from a dead crow in New York in 1999 proved a more virulent phenotype, as it led to higher titered viremia and mortality (Brault et al. 2004); however, this difference was not observed in house sparrows (Langevin et al 2005). More recently, *in vivo* replication within mosquitoes led to significantly shorter extrinsic incubation periods in *Culex* spp inoculated with WN02 genotypes versus NY99 (all were isolates from American crow kidneys, New York) (Moudy et al. 2007). The high virulence of WNV NY99 and its derivatives for New World aviafauna and mosquitoes was the primary factor in driving transmission and dissemination in North America and has been the major difference observed between the patterns of WNV in the Old and the New Worlds. However, an exception to this difference was the increased virulence in birds observed for recent strains isolated from the Middle East in the late 1990s (R. McLean, pers. comm.).

Various studies have evaluated avian host preferences of various WNV mosquito species in the United States. Examination of mosquito blood meals by indirect ELISA, and PCR and DNA sequencing revealed differences in mosquito feeding preferences among common avian hosts. Mosquitoes examined were considered potential vectors for WNV based on previous observations of WNV infection in nature. In New York, New Jersey, and Tennessee, common avian hosts included the American robin (*Turdus migratorius*), northern cardinal (*Cardinalis cardinalis*), northern mockingbird (*Mimus polyglottos*), tufted titmouse (*Baeolophus bicolor*), and brown-headed cowbird

(*Molothrus ater*) (Apperson et al. 2004). Subsequent research in Tennessee revealed the American robin, common grackle (*Quiscalus quiscula*), and northern cardinal as the most commonly fed upon avian host by a variety of mosquito species (Savage et al. 2007). In Maryland and Washington, D.C., the preferred host for *Cx. pipiens* was again the American robin (Kilpatrick et al. 2006). Avian species that were fed upon relatively frequently by mosquitoes, while also proving competent hosts for WNV under experimental conditions (Komar et al. 2003a, 2005), are likely competent hosts of WNV in nature. One study considered a variety of factors (e.g., mortality rates, seroprevalence rates, and host competency) in determination of important WNV amplifying hosts in St. Tammany Parish, Louisiana. Summer resident birds, such as the Northern cardinal and house sparrow, were the principal amplifying hosts, while blue jay (*Cyanocitta cristata*) and northern mockingbird were also deemed important (Komar et al. 2005).

Global avian West Nile virus seroprevalence and surveillance

Collection of serum samples has been a common strategy for disease surveillance, as well as ecological studies pertaining to arboviral infection in birds. However, it should be noted that serological data have limitations, as the timing and location of initial infection of seropositive birds is unknown, and subsequent re-exposures can only be discerned under specific circumstances. Serologic results for free-ranging birds often serve to document survival, while they discount birds that died due to WNV infection (Komar 2001, Komar et al. 2005). Detecting recent infections via live bird serosurveillance is a more complicated task. Collection of multiple serum samples over approximately 2-4 weeks is required to demonstrate recent transmission activity, so that

seroconversion (a fourfold or greater increase in WNV neutralizing antibody titers) following relatively recent infection can be detected (Nemeth et al. 2007b).

Avian serosurveys conducted in the mid-1900s revealed relatively high seroprevalence rates among numerous species of common free-ranging and domestic birds in the Nile Delta (Work et al. 1955, Taylor et al. 1956). Seroprevalence (as determined by neutralization test) for domestic chickens, ducks and geese ranged from 14-27%, and from 25-42% for peridomestic species such as the house sparrow and rock dove, 28% for herons, and 65% for crows (Taylor et al. 1956). In addition, sentinel chicken flocks at various sites in South Africa demonstrated utility for surveillance, and were conducted along with mosquito collection and testing that led to isolation of WNV from mosquitoes at the same sites (McIntosh et al. 1967).

WNV serosurveys [testing by hemagglutination-inhibition (HI); including A and B serologic groups of arboviruses] in 1965-66 in Israel revealed 14.4% (n = 2,294) seroprevalence among 70 migrant and resident species captured along the Mediterranean coast. Turtle doves and coots (*Fulica atra*) were implicated as potentially important reservoir hosts (Nir et al. 1969). WNV was isolated from sera of several turtle doves, which are migratory in Israel, traditionally arriving from the south in April while possibly transporting virus (Nir et al. 1967). Additional avian serosurveys conducted throughout Israel in 1966-67 yielded 18.1% (n = 4,400) seroprevalence among migrants [i.e., turtle dove, starling (*Sturnus vulgaris*), black-headed gull (*Larus ridibundus*)] and residents [i.e., African bulbul (*Pycnonotus capensis*), goldfinch (*Carduelis carduelis*), house sparrow]. Among species tested, seroprevalence rates varied from 6-31% (Nir et al. 1972). Similar rates were observed in birds in Israel from 1998-2001. Neutralizing

antibodies (≥ 1:10 titer) were detected in approximately 12.2% (22/180) of crows trapped throughout the country, in 12-50% (17/159, and 67/128, respectively) of feral pigeons (seroprevalence varied by habitat and elevation), and 53.6% (37/69) of storks. The observation of storks that were seropositive during their first migration suggested that these birds were exposed to WNV while in Europe (Malkinson and Banet 2002).

Serologic studies performed within the former Soviet Union implicated various wild bird species for their involvement in WNV transmission, including the night heron (*Nycticorax nycticorax*), the yellow heron (*Ardeola ralloides*), and the little egret (*Egretta garzetta*). Concurrently, WNV isolates were obtained from the glossy ibis (*Plegadis falcinellus*), hooded crow, bittern (*Botaurus stellaris*), blackbird (*Turdus merula*), nuthatch (*Sitta europea*), and herring gull (*L. argentatus*) (Lvov and Ilichev 1979 cited in Lvov 1994).

Avian serosurveys performed in the Czech Republic from 1984-90 yielded a WNV HI seropositive rate of 5.25% (n = 1,109). Twenty-one species were antibody positive, with highest rates observed in the reed warbler (*Acrocephalus scirpaceus*), sedge warbler (*A. schoenobaenus*), bearded tit (*Panurus biarmicus*), penduline tit (*Remiz pendulinus*), chaffinch (*Fringilla coelobs*), and siskin (*Carduelis spinus*). Many of the seropositive birds were young, suggesting local transmission foci (Juricová and Hubálek 1993). More recent avian serosurveys in southern Moravia of the Czech Republic between 2004-06 were conducted, and revealed none of 122 waterfowl with anti-WNV antibodies, while 3.3% (13/391) of other wild birds (mostly passerines) had anti-WNV antibodies, including both hatch-year and adult birds (Húbalek et al. 2008). Relatively low WNV seropositive rates were observed in house sparrows versus tree sparrows

(*Passer montanus*) in Poland (2.8% and 12.1%, respectively), again likely representing local transmission because these species rarely migrate in the study region. The discrepancy in seroprevalence rates between the two *Passer* species may have been partially due to differences in habitat preferences (Juricová et al. 1998).

Early serosurveys for various flavi- and alphaviruses in the Danube Delta of Romania revealed low HI WNV seroprevalence rates (approximately 1-2.5%) in both resident [i.e., moor hen (*Fulica atra*), cormorant (*Phalacrocorax corbo*)] and migrating [i.e., common heron (*Ardea cinerea*), eastern glossy ibis] birds (Draganescu et al. 1978). Seroconversion to WNV in domestic fowl proved a useful surveillance tool in Romania just prior to the spread of WNV to the Western Hemisphere. In 1996, avian serosurveys in Romania demonstrated relatively high seroprevalence rates in domestic fowl species (30/73; 41%), including chickens, ducks, and turkeys (37%, 38%, 67%, respectively). Seroprevalence was not significantly different between rural and urban sampling sites. At the same time, wild birds sampled (n = 12) had a lower antibody prevalence, with only one robin (*Erithacus rubecula*) testing positive (Tsai et al. 1998, Savage et al. 1999). Sentinel chickens were again successfully used for surveillance during another WNV outbreak in Romania in 1997, with seroconversion rates ranging from 16-40% over a four-week period (Cernescu et al. 2000). Recent (2001-02) WNV activity was detected in Croatia via equine serosurveys, in which 4/980 (0.41%) serum samples were positive for WNV (Madić et al. 2007).

In France, the European magpie, a sedentary species, was recently deemed a potentially useful sentinel for WNV. Seroprevalence (neutralizing antibody titers of ≥ 20) was approximately 10% (n = 271) in magpies in southern France, a region in which

WNV has been documented since the 1960s (Jourdain et al. 2007a). Recent studies of seroprevalence rates of resident birds in Seville, Spain revealed relatively low rates of seropositivity (4/271; 1.5%), suggesting low levels of local WNV circulation (López et al. 2008), while seroconversion was detected among common coots in Doñana, Spain from 2004-05 or 2005-06 (Figuerola et al. 2007). Also in southern Spain, near the bordering countries of Portugal and Morocco, seropositive feral bovids and equids were detected in 2005 (Jiménez-Clavero et al. 2007). Most birds that were seropositive in Germany (53 positive of 3,399 tested; 1.56%) in 2000 and from 2002 to 2005 were either migrants or partial migrants, though one resident goshawk (*Accipiter gentilis*) was seropositive, indicating local transmission (Linke et al. 2007).

Numerous serosurveys have been conducted in North America since the 1999 arrival of WNV to New York City. These serosurveys commenced almost immediately and indicated relatively high seroprevalence among birds in and around Queens, New York in September of 1999. The overall seroprevalence (determined by neutralization test) among birds sampled was 33% (n = 430), with highest rates in the domestic goose (*Anser* sp), domestic chicken (*Gallus gallus*), house sparrow, Canada goose (*Branta canadensis*), and rock dove (Komar et al. 2001b). In October 2000, a similar serosurvey was conducted on Staten Island, New York, and yielded 23% seroprevalence among resident birds (n = 257), with no positives recorded among 96 migrating birds. While high seroprevalence was observed in the northern cardinal and rock dove (69.2% and 54.5%, respectively), lower rates were found in house sparrows and domestic chickens (8.6% and 5.5%, respectively). From these serosurveys, it was evident that many birds survived initial WNV infection (Komar et al. 2001a). Subsequent avian serosurveys

throughout North America, demonstrating widespread and variable rates of exposure among many species, were performed in Louisiana (Komar et al. 2005), Florida (Godsey et al. 2005), Georgia (Gibbs et al. 2006), Illinois (Ringia et al. 2004, Beveroth et al. 2006), North Dakota (Bell et al. 2006, Sullivan et al. 2006), Colorado (Nemeth et al. 2007b), California (Reisen et al. 2005, Stout et al. 2005, Hull et al. 2006, Reisen et al. 2006), Yucatan State (Farfán-Ale et al. 2006) and Tamaulipas State, Mexico (Fernández-Salas et al. 2003), and Ontario, Canada (Gancz et al. 2004). Additional serosurveys have been conducted in the Dominican Republic (Komar et al. 2003b) and Argentina (Diaz et al. 2008).

Serosurveys of free-ranging birds have been conducted for surveillance of various arboviruses (McLean et al. 1983), including WNV (Komar et al. 2001a,b). Due to the difficulty of recapturing live birds to collect serial blood samples within a relatively short time period (e.g., 2-4 weeks), live birds admitted to rehabilitation facilities may offer a more reasonable alternative to free-ranging birds for surveillance purposes. While birds at rehabilitation centers represent a biased sample (i.e., are usually debilitated, injured, or orphaned), they are readily available, and when seropositive, represent naturally-acquired infections. In Colorado, live raptors recently admitted into rehabilitation were used to detect recent seroconversions as well as oral shedding of WNV to contribute to state surveillance efforts (Nemeth et al. 2007b). Captive live pigeons were also examined for their usefulness as WNV sentinels, and determined to be most effective for assessing enzootic transmission levels when housed singly (Deegan et al. 2005). Chickens have most commonly been the live bird of choice for serosurveillance, and yielded variable outcomes regarding their utility in providing an early warning system for human WNV

infection in various regions of the United States and Canada (Cherry et al. 2001, Komar 2001, Blackmore et al. 2003, Drebot et al. 2003).

Another common, ubiquitous, non-native species in North America, the house sparrow, has been the focus of numerous WNV serologic studies. The house sparrow is abundant within many habitat types, and its range includes much of North America (Lowther and Cink 1992). This species was more abundant and more frequently exposed to WNV as compared to other bird species during the 1999 WNV outbreak in New York (Komar et al. 2001b), and in Louisiana, house sparrows were infected with WNV at moderate rates while experiencing low population reductions due to infection (Komar et al. 2005). Further, the house sparrow had a potential role in the short-distance spread of WNV throughout North America (Komar et al. 2001b, Rappole and Hubálek 2003).

Prior to 1999, WNV virulence leading to morbidity and mortality in birds was not well recognized or publicized. Eventually, details regarding an avian morbidity and mortality event in Israel in 1998 were revealed. Storks in southern Israel were apparently weakened from migration, and WNV was isolated from birds of this flock that were dead and dying (Malkinson and Banet 2002). In contrast, WNV-associated disease and death in birds in North America has been well documented, and has in fact become a trademark of this virus in the United States. Langevin et al. (2005) demonstrated mortality in house sparrows that were experimentally infected with both the NY99 strain and a Kenyan strain of WNV, suggesting that avian mortality due to WNV in the Old World may be overlooked.

Avian mortality surveillance has proven a useful and sensitive indicator of WNV activity in many locations in the United States and Canada (Eidson et al. 2001, Julian et

al. 2002, Blackmore et al. 2003, Drebot et al. 2003, Guptill et al. 2003, Mostashari et al. 2003, Reisen et al. 2006). The use of avian carcasses for surveillance has both advantages (e.g., potential for early detection, passive data collection) and disadvantages (e.g., reliance on outside sources for reporting and submitting dead birds with associated biases and difficulty of coordinating dead bird reports and collection) (Eidson 2001, Komar 2001, CDC 2003, Hochachka et al. 2004, Ward et al. 2006). The American crow became the focus of early surveillance efforts involving carcass testing in New York State, comprising approximately 67% of nearly 4,000 birds (of 63 species) tested in 2000 (Eidson et al. 2001). Further, 47% (n = 1,687) of American crows tested during the 2000 WNV transmission season in New York were positive for WNV by RT-PCR (Bernard et al. 2001). West Nile virus prevalence rates were relatively high among carcasses of numerous corvid species in California [yellow-billed magpie (*Pica nuttalli*), 81.5%; Stellar's jay (*Cyanocitta stelleri*), 48.9%; Western scrub jay (*Aphelocoma californica*), 69.8%; and American crow, 56.4%] (Koenig et al. 2007). The American crow was also deemed the most valuable species for avian mortality surveillance in Colorado, while other useful species included the American kestrel (*Falco sparverius*), black-billed magpie (*Pica hudsonia*), house finch (*Carpodacus mexicanus*), house sparrow, blue jay, red-tailed hawk (*Buteo jamaicensis*), and great horned owl (*Bubo virginianus*); the most sensitive option for this type of surveillance was to test many avian species (Nemeth et al. 2007a). Common urban or suburban species (e.g., house sparrow, house finch) were useful for WNV avian mortality surveillance in areas of New York State where the density of corvid (e.g., the American crow) populations was low (Stone et al. 2004). In Florida, Columbiformes were most commonly reported as carcasses found by the public,

followed by corvids. Dead crow reports appeared to be higher in areas of known WNV transmission foci, while such a correlation was less clear for Columbiformes (Blackmore et al. 2003), which are not believed to exhibit high rates of WNV-associated mortality (Nemeth et al. 2007a, Gerhold et al. 2007. In Texas and Louisiana, blue jays were the most common species submitted for carcass testing (n = 104), of which nearly 80% were positive by virus isolation from brain tissue (Siirin et al. 2004). The American crow and blue jay were the most common species submitted for testing and had among the highest proportions of WNV positive carcasses by RT-PCR (approximately 65% and 49%, respectively) in New York State from 2003-04 (Stone et al. 2004). Some raptor species (e.g., red-tailed hawk, American kestrel, great horned owl) also had relatively high proportions of virus or RT-PCR positive carcasses in various geographic locations (e.g., New York State, Colorado), and have potential use in surveillance (Bernard et al. 2001, Stone et al. 2004, Nemeth et al 2007a). Recently, American crow carcasses were found beneath a roost in December in New York, and 13% (n = 98) of brains were WNV positive by TaqMan RT-PCR, with 6.7% (n = 45) of fecal samples positive. The source of these infections remains unknown (there was no evidence of mosquito activity or hibernacula), but the authors suggested that bird-bird transmission may have played a role (e.g., fecal-oral; Dawson et al. 2007).

West Nile virus pathogenesis, virulence, and immune responses in birds

West Nile virus caused an outbreak in a flock of young domestic geese in Israel in 1997. This outbreak was associated with unexpectedly high levels of morbidity and mortality, with birds presenting acutely with paresis and other neurological abnormalities. In subsequent years (1998-2000), recurrences of WNV-associated disease in goose flocks

were observed in Israel (Malkinson and Banet 2002). Experimental inoculation of young geese with a WNV isolate from a goose in Israel (WN-Isr98) resulted in peak viremia titers at 2-4 DPI, decreasing to undetectable levels by 6 DPI in all birds except for one bird that had a detectable viremia on 8 DPI; this bird died on 10 DPI (Banet-Noach et al. 2003). Half of the inoculated geese (n = 10) died between 7-10 DPI. Various isolations of WNV during the same time period in Israel suggested further associated morbidity and mortality; species involved were feral pigeons, collared doves, a rosella, white storks, lappet-faced vultures, and a white-eyed gull. The latter became paralyzed while living in a zoo colony of gulls. Several more WNV isolates were obtained from injured and morbid storks from a flock that had gone wayward during migration (Malkinson and Banet 2002).

Since the arrival of WNV to North America, North American strains have been used in experimental infections to define pathogenesis, supplemented by careful observations in naturally infected birds that have focused on not only pathogenesis, but also on clinical aspects of disease. Experimental inoculations were performed on bird species ranging from domestic chickens and turkeys (Senne et al. 2000, Swayne et al. 2000, Langevin et al. 2001, Swayne et al. 2001), to raptors (Nemeth et al. 2006a,b), corvids such as crows and blue jays (Komar et al. 2003a, Weingartl et al. 2004), and others (Komar et al. 2003a, Komar et al. 2005, Clark et al. 2006, Owen et al. 2006, Reisen and Hahn 2007). Observations from the field, commercial operations, and zoological or rehabilitation settings have provided further insight into WNV-associated morbidity and mortality in birds in North America (Steele et al. 2000, Ludwig et al. 2002, Caffrey et al. 2003, Bertelsen et al. 2004, D'Agostino and Isaza 2004, Gancz et al. 2004,

Wünschmann et al. 2004, 2005, Gibbs et al. 2005a, Joyner et al. 2006, Meece et al. 2006, Ellis et al. 2007, Lopes et al. 2007, Saito et al. 2007, Wojnarowicz et al. 2007). Corvids have proven highly susceptible, with species differences within the Corvid family (Komar et al. 2003a, Turrel et al. 2003); American crows are most severely affected, with mortality rates approaching 100% (McLean et al. 2001, Komar et al. 2003a, Brault et al. 2004, Bunning et al. 2007). Relatively low seroprevalence rates among free-ranging American crows in Georgia support the apparently low survival rate of this species to WNV infection in North America (Wilcox et al. 2007). Raptors are also susceptible to WNV infection, with differences in susceptibility to clinical disease observed among species (Joyner et al. 2006, Saito et al. 2006, Nemeth et al. *in review*); for example, owl species with a more northern distribution appear to be more susceptible (Gancz et al. 2004). Some research has suggested that WNV has had negative population impacts on bird populations (Hochachka et al. 2004, Yaremych et al. 2004, Caffrey et al. 2005, Joyner et al. 2006, Saito et al. 2006, Koenig et al. 2007, LaDeau et al. 2007).

Virulence levels of North American WNV strains have been compared with those of earlier strains. House sparrows inoculated with New York (NY99-4132) and Kenyan (KEN-3829) WNV strains exhibited similar and relatively higher viremia profiles and mortality rates than those inoculated with an Australian strain (Kunjin-6453), leading to the conclusion that the New York and Kenyan strains of WNV were more virulent in house sparrows than the Australian strain (Langevin et al. 2005). A single amino acid substitution in low virulence parental WNV strains generated a highly virulent recombinant phenotype for American crows; this same site of mutagenesis demonstrated a tendency for adaptive evolution. The recombinant strains led to earlier onset of

significantly higher titers of viremia, and higher rates and earlier onset of mortality among inoculated American crows. This type of point mutation could be responsible for the increased virulence of WNV in North America for some species of birds (Brault et al. 2007).

In addition to providing information about morbidity, mortality and viremia profiles (Bowen and Nemeth 2007), experimental WNV inoculations in birds have revealed information regarding serological responses to infection. In general, birds produce detectable neutralizing antibodies within 5-10 DPI (Langevin et al. 2001, Nemeth et al. 2006a,b, Nemeth and Bowen 2007). These antibodies showed little variation in titer over a 60-week period in rock pigeons (Gibbs et al. 2005b), a 12-month period in fish crows (*Corvus ossifragus*) (Wilcox et al. 2007) and 51 months in raptors (Nemeth et al. 2008a). The protective nature of WNV antibodies in birds has not been well documented, but pre-existing WNV antibodies protected against a recurrence of clinical disease in raptors (Nemeth et al. 2008a), and WNV maternal antibodies protected against viremia in chicken and sparrow chicks for a finite period post-hatch (Nemeth and Bowen 2007, Nemeth et al. 2008b).

Age-associated response to West Nile virus infection in birds

The response of young birds to WNV infection is important to understanding transmission because younger birds generally experience greater levels of viremia and rates of morbidity in response to viral infection (McLean 1991). In early experimental WNV inoculation studies using Egyptian strains, chicken chicks developed higher viremia titers than older chickens, and older chickens became refractory to infection via mosquito bite (Taylor et al. 1956). Decades later, during WNV outbreaks in Israel from

1997-2000, young geese (but not adults) were observed with acute neurological disease (Malkinson et al. 1998). After WNV had been introduced to North America, younger geese (*Anser anser domesticus*), aged 6-weeks, within a domestic flock were more affected by WNV disease than older geese in southern Manitoba (Austin et al. 2004). These results were confirmed through an experimental infection study of 2-week old goslings, inoculated with WNV NY99, after which some of them demonstrated lethargy, depression, and weight loss (Swayne et al. 2001). Additional studies using WNV NY99 strains supported the notion that very young chicken chicks were more susceptible to higher mortality rates and viremia profiles with higher peak levels and longer duration than older chickens (Turell et al. 2000, 2001, 2002, Langevin et al. 2001, Nemeth and Bowen 2007).

Passive transfer of maternal antibodies to West Nile virus and other Flaviviruses in birds

Maternally-derived antibodies circulate within blood of newly hatched chicks to provide temporary protection (Tizard 2002). These immunologically immature birds of some species develop higher West Nile viremia levels and more severe associated disease than older individuals (Austin et al. 2004, Nemeth and Bowen 2007). Information regarding the presence, prevalence, duration, and level of protection provided by WNV maternal antibodies in birds would aid in interpreting field data and sentinel flock status, as well as in understanding WNV transmission as the virus continues to expand and establish itself in the New World (Nemeth and Bowen 2007).

Passive transfer of maternal antibodies in birds has been documented for a number of viruses within the family Flaviviridae, including Murray Valley encephalitis

virus, Japanese encephalitis virus (JEV), and St. Louis encephalitis virus (SLEV) (Reeves et al. 1954, Sooter et al. 1954, Warner 1957, Buescher et al. 1959, Bond et al. 1965, Ludwig et al. 1986). In addition, maternally derived neutralizing antibodies to WNV were detected in sera of rock pigeon squabs of naturally infected parents, and persisted for 19–33 days post-hatch (PH) (Gibbs et al. 2005b). Eastern screech owlets (*Megascops asio*) from a captive colony of naturally infected, WNV seropositive adults had circulating maternal antibodies when sampled between 1 and 27 days PH (Hahn et al. 2006). There may be differences in patterns of passive transfer of WNV antibodies among avian individuals and species, and these differences could be associated with varying immune investment in offspring due to physiological trade-offs associated with life history traits of both adults and young (Lowther and Cink 1992, Lochmiller and Deerenberg 2000, Grindstaff et al. 2005).

West Nile virus persistence in birds and mammals

The concept of arbovirus persistence within vertebrate hosts was historically exciting because it could provide a mechanism for which arboviruses maintain themselves in temperate regions during the winter. Persistent virus has been documented in vertebrates for most families of arboviruses (Kuno 2001), including members of the family Flaviviridae, such as JEV (Chunikhin and Takahashi 1971, Mather et al. 1986), SLEV (Slavin 1943, Chamberlain et al. 1957), and WNV (Fedrova and Stavskiy 1972, and Semenov et al. 1973 cited in Kuno 2001, Pogodina et al. 1983). One potential mechanism for the survival of arboviruses through the winter months is persistent or latent infections within vertebrates, with subsequent recrudescence of infectious virus that reinitiates transmission (McLean 1991). Supporting evidence for this theory has been

weak (Emord and Morris 1984, Crans et al. 1994, Gruwell et al. 2000). However, WNV has been isolated from bird carcasses in the winter in the northeastern United States when mosquitoes were inactive (Garmendia et al. 2000, Dawson et al. 2007). Further, experimental inoculation of birds with Western equine encephalitis virus (WEEV) led to isolation of low levels of virus from tissues (i.e., blood, gall bladder, lung, brain, liver, spleen) of various species [Brewer's blackbird (*Euphagus cyanocephalus*), cowbird (*Molothrus* sp), house finch, house sparrow, tricolored blackbird (*Agelaius tricolor*)] between 133 and 306 DPI (Reeves 1990).

Persistent WNV infection has been documented in experimentally inoculated mammals, including rhesus macaques (*Macacus rhesus*; Pogodina et al. 1983), laboratory mice (Brinton et al. 1985), and golden hamsters (*Mesocricetus auratus*; Xiao et al. 2001). Viremia was detected in symptomatic macaques experimentally inoculated (either intracerebrally or subcutaneously) with either Egypt-101 strain, W-956 (Uganda), Hp-94 (European USSR), or Ig-2266 strain (India) as late as 18 DPI, and for up to 11 DPI in asymptomatic macaques. These 'persistent' viremia detections are not sufficient to support over-wintering of WNV in vertebrates, but demonstrate detections of WNV beyond the acute phase of infection. However, WNV was detected at much later time points in the brain of seven macaques at 39, 59, 100, and 167 DPI (strains used for inoculation of these individuals included: Egypt (Eg-101), Russia (Hp-94), Uganda (W-956), India (Ig-2266), poorly pathogenic clones 66 or 64, clone 41/WN+JE+, clone 176/WN+JE+) (Pogodina et al. 1983). Persistent WNV infection in kidneys of experimentally inoculated hamsters (Ding et al. 2005) has led to persistent shedding of WNV in urine for up to 247 DPI. In addition, WNV antigen was observed through

immunohistochemistry in renal tubular epithelial cells and vascular endothelial cells (Tesh et al. 2005). Infectious WNV was also cultured from brains of asymptomatic hamsters on 53 DPI (Xiao et al. 2001).

The potential for persistent WNV infection has been contemplated to occur in free-ranging birds (Garmendia et al. 2000, Yaremych et al. 2004), and may provide a mechanism for reoccurring or continued transmission to vector mosquitoes at some time following initial viremia and apparent viral clearance (Fedrova and Stavskiy 1972 and Semenov et al. 1973 cited in Komar et al. 2003a). Experimentally inoculated birds [i.e., American kestrel, Japanese quail (*Coturnix japonicus*), killdeer (*Charadrius vociferous*), mourning dove (*Zenaida macroura*), budgerigar (*Melopsittacus undulatus*), blue jay, fish crow, red-winged blackbird (*Agelaius phoeniceus*), common grackle, house sparrow] that were sacrificed at 14 DPI yielded infectious WNV in various tissues (Komar et al. 2003a). However, this phenomenon is difficult to definitively demonstrate in nature. Recurrence of presumed WNV-associated illness after apparent recovery has also been reported in some raptors in a rehabilitation setting (Lopes et al. 2007).

Ecological impacts of WNV on birds in North America

There is some evidence that WNV has caused population reductions in some bird species in regions of the United States (Koenig et al. 2007, LaDeau et al. 2007). Researchers have evaluated data that were collected during North American Breeding Bird Surveys to examine annual trends in bird count numbers from both before and after the arrival of WNV to geographic regions of North America. Koenig and colleagues (2007) demonstrated a correlation between susceptibility to WNV as determined by avian carcass testing and population change among bird species after the arrival of WNV to

California. This correlation was attributed mostly to declines in four corvid species: yellow-billed magpie, Stellar's jay, western scrub jay, and American crow. In addition, trends in estimated population numbers of 20 bird species were evaluated over a 26-year period in regions throughout the United States. Seven species showed declines, either sharp, multi-year declines [American crow, American robin, chickadee (*Poecile* spp), eastern bluebird (*Sialia sialis*)], or 1- to 2-year declines following WNV epidemics [blue jay, tufted titmouse, house wren (*Troglodytes aedon*)] (LaDeau et al. 2007).

The ecological impacts of WNV in North America are difficult to document, and fluctuations in numbers of some bird species in regions throughout the United States and Canada may become more evident over time. In addition, the effects of WNV on avian communities in Mexico, Central and South America, as well as other areas of the tropics, are unknown. Certainly the presence of WNV in the United States and Canada has had an alarming affect on native bird species (Marra et al. 2004), and the subsequent changes to ecosystems that are already undergoing many human-associated alterations should not be ignored. Avian responses to WNV infection, including viremia profiles, morbidity and mortality, humoral immune response, dynamics of transovarial transfer of protective antibodies to offspring, and potentially persistent, chronic infections, have implications for the population health of birds in North America, as well as for the larger and more complex ecological picture.

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CHAPTER 2

WEST NILE VIRUS ANTIBODY PERSISTENCE IN HOUSE SPARROWS AND RAPTORS

HUMORAL IMMUNITY TO WEST NILE VIRUS IS LONG-LASTING AND PROTECTIVE IN A COMMON PASSERINE (*PASSER DOMESTICUS*)

ABSTRACT

The house sparrow (*Passer domesticus*) is a common and abundant passerine that is a competent reservoir host of West Nile virus (WNV) and likely contributed to its continued spread and circulation in North America. In addition, many house sparrows survive WNV infection and develop humoral immunity. We performed a controlled study to examine the duration and protection provided by anti-WNV antibodies in house sparrows. Antibodies remained at a relatively constant titer for ≥ 36 months (n = 42) and were protective against viremia upon re-inoculation at 6-, 12-, 24- and 36-months post-inoculation (PI) in all but one individual (98.6%; 70/71). The peak viremia titer in this individual ($10^{2.4}$ PFU/ml serum) was not considered infectious to mosquitoes, and was > 1,000-fold less than peak viremia titers in non-immune sparrows (range $10^{4.5-10.2}$ PFU/ml serum). Serologic responses to secondary exposure in most sparrows (72.9%; 51/70) consisted of a 4-fold or more increase in $PRNT_{90}$ antibody titer by 14 days PI, while 55% (55/100) underwent a 4-fold or more decrease in titer between 1-6 months PI. Mortality during acute WNV infection varied between sparrows housed in cages and bled for 6-7

consecutive days (27.8%; 5/18) and those in a free-flight aviary and not handled following inoculation (8.4%; 9/107). Our results imply that house sparrows are protected from secondary WNV infection for at least three years following initial exposure, which has implications for WNV transmission ecology, as well as the interpretation of serosurveys and diagnosis of WNV in birds. Additionally, WNV-associated mortality rates of free-ranging birds may be less than observed in experimental studies.

INTRODUCTION

West Nile virus (WNV; family *Flaviviridae*, genus *Flavivirus*) has reached endemic status in much of the United States (Bertolotti et al. 2007), and birds likely played a role in its rapid geographic expansion and establishment (Peterson et al. 2003, Rappole and Hubálek 2003, Owen et al. 2006, Rappole et al. 2006). Since its arrival to the Western Hemisphere in 1999, WNV has caused mortality of tens of thousands of birds (Marra et al. 2004), while survivors respond with sufficient anti-WNV antibody production and overcome infection (Fang and Reisen 2006). Seroprevalence rates of various avian species have been recorded within many geographic regions of the United States (Komar et al. 2001a and 2005, Godsey et al. 2005, Reisen et al. 2005c, Stout et al. 2005, Bell et al. 2006, Beveroth et al. 2006, Gibbs et al. 2006, Sullivan et al. 2006), while antibody titers have been measured over time in captive birds. Anti-WNV antibody titers showed relatively little variation over a 60-week period in rock pigeons (*Columba livia*) (Gibbs et al. 2005), and were relatively stable in fish crows (*Corvus ossifragus*) for 12 months (Wilcox et al. 2007) and raptors for up to 51 months (Nemeth et al. 2008). Traditionally, a ≥4-fold increase in antibody titer over several weeks to months indicates

a recent infection, a pattern which aids in interpretation of surveillance or diagnostic data (Beaty et al. 1995).

The duration and protectiveness of primary immunity to WNV in birds over multiple transmission seasons has yet to be characterized in a controlled setting. This information is critical to understanding WNV transmission dynamics, as well as the long-term affects of WNV on avian population health. Herd immunity to WNV could potentially play a role within some geographic regions, depending upon the average longevity of the bird species, transmission intensity, and duration of protective immunity (Kramer and Bernard 2001, Blackmore et al. 2003, Bell et al. 2006). A lapse in WNV immunity might also support the hypothesis that persistent virus infections in birds may serve as an over-wintering mechanism for some arboviruses, such as WNV, within temperate areas (Reeves 1990). In addition, data regarding long-term duration of antibodies, as well as response to challenge, in a variety of bird species would aid in interpretation of WNV serosurveys and understanding the epidemiology and ecology of WNV. Therefore, we performed a 36-month controlled study of WNV infection in house sparrows (*Passer domesticus*), which are a common and ubiquitous passerine species, are a competent reservoir host of WNV, and have likely played a role in its spread across the United States (Komar et al. 2001b, Peterson et al. 2003, Rappole and Hubálek 2003, Hamer et al. 2008).

To determine the duration of WNV neutralizing antibodies in house sparrows and characterize their viremic and serologic responses following secondary exposure at various time points post-infection, we studied a captive population of house sparrows of known WNV exposure history for a three year period. Our objectives were: 1. to follow

anti-WNV antibody titers of experimentally-inoculated house sparrows for 36 months, 2. to assess the protectiveness of anti-WNV antibodies in sparrows over time, 3. to assess serologic responses to primary and secondary exposure in sparrows, 4. to determine whether contact transmission occurs among communally housed sparrows over a 3-year period, and 5. to compare morality rates among sparrows caged and handled throughout the period of acute WNV infection (1-7 days post-inoculation; PI) with those within a free-flight aviary and not captured during this time.

MATERIALS AND METHODS

Sparrow collection and husbandry

From January-March, 2005, 179 house sparrows were captured by mist net in northern Colorado and immediately transported to Colorado State University in Fort Collins, Colorado. Upon arrival, birds were leg-banded with color (Avinet, Darvic, size XCSD) and numbered bands (AOU band 1b aluminum bands), weighed, and bled from the jugular vein.

Sparrows were housed free-flight, divided equally between two rooms (each 3.7m x 3.7m x 5.5m L) containing tree branches, ropes, tree stumps, sand baths, cuttlefish bone, and multiple food and water stations. Fresh water and food were provided *ad libitum*; food consisted of a dry mix of millet, milo, cracked corn, cracked sunflower seed, and oats (in equal parts), as well as live mealworms 1-2 times/week. Oxytetracycline was administered in the water for 12-14 days (700 mg/gallon) following arrival. Sparrows were acclimated to their captive surroundings for several weeks to several months prior to experimental inoculation. At the 12-month post-inoculation (PI) capture, all remaining sparrows were combined into one 2.57m high x 3.24m wide x

12.12m long room. Birds that exhibited signs of illness (lethargy, fluffed feathers, anorexia) at any time point prior to or during the study were euthanized via sodium pentobarbital overdose administered intravenously.

Following WNV inoculation, a subset of immune and non-immune birds were caged separately, with 2-5 birds per cage, with cage dimensions either 0.38m high x 0.41m wide x 0.61m long or 0.43m high x 0.46m wide x 0.76m long.

Experimental groups, inoculation, and sample collection

Sparrows were divided into three experimental groups based on initial WNV serostatus. Groups included WNV seronegative birds for experimental inoculation (hereafter, deemed "experimentally immune;" n = 114), naturally-infected birds with pre-existing anti-WNV antibodies (hereafter, deemed "naturally immune;" n = 21), and WNV seronegative birds to serve as antibody-negative controls (hereafter, deemed "non-immune;" n = 20). The former two groups were experimentally inoculated with WNV, while the latter served as seronegative, contact controls, some of which were later used during challenge studies as non-immune controls.

On the day of inoculation, all birds were again bled as before for confirmation of WNV neutralizing antibody status prior to inoculation. Sparrows were inoculated subcutaneously over the chest with approximately 1,000-2,000 plaque forming units (PFU) of WNV strain NY99-4132 (originally isolated from an infected crow and passaged once in Vero cells, once in C6/36 mosquito cells, and once in baby hamster kidney-21 cells), administered in 0.1 ml BA1 (M199-Hank's salts, 1% bovine serum albumin, 350 mg/L sodium bicarbonate, 100 units/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL amphotericin B in 0.05 M Tris, pH 7.6).

Following initial WNV inoculation, all but 14 sparrows were housed free-flight within the rooms previously described. These 14 sparrows were caged for seven days prior to joining the free-flight group. The caged sparrows consisted of seven naturally immune birds and seven birds that were WNV antibody negative upon arrival (and became part of the experimentally immune group). These birds were bled daily from 1-6 days PI to assess viremic responses to infection. Daily blood collections following inoculation involved removal of 0.1 ml of blood via jugular venipuncture, with blood immediately added to 0.45 ml BA1 with 20% fetal bovine serum (FBS) for an approximate 1:10 serum dilution. Diluted blood samples were held at room temperature for approximately 20-30 minutes for coagulation, centrifuged for 10 min at 2000 x G, and then sera extracted and frozen to -80°C until testing by Vero cell plaque assay. Any sparrows that died <10 days following WNV inoculation and had WNV isolated from numerous tissues was considered to have experienced acute WNV-induced mortality.

Non-immune sparrows (n = 20) were not experimentally inoculated with the initial group, but remained among the inoculated birds in the free-flight aviary to assess potential contact-exposure and serve as seronegative controls for subsequent challenge experiments.

All birds were captured by hand-held nets and bled 0.2 cc at 1-, 6-, 12-, 18-, 24-, 30-, and 36-months PI. Blood samples collected at these intervals were placed undiluted into serum separator tubes, allowed to coagulate at room temperature for 1-2 hours, centrifuged for 3 min at 16,000 x G, and sera frozen to -20°C.

At 6-months PI, the 21 naturally immune sparrows that had been challenged 6 months prior were bled and euthanized.

For challenge experiments, ten experimentally immune sparrows were placed into cages with 2-4 birds/cage, allowed to acclimate for several days, then challenged with WNV (or secondarily exposed in the case of experimentally immune birds) by needle-inoculation with 2,500-3,500 PFU of WNV strain NY99-4132. At each 6- and 12-months PI, ten experimentally immune sparrows with relatively low antibody titers (reciprocal $PRNT_{90}$ titers from 10-40) as determined by the most recent sampling time point (1- and 6-months PI, respectively) were challenged. At 24-months PI, ten experimentally immune sparrows (18-month PI $PRNT_{90}$ titers from 20-320) were challenged, and at 36-months PI all remaining experimentally immune sparrows (30-month PI $PRNT_{90}$ titers from 10-640) were challenged. In addition, with each challenge experiment, non-immune sparrows were also inoculated to serve as non-immune controls. At the 36-month time point, five non-immune sparrows were inoculated, while at all other time points, two non-immune sparrows were inoculated. During challenge experiments, non-immune and experimentally immune sparrows were housed separately.

Following challenge inoculation (or initial inoculation for non-immune controls), birds were bled 0.1 ml for 1-7 days PI, and then bled and euthanized on 14 days PI. Blood samples collected from 1-7 days PI were processed as described above for daily blood collections following inoculation. Blood samples collected on 14 DPI were left undiluted and processed as described for blood samples collected at 1-, 6-, 12-, 18-, 24, 30- and 36-months PI.

Sparrows that died or were euthanized due to morbidity at < 10 DPI were necropsied immediately when possible or refrigerated and necropsied within 24 hours. At the time of necropsy, oral swabs were collected by swabbing the oropharyngeal cavity

with a cotton-tipped swab and placing the swab in 1 ml BA1 with 20% FBS. Tissues (spleen, kidney, heart, and brain) were collected and placed in 1 ml BA1 with 20% FBS for an approximate 10% tissue suspension. A single copper-coated steel 4.5 mm ball bearing ("BB") pellet was added to each tissue vial, and tissue samples were homogenized via a Qiagen mixer mill (Qiagen, Valencia, CA) run at 25 cycles/second for 5 minutes, after which homogenates were clarified by centrifugation (12,000 x G for 3 minutes). Samples were stored at -4°C if testing occurred within 24 hours; otherwise, they were frozen to -80°C until thawing for testing.

Vero cell plaque assay and plaque reduction neutralization test

To assess for the presence of WNV neutralizing antibodies in sera, samples were tested by plaque reduction neutralization test (PRNT; Beaty et al. 1995) at a 1:10 dilution using WNV strain NY99-4132 (the same strain used for all inoculations). Antibody positive serum samples were serially diluted 2-fold (beginning at 1:10) and tested in duplicate to determine reciprocal endpoint 90%-neutralization ($PRNT_{90}$) titers. Significant anamnestic antibody responses to secondary exposure were signified by a $\geq$ 4-fold increase in $PRNT_{90}$ titers measured between two weeks and one month PI. The same anti-WNV antibody positive control serum was used in all PRNT assays.

Serum samples collected between 1-7 days PI, as well as oral swabs and tissue homogenates (kidney, spleen, heart, and brain) from birds dying within 10 days PI were tested for infectious virus by Vero cell plaque assay as previously described (Bunning et al. 2002). Briefly, Vero cell monolayers in six-well plates were inoculated in duplicate with 0.1 ml of sample per well. After 1 hour of incubation at 37°C, the cells were overlaid with 3 ml/well of 0.5% agarose (in M-199 medium supplemented with 350 mg/l

sodium bicarbonate, 29.2 mg/l L-glutamine, and antibiotics as with BA1). Two days later, cells were overlaid with 0.5% agarose with 0.004% neutral red dye (Sigma Chemical Corp, St. Louis, Missouri, USA). Viral plaques were counted after 3 and 4 days of inoculation of wells.

Detection of infectious WNV plaques was reconfirmed through reisolation, while the identity of the plaques was verified by VecTest WNV Antigen Assay (VecTest; Medical Analysis Systems, Camarillo, CA) as previously described (Nemeth et al. 2007).

Mathematical and statistical analyses

To assess the variation in $PRNT_{90}$ titers among the sparrow group throughout each time point of the study, the multiple-fold decrease or increase in titer for each individual bird present for a given time point and the one immediately following was represented by a numerical value (e.g., -2 for a two-fold decrease, 0 for no change in titer, +2 for a two-fold increase). These multiple-fold value changes between two chronological time periods were then averaged among all individual birds for each of the later time points to determine average changes in titer over time for all birds present at each time period (Table 2.2). This calculation avoided eliminating individuals from the analysis that were not present throughout all time points in which $PRNT_{90}$ titers were determined (1-, 6-, 12-, 18-, 24-, 30-, and 36-months PI).

A Chi-Square test ($\alpha = 0.05$) was used to compare mortality rates (as proportions) among caged, frequently captured and sampled sparrows versus free-flight sparrows that were not handled following inoculation. Peak viremia titers (in $\log_{10}$ PFU/ml) were analyzed as a function of disposition (death vs. survivor) using general linear model procedure (Proc GLM). Peak viremia titers were treated as a dependent variable and

disposition was a fixed variable. Statistics were calculated in SAS/STAT MULTTEST software, version 9.1 (SAS Institute, Inc., Cary, North Carolina 27513, USA).

RESULTS

Initial serology and mortality

A total of 179 sparrows were brought into captivity, 31 (17.3%) of which had WNV neutralizing antibodies upon arrival (ten of these seropositive sparrows were excluded from the study, while 21 were challenged and euthanized 6 months later). Upon arrival, sparrows with $\leq$ 60% or less WNV neutralization by PRNT at a 1: 10 dilution were considered seronegative, while all others had 100% WNV neutralization at a 1:10 serum dilution and were considered seropositive.

Of the seronegative birds brought into captivity, 14 died or were euthanized prior to initiation of the study. In addition, three experimentally immune birds were euthanized at 6-, 12-, 18- and 24-months PI for a separate study, and 10 experimentally immune birds and 2 non-immune birds were challenged and euthanized at each of the 6-, 12-, and 24-month PI time points. Additional deaths (n = 23) occurred over the 3-year study due to apparently natural causes, aviary or bird-induced trauma, or husbandry- or capture-related causes. At 36-months PI, 52 sparrows remained alive (42 experimentally-inoculated seropositive sparrows, and 10 seronegative controls); one of the experimentally immune sparrows died at the 36-month PI capture, leaving 41 experimentally immune birds for challenge.

None of the 21 naturally immune sparrows challenged with WNV exhibited morbidity or mortality within 6 months PI, at which time they were euthanized. No

experimentally immune birds died within 14 days of challenge except one sparrow that died on 5 days post-challenge at 36-months PI.

There was a significant difference between WNV-associated mortality of sparrows caged and handled following inoculation (5/18; 27.8%) versus those that were free-flying and not handled (9/107; 8.4%) ($n = 125$, $\chi^2 = 5.81$, $P = 0.016$; Odds ratio 23.88, 95% CI: 0.0693, 0.8225).

Viremic and clinical responses of immune sparrows when challenged with WNV

None of the seven naturally infected seropositive sparrows that were bled daily from 1-6 days following experimental challenge with WNV had detectable viremia.

Upon challenge at 6-months PI, 9/10 birds had no detectable viremia (pre-challenge $PRNT_{90}$ titers ranged from 10-80). In one sparrow (with a pre-challenge $PRNT_{90}$ titer of 10), viremia was from $10^{1.7-2.4}$ PFU/ml on 3-5 days post-challenge. No experimentally immune birds challenged at 12-, 24- or 36- months PI had detectable viremia or any clinical signs of disease, except for one sparrow that died on 5 days post-challenge at 36 months PI (having a $PRNT_{90}$ titer of 80 at 30-months PI). This bird had no detectable virus in sera or tissues (heart, spleen, brain, and kidney), but did have a low titer (1.5 PFU/swab) of infectious WNV in oropharyngeal swab collected after death.

Serologic responses of naturally immune sparrows following WNV challenge

Pre-challenge $PRNT_{90}$ titers of naturally immune sparrows ranged from 10-320, while 1-month PI titers were between 80-2,560; 38.1% (8/21) of sparrows exhibited a 4-fold or greater increase in titer at 1-month post-challenge (Table 2.1). At 6-months PI,

$PRNT_{90}$ titers ranged from 40-1,280, with 19.0% (4/21) exhibiting a 4-fold or more decrease from the 1-month PI titer.

Acute serologic responses of experimentally immune sparrows following WNV challenge

The single experimentally immune sparrow that had detectable viremia following challenge at 6 months PI had a marked anamnestic antibody response by 14 DPI, with a $PRNT_{90}$ increase from 10 to 2,560. Approximately 73% (51/70) of experimentally immune sparrows responded with a $\geq$ 4-fold increase in $PRNT_{90}$ titer by 14 days post-challenge. These immune responses varied from no change to a 512-fold increase at 14 days following secondary exposure (Table 2.1). Nine of 40 immune birds challenged at 36-months PI had a marked anamnestic response with $\geq$ 32-fold increase in titer (e.g., the $PRNT_{90}$ of one individual increased from 40 to 20,480). The single experimentally immune sparrow that died on 5 days PI had not mounted a rise in titer by the time of death.

Long-term serologic patterns following primary WNV inoculation

All sparrows seroconverted that were initially negative for anti-WNV antibodies and then experimentally inoculated; $PRNT_{90}$ titers at 14 days to 1-month PI ranged from 40-2,560. Fifty-five percent (55/100) of sparrows had a 4-fold or more decrease in titer between 1- and 6-months PI, and the range of titers at the latter time point was 10-2,560. However, subsequent to the 6-month PI antibody titer assessment, $PRNT_{90}$ titers of most sparrows from subsequent time points did not vary more than two-fold through 36-

months PI. At 12-, 18-, 24-, 30- and 36-months PI, sparrow $PRNT_{90}$ titers of the majority of sparrows ranged from approximately 20-160 (Table 2.2).

Viremia, morbidity, and tissue tropism of non-immune sparrows

All seronegative controls remained seronegative throughout the study. Ten of the twenty sparrows were alive at 36-months PI; six had been sacrificed during previous challenge experiments, while four died due to apparent natural causes during the study.

All non-immune control sparrows inoculated during challenge studies reached detectable viremia titers of variable duration between 1-7 days PI, with peak viremia titers among those that showed no signs of morbidity and survived to 14 DPI ranging from $10^{4.5-7.6}$ PFU/ml serum. Five of 18 (27.8%) had clinical signs, including lethargy, fluffed feathers, anorexia, and/or hind limb rigidity; some died and others were euthanized between 5-9 DPI. The range of peak viremia titers among those that died or were euthanized was $10^{5.5-10.2}$ PFU/ml serum, with death occurring from 1-5 days after peak viremia. There was a significant difference in peak viremias of those that experienced WNV-associated morbidity and mortality vs. those that survived acute infection (n = 18, P = 0.006, 95% CI: 0.694, 3.401). All of the individuals that died or were euthanized had WNV isolated from the oral swab ($10^{2.2-6.2}$ PFU/swab), heart ($10^{1.7-6.5}$ PFU/0.5 cm^3), and kidney ($10^{2.3-7.1}$ PFU/0.5 cm^3), and 4/5 birds also had virus isolated from brain ($10^{4.2-6.6}$ PFU/0.5 cm^3) and spleen ($10^{4.4-7.1}$ PFU/0.5 cm^3), with higher titers corresponding to those that died earlier following inoculation (5-6 DPI). Virus isolation from swabs and tissues collected from the eight individuals that had no clinical signs and survived to 14 days PI was rare and at low titers (spleen from two individuals at $10^{1.3-2.0}$ PFU/cm^3, and kidney $10^{1.0}$ PFU/cm^3 and heart $10^{0.7}$ PFU/cm^3 from another individual).

DISCUSSION

An understanding of the duration and protection provided by WNV immunity in passerine birds is important, because numerous members of this large taxonomic group have been deemed competent reservoir hosts (Komar et al. 2003), and are commonly fed upon by mosquitoes (Apperson et al. 2004, Kilpatrick et al. 2006, Savage et al. 2007). While many passerines experience relatively high viremia titers following WNV inoculation, some also mount an effective immune response and survive infection (Komar et al. 2001b, Komar et al. 2003, Beveroth et al. 2006, Gibbs et al. 2006). While the duration, variation, and protection provided by anti-WNV antibodies may vary among birds, naturally-induced WNV neutralizing antibodies were detectable and remained at relatively consistent levels for at least 1-4 years in some non-passerine and passerine species (Gibbs et al. 2005, Wilcox et al. 2007, Nemeth et al. 2008). Information on the duration of WNV immunity in the house sparrow adds to existing knowledge, which collectively may be used to evaluate potential population-level WNV transmission dynamics and health effects on free-ranging birds.

The house sparrow is an abundant North American passerine and commonly lives within human-altered habitats, including residential and urban areas (Lowther and Cink 1992). This species has been implicated in the epizootic cycles of numerous arboviruses in the United States (e.g., Eastern equine encephalitis, St. Louis encephalitis, Venezuelan equine encephalitis, and Western equine encephalitis viruses) (Kruszewicz 1995). The house sparrow is a highly competent WNV amplifying host and readily transmits the virus via mosquitoes (Komar et al. 2003, Reisen et al. 2005b, Kilpatrick et al. 2007). Hatch-year house sparrows were deemed important in the amplification of epizootic

WNV transmission, as well as contributing to local virus amplification and thereby increasing human risk of infection (Hamer et al. 2008). While house sparrows were deemed to be avoided by feeding mosquitoes relative to other bird species, theoretical removal of sparrows from the population decreased "community WNV reservoir competence" (i.e., WNV transmission) (Kilpatrick et al. 2006). Further, high WNV seroprevalence rates have been observed in house sparrows in some areas of the United States (Komar et al. 2001b, Godsey et al. 2005, Komar et al. 2005, Beveroth et al. 2006). These findings collectively suggest that within areas of high rates of local transmission, many house sparrows are exposed, potentially transmit, and survive WNV infection.

Some have suggested that widespread mortality of wild birds, e.g., corvid species, has been an important factor in the emergence of WNV in North America (Foppa and Spielman 2007). In turn, survival of birds, and possibly differential survival rates of some species over others, may be important in the continued maintenance and emergence of WNV. For other zoonotic pathogens, such as avian influenza viruses, transmission rates could increase due to pre-existing immunity among poultry and other birds. Immunity to one subtype may provide some level of cross-protection in some birds, masking disease but permitting shedding (Seo et al. 2001). Further, the existence of a proportion of immune individuals within a given population may lead to 'epidemic enhancement,' which may facilitate pathogen persistence. The epidemic is of extended duration and/or larger magnitude than it would have been if the population had been naïve (Pulliam et al. 2007). West Nile virus transmission patterns can be unpredictable and difficult to control (Gubler 2007), and existing immunity in some bird populations could in part shape these patterns.

Elevated levels of WNV transmission may lead to high exposure rates among birds, resulting in a proportion of immune birds that would be dead-end hosts within the same or subsequent transmission seasons, thereby dampening transmission rates (Reisen et al. 2003). Therefore, the proportion of WNV-immune birds would be expected to increase over time (Gibbs et al. 2005), leading to potentially lower transmission levels and mortality rates (Kramer and Bernard 2001). A given population could theoretically reach herd immunity, a concept that pertains to the overall protection of a given population against infection due to some proportion of immune individuals within the population; the extent of herd immunity needed to protect a given population may vary, and in some cases partial resistance may lead to some reduction in disease frequency (Fine 1993). However, with the relatively high turnover rate of house sparrows and many other passerines (e.g., short lifespan and high reproductive rate) (Lowther and Cink 1992), herd immunity may not be attainable. While annual survival of hatch-year sparrows is only 20%, annual survival of adults is 57%, and longevity of a free-ranging sparrow has reached 13 years, 4 months (Lowther and Cink 1992). In addition, sparrow mark-recapture data revealed an average of 559 days (range 502-649) between recaptures in southern California, demonstrating that some free-ranging sparrows likely live through multiple transmission seasons (Gruwell et al. 2000). It has also been demonstrated that free-ranging birds with greater body size had higher WNV seroprevalence, attributed in part to longer life spans and relative vector attraction to larger-sized birds (Figuerola et al. 2008).

Along with the duration of circulating anti-WNV antibodies, the protection provided is relevant to continued transmission and avian population health. Anti-SLEV

neutralizing antibodies began a rapid then gradual decline after three months PI, but persisted in 36% of house sparrows for up to two years, when antibodies protected against viremia upon secondary exposure (McLean et al. 1983). Neutralizing anti-SLEV antibodies were also undetectable in some house finches (*Carpodacus mexicanus*) by 6-12 months PI; however, even with undetectable SLEV-neutralizing antibodies, some experimentally inoculated house finches were protected from viremia at 6 and 12 months PI, with a strong anamnestic antibody response to challenge in the latter group (Reisen et al. 2001, 2003). Undetectable titers of anti-WNV maternal antibodies also protected chicken chicks from viremia upon secondary exposure (Nemeth and Bowen 2007). In the present study, all but one sparrow challenged by needle-inoculation at 6-, 12-, 24-, and 36-months following initial infection demonstrated sterilizing immunity. The significance of the single sparrow that experienced a relatively low titer viremia is unknown, but this level is not considered infectious to most mosquitoes (Komar et al. 2003, Kilpatrick et al. 2007) and is substantially lower than peak viremia titers observed in non-immune sparrows following infection (Table 2.1). The antibody titer of this bird five months prior to challenge was also relatively low ($PRNT_{90} = 10$), though other sparrows with the same titer upon challenge had no detectable viremia. One experimentally immune sparrow died unexpectedly following challenge inoculation (5 DPI); WNV was unlikely associated with this death due to lack of virus detection in serum and tissues. However, low levels of virus were detected in the oropharyngeal cavity upon death, the significance of which is unknown.

In further support of the protection provided by anti-WNV antibodies against homologous challenge, cross neutralization occurs among various flaviviruses, including

SLEV, WNV and Japanese encephalitis virus (JEV), suggesting that there may also be partial or complete protection provided by antibodies to one virus against the others (Calisher et al. 1989). Cross-protection among flaviviruses (e.g., WNV, JEV, and SLEV) has been supported by experimental infection studies of mammals and birds (Ilkal et al. 1988, Goverdhan et al. 1992, Ilkal et al. 1994, Fang and Reisen 2006, Patiris et al. 2008). As with WNV and SLEV transmission, birds are accepted as amplifying hosts in the transmission cycle of JEV (Buescher et al. 1959), an emerging virus that has not yet been documented in North America (Mackenzie et al. 2004). Pre-existing WNV neutralizing antibodies in birds may dampen JEV transmission in the event of its introduction into WNV endemic areas, such as the United States. If pre-existing immunity to WNV leads to partial or sterilizing immunity in birds, transmission of other flaviviruses would likely be reduced if there are WNV-immune individuals within the population (Reisen et al. 2005b).

The persistence of anti-WNV antibodies in birds should be considered in the interpretation of serologic studies of free-ranging birds (Gibbs et al. 2005), as well as WNV diagnosis in individuals. For example, WNV seroprevalence rates were as high as 69.2% in northern cardinals (*Cardinalis cardinalis*) in New York (Komar et al. 2001a), and 77.8% in northern mockingbirds (*Mimus polyglottos*) in Louisiana (Komar et al. 2005), suggesting that transmission rates were potentially high in these areas prior to sample collection, and many individuals of these species survived exposure. However, the timing and location of initial infection in these birds, as well as subsequent exposures, are difficult or impossible to decipher from analysis of a single serum sample (Nemeth et al. *in review*). If birds in these studies remained local and were relatively long-lived, a

single season of high transmission would be reflected in serosurveys for multiple years. Secondary WNV exposure in birds has typically been associated with a ≥ fourfold increase in pre-exposure antibody titers within 2-4 weeks of secondary exposure. However, results from the present study suggest that not all birds demonstrate an anamnestic rise in antibody titer within this time frame. A similar observation was made in house finches immune to SLEV at 2- and 6-weeks following homologous challenge. However, in the same study, all of four WNV-immune finches challenged with WNV exhibited a ≥ 4-fold increase in WNV $PRNT_{80}$ titer (Fang and Reisen 2006). Birds lacking a significant rise in antibody titer may have experienced a delayed rise or existing immunity was sufficient to control subsequent exposures. Most sparrows experienced a ≥ 4-fold decrease in antibody titer between 1- and 6-months PI, likely reflecting a decline in antibody titer after the initial peak that follows primary infection, a pattern that could be used to indicate recent WNV infection in birds (Nemeth et al. 2008). It should be recognized that the inoculation method (needle) in the present study could have affected the sparrows' immune responses; however, mosquito versus needle inoculation did not significantly affect overall patterns of infection observed in chickens or house finches inoculated with various arboviruses, including WNV (Reisen et al. 2005a, Styer et al. 2006).

Lack of contact transmission as evidenced by lack of seroconversion among non-immune sparrows in this study was not surprising, despite its occurrence among other captive birds in experimental settings (Swayne et al. 2001, McLean et al 2002, Komar et al. 2003). In the present study, sparrows were not housed in close quarters as in some previous studies, but within relatively large aviaries that provided many options for

perching, and numerous feeding and water stations. Further, water receptacles contained > 1 gallon of water, thereby diluting any virus shed from oropharyngeal cavities of sparrows while drinking. This setting was likely more reflective of natural conditions, including behavior of the sparrows. While WNV is shed at relatively high titers in oral secretions and feces, contact transmission may be relatively rare among free-ranging birds. However, with the relatively close oral and fecal contact between parent and nestling birds, WNV inter-family transmission may be more common during the breeding season (Komar et al. 2003).

Much of the currently available information regarding avian mortality rates associated with North American strains of WNV has been derived from studies involving caged birds that were frequently handled. These WNV-associated morbidity and mortality rates may over-estimate those of free-ranging birds. Among caged house sparrows inoculated with WNV NY99 and then bled daily, mortality rates ranged from 38% (3/8; Langevin et al. 2005) to 50% (3/6; between 3-6 DPI; Komar et al. 2003). In the present study, the mortality rate of caged birds handled daily was 5/18 (27.8%), significantly higher than that among birds allowed free-flight in an aviary and spared the handling stress of capture and blood collection (7.5%; 8/107). The stress of confinement and repeated close contact and handling by humans should not be discounted in the interpretation of captive bird studies including pathogenesis and morbidity and mortality, especially when extrapolating these results to free-ranging birds. Not surprisingly, peak viremia titers of birds that had WNV-associated morbidity and/or mortality were significantly higher than of those that survived infection and showed no clinical signs. This finding supports the notion that some individuals are less able to control WNV

infection, having higher levels of viral replication in the blood, and fatal outcomes are more likely in these individuals.

In conclusion, WNV transmission dynamics are greatly dependent upon avian amplifying hosts that are crucial to successful transmission of this virus in nature. Knowledge of the duration of protective immunity to WNV, which appears to last beyond multiple transmission seasons in a common passerine species, aids in both understanding possible ecological roles of birds in various geographic regions as WNV persists in North America, as well as predicting future transmission patterns. Finally, whether a given avian population is able to reach herd immunity depends upon many factors, including evolution and natural history of birds, as well as vectors and the virus, but is presumably less likely in relatively small-bodied, short-lived birds (Figuerola et al. 2008).

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TABLE 2.1. Serologic responses among non-immune and immune house sparrows following experimental West Nile virus inoculation.

		Pre-inoculation	Viremia profiles		14 days PI*	
Experimental Group, time post-inoculation (PI)	n	$PRNT_{90}$† range	% viremic	Peak range (PFU/ml serum)	$PRNT_{90}$ titer range	% ≥ 4-fold increase in titer
Non-immune controls	18	< 10‡	100%	$10^{4.5\text{-}10.2}$	40-2,560§	NA
Naturally immune	7	10-320	0 %	$< 10^{1.7}$	80-2,560¶	38%
Experimentally immune, 6 mo PI	10	10-80	10%	$10^{1.7\text{-}2.4}$	80-2,560	80%
Experimentally immune, 12 mo PI	10	< 10-80	0%	$< 10^{1.7}$	20-2,560	80%
Experimentally immune, 24 mo PI	10	10-320	0%	$< 10^{1.7}$	40-2,560	60%
Experimentally immune, 36 mo PI	41	10-640	0%	$< 10^{1.7}$	80-≥5,120	73%

* PI = post-inoculation.
† $PRNT_{90}$ = endpoint 90% neutralization titer.
‡ $PRNT_{90}$ < 10 represents birds that were seronegative.
§ The $PRNT_{90}$ titer range was determined for nine sparrows.
¶ One month PI.

TABLE 2.2. Antibody profiles of a captive group of house sparrows following experimental inoculation with West Nile virus.

	Time post-inoculation (months)						
	1	6	12	18	24	30	36
n*	104	100	82	69	65	45	42
$PRNT_{90}$ range	40-2,560	10-2,560	<10-320	10-640	20-1,280	10-640	10-640
% with $PRNT_{90}$ 20-160	79.8†	62	79.2	84.1	81.5	86.7	85.7
Overall change in $PRNT_{90}$‡	—	-4.7	-2.4	0.0	1.1	-1.6	0.3

* The sample size represents the number of sparrows still alive, and therefore sampled, during each time point, and is the number included in $PRNT_{90}$ analyses.
† The range of $PRNT_{90}$ titers at 1-month PI reflected in the % sparrow calculation was 160-640.
‡ The overall change in $PRNT_{90}$ titers reflects the number-fold increase or decrease in titer from the closest previous time point to the current time point.

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NATURALLY-INDUCED HUMORAL IMMUNITY TO WEST NILE VIRUS INFECTION IN RAPTORS

ABSTRACT

West Nile virus (WNV) infection can be fatal to many bird species, including numerous raptors, though population- and ecosystem-level impacts following introduction of the virus to North America have been difficult to document. Raptors occupy a diverse array of habitats world-wide and are important to ecosystems for their role as opportunistic predators. We documented initial (primary) WNV infections and then regularly measured WNV-specific neutralizing antibody titers in 16 resident raptors of seven species, plus one turkey vulture. Most individuals were initially infected and seroconverted between July and September of 2003, though three birds remained seronegative until summer 2006. Many of these birds became clinically ill upon primary infection, with clinical signs ranging from loss of appetite to moderate neurological disease. Naturally-induced WNV neutralizing antibody titers remained essentially unchanged in some birds, while eight individuals experienced secondary rises in titer presumably due to additional exposures at one, two or three years following primary infection. No birds experienced clinical signs surrounding or following the time of secondary exposure, and therefore antibodies were considered protective. Results of this study have implications for transmission dynamics of WNV and health of raptor populations, as well as the interpretation of serologic data from free-ranging and captive birds. Antibodies in raptors surviving WNV may persist for multiple years and protect against potential adverse effects of subsequent exposures.

INTRODUCTION

West Nile virus (WNV; family Flaviviridae, genus *Flavivirus*) infection causes severe disease leading to population reductions in many species of North American birds, including raptors (Marra et al. 2004, Joyner et al. 2006, McLean 2006, LaDeau et al. 2007, Saito et al. 2007, Nemeth et al. *in review*). Experimental WNV infections in raptors suggest that numerous species are reservoir competent (develop infectious-titered viremia) and in contrast to other competent hosts such as the American crow (*Corvus brachyrhynchos*), often survive infection (Komar et al. 2003, Nemeth et al. 2006a,b). Birds that survive experimental WNV infection circulate antibodies that protect against future infection (Brault et al. 2004). Raptors are a potentially long-lived group of birds (Newton 1979) and therefore, both individuals and populations may benefit from long-term WNV antibody duration and protection.

The duration of WNV-antibody persistence in most bird species is poorly characterized, particularly in raptors. Furthermore, the antibody response to a secondary WNV exposure has yet to be adequately described for any vertebrate, and protection provided by pre-existing antibodies is difficult to document in wildlife species. Because it is difficult to ascertain whether re-exposure has occurred in seropositive free-ranging birds, determining precise exposure rates for WNV among birds can be problematic (Komar 2001). Knowledge of duration of detectable antibodies in birds would aid in the interpretation of serologic data, increase our understanding of WNV transmission dynamics, and help reveal the potential effects of WNV on raptor populations and their respective ecosystems.

We opportunistically followed up on a preliminary observation that approximately 80% of a small cohort of outdoor-held raptors seroconverted to WNV in 2003. By serially sampling these individuals, we could address the question of antibody duration in birds, and more specifically, raptors. We expected that WNV-induced humoral antibodies would persist over time, and that immune birds would respond to secondary exposure with elevation of WNV-specific antibody titers. Accordingly, we monitored WNV-antibody titers and clinical status for up to 51 months in 16 recently infected raptors (plus one turkey vulture; *Cathartes aura*).

MATERIALS AND METHODS

Bird Origin and Husbandry

Birds in this study (Table 2.3) had previously been in rehabilitation but were deemed non-releasable, and therefore resided as educational birds at the Rocky Mountain Raptor Program (RMRP) in Fort Collins, Colorado. All but one of the individuals in the study was an educational bird at RMRP prior to the initiation of the study, while one red-tailed hawk (*Buteo jamaicensis*, 197) was admitted to rehabilitation in the summer of 2003, deemed non-releasable, and shortly thereafter began training as an educational bird. All birds were housed outdoors in flight cages year-round and offered a variety of fresh prey items and water daily. Covering cages with mosquito-proof netting was not feasible due to financial constraints. When birds demonstrated abnormal behaviors or clinical signs (e.g., lethargy, anorexia, ataxia, increased excitability), they were moved to indoor isolation cages and provided supportive and/or medical care until sufficient clinical improvement permitted their return to normal husbandry. No birds in this study had been vaccinated against WNV or any other pathogen.

Sample Collection

Serial blood samples were collected from each of 16 resident educational raptors, plus one turkey vulture. Sample collection from most birds began in April 2003 (seven birds were also sampled between November 2002 and January 2003) and continued through October 2007. Samples were collected monthly between April and October, and then in December and February, except for the turkey vulture, from which continuous serial sample collection did not begin until May 2005 (though additional samples had been collected in January 2003 and April 2004). Sampling was less frequent than monthly during the winter due to the unlikelihood of WNV transmission during extended periods of cold weather. Blood (0.3-0.7 cc) was collected via ulnar venipuncture into Microtainer® serum separator tubes, and stored at -20°C after centrifugation for separation of serum.

Plaque Reduction Neutralization Test

The plaque reduction neutralization test (PRNT) of serum samples was performed using Vero cell monolayers as previously described (Beaty et al. 1995) with a challenge dose of approximately 100 PFU of WNV strain NY99-4132. Serum samples were screened at a 1:10 dilution. Sera that reduced the challenge dose by at least 80% were suspect positives, and these were titrated in duplicate by testing serial two-fold dilutions (beginning at 1:10) to determine reciprocal endpoint 90% neutralization ($PRNT_{90}$) titers. For titration of samples from individuals determined to have WNV neutralizing antibodies, the current month's serum sample was tested concurrently with the most recent previously collected sample to detect changes in $PRNT_{90}$ antibody titers. Primary WNV infection was identified by seroconversion from negative status ($PRNT_{90}$ titer <10)

to positive status ($PRNT_{90}$ titer ≥20, with a requisite ≥four-fold greater titer as compared with SLEV-neutralizing antibodies (strain TBH-28 was used for SLEV PRNT). Secondary WNV exposure was presumed in cases of four-fold or greater increase in $PRNT_{90}$ between two consecutive months. All apparent secondary exposures were reconfirmed with a repeat test.

RESULTS

Of 17 birds, 100% seroconverted to WNV positive antibody status, with 13 demonstrating initial serologic evidence of infection in July and August of 2003, and the remainder of birds in August and September of 2006. In the case of the turkey vulture, seronegative status in January 2003 with seropositive status in April 2004 suggested probable primary infection during the 2003 transmission season. Antibody titers did not change by more than two-fold among consecutive months throughout the study, except in eight birds for which serological evidence of presumed secondary WNV exposure was observed. One of these eight individuals was presumably WNV-exposed a third time. Primary infections and secondary exposures occurred between the months of July and September. Post-infection $PRNT_{90}$ titers became stable (termed "maintenance titers") and ranged from 10-2,560. Maintenance titers of the nine birds with only primary infections endured for the duration of the study or the lifetime of the bird, whichever ended first, with maximum observed duration of 51 months (Table 2.3, Fig. 2.1). Infection with SLEV was ruled out for all birds.

Upon primary WNV infection, most birds had clinical illness at or near the time of seroconversion that was attributed to WNV infection (Table 2.3). Ten birds had neurologic signs upon primary infection, including two golden eagles (*Aquila*

chrysaetos), one bald eagle (*Haliaeetus leucocephalus*), four great horned owls (*Bubo virginianus*), two red-tailed hawks, and one ferruginous hawk (*Buteo regalis*), while most birds had mild, non-specific signs (dehydration, reduced appetite, and pinched-off feathers). Neurological signs ranged from subdued attitude to ataxia, seizures, and partial paralysis. Clinical syndromes associated with WNV infection in the birds in the present study are described elsewhere (Nemeth et al. *in review*).

Following primary WNV infection in 2003, secondary WNV exposures were documented in eight birds as evidenced by eight- to 64-fold increases in serum $PRNT_{90}$ titers (Fig. 1). One of these, a golden eagle (170), was re-exposed in 2005 and again in 2007. Following sharp increases in antibody titers upon secondary exposure, subsequent maintenance titers were four-fold higher in 7 of the 8 birds. None of these birds exhibited clinical signs associated with secondary or tertiary exposures. Two birds were euthanized during the study due to chronic poor health; one great horned owl (109) was euthanized in April 2005 and another (176) in December 2006. A possible association between previous WNV infection and failure to thrive in these owls remains unknown.

Some infections and exposures resulted in an acute drop in neutralizing antibody titer (four-fold or greater within three months of exposure). This acute drop in titer was observed in 6/14 (43%) of primary infections and 3/8 (38%) of secondary exposures. For all nine instances where a four-fold decrease was observed, a ≥four-fold increase indicating recent exposure had occurred within two months prior.

DISCUSSION

As top predators, raptors are important to ecosystem diversity and sustainability and are sensitive indicators of environmental health (Anderson 2001, Sergio et al. 2006).

Within some areas of the United States and Canada, high proportions of raptors infected with WNV may jeopardize the well-being of raptor populations and hence, the surrounding ecosystems (Gancz et al. 2004, Joyner et al. 2006, Saito et al. 2007, Nemeth et al. *in review*). WNV transmission levels were relatively high in our study area in northern Colorado in 2003, when numerous WNV-positive raptor fatalities were reported (with WNV detection rates in carcasses of raptors ranging from 23-45% depending on the species; Nemeth et al. 2007a) and approximately 23% of raptors admitted to a local rehabilitation facility tested positive for acute WNV infection (Nemeth et al. 2007b). Survival of WNV-infected free-ranging raptors presumably occurs, as evidenced by detection of seropositive birds captured during serosurveys (Banet-Noach et al. 2004, Stout et al. 2005, Hull et al. 2006). Adaptation of raptors to survive the epizootic threat of WNV in the Western Hemisphere relies in part upon their immune systems. While exact mechanisms are poorly understood, innate immunity is believed to play a role in protection against primary WNV infection; however, humoral immunity (i.e., development of specific antibodies) is known to be an essential component of immune control over primary infection and protection against subsequent infections (Diamond et al. 2003). Little is currently known about the duration, dynamics, or protective effects of antibody responses following natural WNV infection in non-human vertebrates (Marra et al. 2004), though information exists for closely related St Louis encephalitis virus (SLEV; McLean et al. 1983, Gruwell et al. 2000).

Duration of antibodies to WNV is generally thought to be life-long in vertebrate hosts (Komar 2000) but few studies address this issue. WNV-specific antibodies persisted for at least 36 months in naturally infected pig-tailed macaques (*Macaca nemestrina*)

(Hukkanen et al. 2006), and WNV IgM antibodies were detectable in humans for up to 16-17 months after onset of clinical illness (Roehrig et al. 2003). WNV-neutralizing antibodies in naturally infected rock pigeons (*Columba livia*) and fish crows (*Corvus ossifragus*) endured for at least 12 months (Gibbs et al. 2005, Wilcox et al. 2007), while antibodies to SLEV persisted for <6 months and failed to protect 50% of house finches (*Carpodacus mexicanus*) (Reisen et al. 2001). In the present study, raptors of four species (barn owl, great horned owl, red-tailed hawk, Swainson's hawk) demonstrated stable antibody titers for more than four years (Table 2.3, Fig. 2.1). This finding represents progress toward understanding WNV antibody duration in raptors and vertebrates in general. However, many aspects of the anti-WNV humoral immune response remain poorly understood and understudied, especially in birds.

One issue of particular importance is the level of protection provided by humoral immunity. The antibody titer necessary to impart sterilizing immunity against infection with WNV (and other arboviruses) remains largely unexplored. We documented the patterns of circulating antibody titers for eight individual raptors that presumably experienced secondary, and in one case tertiary, exposure to WNV. These re-exposures took place approximately one, two or three years following primary infection (Table 2.3). In these birds, pre-existing antibody titers increased sharply during periods of peak transmission and then declined over subsequent months, though they generally remained elevated above antibody maintenance levels resulting from primary WNV infection. One golden eagle that experienced both secondary and tertiary WNV exposure, two years apart, experienced a lesser increase in antibody titer upon tertiary exposure (Fig. 1). Protection (defined here by lack of clinical signs) upon secondary WNV exposure

occurred with $PRNT_{90}$ titers as low as 10 and as high as 640, so we were unable to determine a threshold titer indicative of protection. WNV maternal antibody titers of <10 protected against viremia in experimentally inoculated chicken chicks (*Gallus gallus domesticus*) (Nemeth and Bowen 2007), while several house sparrows (*Passer domesticus*) with titers of 10 or <10 failed to develop viremia following experimental inoculation with WNV (Nemeth and Bowen, unpub data). WNV appears to be sufficiently immunogenic to elicit protective antibody responses in raptors for at least several years duration. The threshold titer for protection is probably below the level of detection using the 90% neutralization cutoff in serum diluted 1:10 as a diagnostic criterion.

The consequences of long-term protective antibodies extend beyond the level of individuals, as these antibodies also affect the susceptibility of populations to disease-related impacts, which is relevant in the case of WNV infection in raptors and other birds (Gancz et al. 2004, Joyner et al. 2006, Nemeth et al. 2006a, Saito et al. 2007, Nemeth et al. *in review*). In general, as the number of WNV survivors accumulates in the population, a decreasing proportion remains susceptible and reservoir-competent. The immune survivors absorb a proportional share of infectious mosquito bites, thus causing a reduction in the basic reproduction ratio of infection (R_0) of the virus. Once R_0 falls below unity, the zooprophylactic effect of immune birds represents herd immunity, and theoretically, transmission fails to persist (Diekmann et al. 1990).

In individual raptors that survive WNV infection, antibody duration may contribute to long-term survival, and therefore reproductive output, including fecundity and recruitment. The potential effects of WNV infection on the health of individual free-

ranging hawks have been examined, and clearly, some individuals survive and remain in healthy condition (Hull et al. 2006). Larger-bodied raptors are longer-lived and are relatively more sensitive to population-level effects due to later age at first reproduction, lower reproductive rates, and longer development times of progeny as compared to smaller raptors and other birds (Newton 1979). These traits should correlate to lower numbers and proportions of susceptible birds entering the ecosystem (Altizer et al. 2006), and are often associated with greater investment in immune defenses (Martin et al. 2006). In contrast, shorter-lived (often smaller-bodied) avian species with higher rates of reproductive output, and therefore population turnover, would benefit less from long-term antibody duration and may lack the potential of reaching herd immunity.

The long-term persistence of WNV antibodies in raptors may have additional ecological ramifications. First, while antibodies protect against vector-borne transmission, they should also protect against food-borne transmission, which has been documented in kestrels and owls (Komar et al. 2003, Nemeth et al. 2006a,b). The role of raptors as predators not only provides an additional exposure route to WNV, but confers importance to raptors for ecosystem stability. Second, passive transfer of antibodies from seropositive females would further protect nestlings and enhance population recruitment (Stout et al. 2005, Hahn et al. 2006). If an immune female reproduces for several or more seasons after surviving primary infection, multiple clutches of her offspring may be protected (temporarily) from the effects of WNV infection. Third, WNV infection may alter a raptor's future ability to migrate, an issue that requires further investigation.

The long-term stability of antibodies observed in several species of raptors and a turkey vulture (representing three taxonomic orders) suggests that humoral immunity to

WNV may be long-lasting in most or all birds that survive infection. If true, several implications are worth noting. First, herd immunity may develop in local patches, and thus transmission foci should be ephemeral. However, numerous transmission foci seem stable over time (e.g., New York City; Deegan et al. 2005), implying that other factors overwhelm immunity in determining transmission potential. Second, long-lasting antibodies in birds favor survival of populations facing pathogens such as WNV. Thus, humoral immunity functions as a buffer against ecologic disruption. On the contrary, even greater population reductions in naïve or unprotected birds would occur due to WNV transmission, resulting in more extreme downstream effects in trophic cascades of numerous species. Third, long-term duration of antibodies, assuming they are detectable by available diagnostic testing methods, lends credence to widely used assumptions inherent in the interpretation of surveillance data derived from free-ranging bird sampling.

In summary, duration of WNV-specific antibodies in avian amplifying hosts (which include some raptors) will impact herd immunity and therefore, at least to some extent, the biology and geography of WNV transmission. Long-lasting antibodies (> 4 years) in hawks and owls supports the notion that humoral immunity to WNV is likely to be lifelong in many birds and implies that false negative serologic test results in birds are rare, and that antibodies could play a suppressive role in transmission dynamics thereby buffering larger-scale ecologic disruptions. Our observation of acute four-fold reductions in neutralizing antibody titer following secondary exposures suggests that in addition to rises in antibody titer, reductions also signal recent exposure in some birds.

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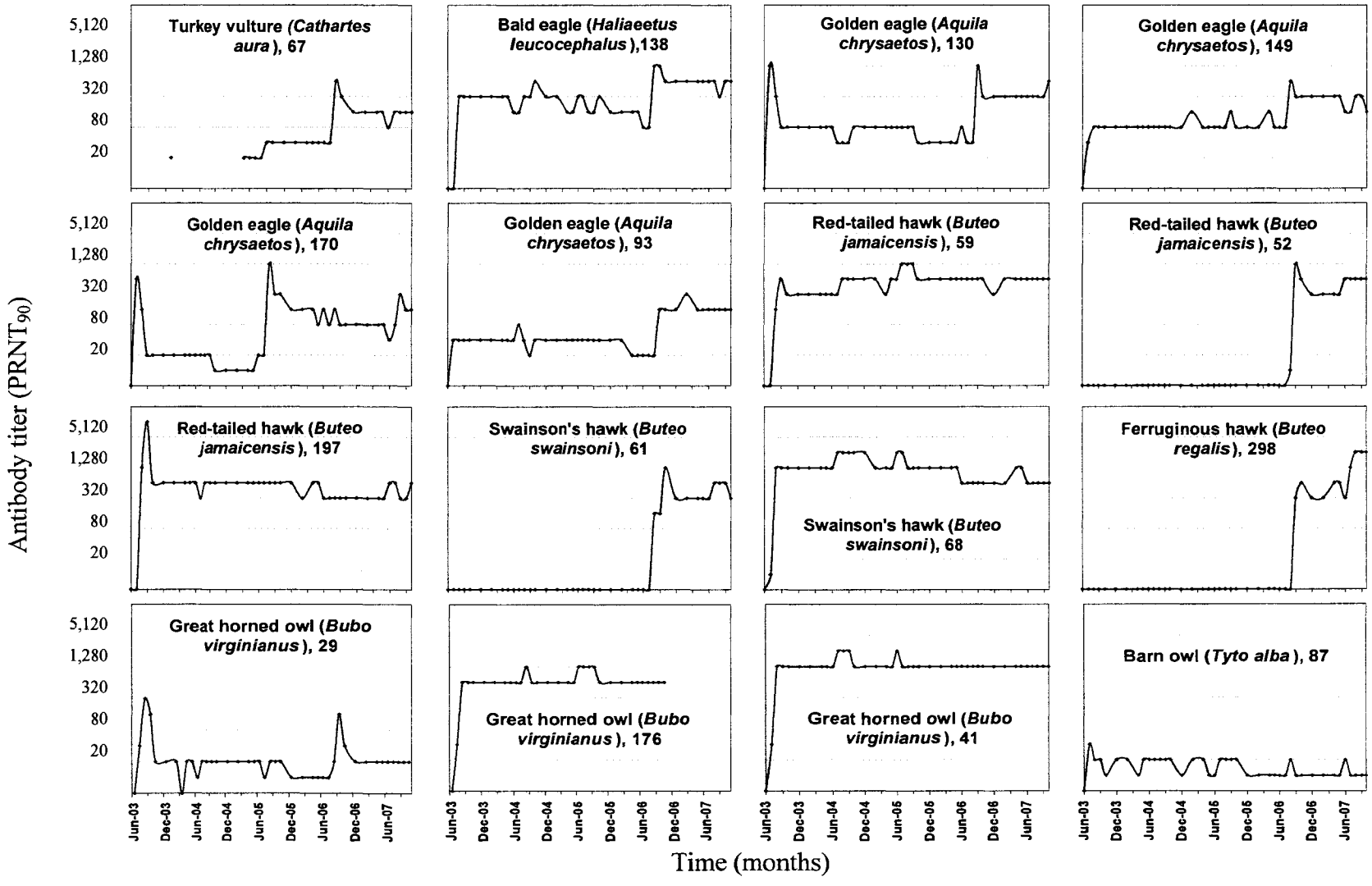
Antibody titer (PRNT90)
5,120
1,280
320
80
20
Turkey vulture (Cathartes aura), 67
Bald eagle (Haliaeetus leucocephalus),138
Golden eagle (Aquila chrysaetos), 130
Golden eagle (Aquila chrysaetos), 149
Golden eagle (Aquila chrysaetos), 170
Golden eagle (Aquila chrysaetos), 93
Red-tailed hawk (Buteo jamaicensis), 59
Red-tailed hawk (Buteo jamaicensis), 52
Red-tailed hawk (Buteo jamaicensis), 197
Swainson's hawk (Buteo swainsoni), 61
Swainson's hawk (Buteo swainsoni), 68
Ferruginous hawk (Buteo regalis), 298
Great horned owl (Bubo virginianus), 29
Great horned owl (Bubo virginianus), 176
Great horned owl (Bubo virginianus), 41
Barn owl (Tyto alba), 87
Jun-03
Dec-03
Jun-04
Dec-04
Jun-05
Dec-05
Jun-06
Dec-06
Jun-07
Time (months)

FIGURE 2.1. West Nile virus endpoint 90% neutralization antibody titers ($PRNT_{90}$) of 16 educational birds from June 2003-October 2007. Great horned owl 109 was euthanized in April 2005 and is not included in the Figure. Great horned owl 176 was euthanized in December 2006.

TABLE 2.3. Timing of primary and secondary West Nile virus (WNV) exposures in 17 raptors, resulting stabilized antibody titers ("maintenance titers"), and presence or absence of clinical signs.

	Primary infection			Secondary exposure*		
Species, ID	Month/year	Clinical signs	Maintenance titer†	Month/year	Clinical signs	Maintenance titer‡
Turkey vulture, 67 *Cathartes aura*	Unknown§	No	40	Sept 2006	No	160
Bald eagle, 138 *Haliaeetus leucocephalus*	Aug 2003	Yes	160-320	Aug 2006	No	640
Golden eagle, 130 *Aquila chrysaetos*	July 2003	Yes	80	Sept 2006	No	320
Golden eagle, 149 *Aquila chrysaetos*	July 2003	Yes	80	Aug 2006	No	320
Golden eagle, 170 *Aquila chrysaetos*	July 2003	Yes	20	Aug 2005	No	80
Golden eagle, 93 *Aquila chrysaetos*	Aug 2003	No	40	Sept 2006	No	160
Red-tailed hawk, 59 *Buteo jamaicensis*	Sept 2003	Yes	640	None	NA	NA
Red-tailed hawk, 52 *Buteo jamaicensis*	Aug 2006	No	320-640	None	NA	NA
Red-tailed hawk, 197 *Buteo jamaicensis*	Aug 2003	Yes	320-640	None	NA	NA
Swainson's hawk, 61 *Buteo swainsoni*	Aug 2006	Yes	320	None	NA	NA
Swainson's hawk, 68 *Buteo swainsoni*	Aug 2003	No	1,280	None	NA	NA
Ferruginous hawk, 298 *Buteo regalis*	Sept 2006	Yes	320-640	July 2007	No	2,560

	Primary infection			Secondary exposure*		
Species, ID	Month/year	Clinical signs	Species, ID	Month/year	Clinical signs	Species, ID
Great horned owl, 29 *Bubo virginianus*	Aug 2003	Yes	10-20	Sept 2006	No	20
Great horned owl, 176¶ *Bubo virginianus*	July 2003	Yes	640	None	NA	NA
Great horned owl, 109‖ *Bubo virginianus*	Aug 2003	Yes	2,560	None	NA	NA
Great horned owl, 41 *Bubo virginianus*	July 2003	Yes	1,280	None	NA	NA
Barn owl, 87 *Tyto alba*	July 2003	No	10-20	None	NA	NA

* Secondary exposure was indicated by ≥ four-fold increase in WNV antibody titer over a one month period.
† Represents the predominant reciprocal $PRNT_{90}$ titer, which often varied within two-fold from month-to-month.
‡ Antibody titers were measured for the final time in birds still alive in October 2007.
§ A seronegative serum sample from January 2003 and seropositive sample from early April 2004 are consistent with an initial exposure during the 2003 transmission season, but the month of primary infection is unknown.
¶ Euthanized in December 2006 due to chronic poor health.
‖ Euthanized in April 2005 due to chronic poor health.

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CHAPTER 3

WEST NILE VIRUS PASSIVE TRANSFER IN DOMESTIC CHICKENS AND HOUSE SPARROWS, AND AGE-ASSOCIATED RESPONSE TO INFECTION IN DOMESTIC CHICKENS

DYNAMICS OF PASSIVE IMMUNITY TO WEST NILE VIRUS IN DOMESTIC CHICKENS (*GALLUS GALLUS DOMESTICUS*)

ABSTRACT

Birds are the principle amplifying hosts for West Nile virus (WNV) and understanding the acquisition and decay of passive immunity is important to avian surveillance and diagnostics. We characterized passive transfer of WNV-neutralizing antibody from chicken (*Gallus gallus domesticus*) hens to eggs and chicks, and the protective efficacy and decay of maternally-acquired antibody over time. We also characterized age-associated changes in magnitude of viremia and examined the possibility of vertical transmission of WNV. All egg yolks and chicks from seropositive hens were maternal antibody positive. Maternal antibodies were undetectable in most chicks by 28 days post-hatch (PH), but some chicks remained protected as late as 42 days PH. By 56 days PH, chicks from immune hens had viremia profiles similar to control chicks. There were significant age-related differences in WNV-attributed morbidity and viremia levels of unprotected chicks. Vertical transmission of WNV was not detected.

INTRODUCTION

Passive transfer of maternal antibody in birds has been documented for several members of the Japanese encephalitis virus serocomplex of flaviviruses, including Murray Valley encephalitis virus, Japanese encephalitis virus, and St. Louis encephalitis virus (SLEV) (Reeves et al. 1954, Sooter et al. 1954, Warner 1957, Buescher et al. 1959, Bond et al. 1965, Ludwig et al. 1986). In the case of West Nile virus (WNV; family *Flaviviridae*, genus *Flavivirus*), maternally-derived neutralizing antibodies were detected in a colony of wild-caught rock pigeons (*Columba livia*) that bred in captivity and in a captive colony of Eastern screech owls (*Megascops asio*), both following natural WNV infection of adults. Maternal antibodies to WNV in pigeon squabs persisted for 19--33 days post-hatch (PH) while Eastern screech owlets had circulating maternal antibody when sampled between 1-27 days PH, although in neither case was the protective nature of these antibodies investigated (Gibbs et al. 2005, Hahn et al. 2006). Maternally-derived antibodies to SLEV in house sparrow (*Passer domesticus*) chicks reached undetectable levels by 16 days PH, and upon challenge, the responses of chicks with maternal antibody versus those without were not significantly different except for enhanced viremia titers in the former (Ludwig et al. 1986).

Although there has been speculation as to the prevalence and effects of maternal WNV antibodies in birds (Nasci et al. 2002, Reisen et al. 2005, Stout et al. 2005), this phenomenon has not been examined under controlled conditions. The response of young birds to WNV infection is one key to understanding transmission because some unprotected nestling-age birds experience greater levels of WNV viremia and morbidity than older birds (Swayne et al. 2001, Turell et al. 2001, Austin et al. 2004). Details

regarding the presence, prevalence, duration, and level of protection afforded by WNV maternal antibodies in birds would aid in interpreting field data and sentinel flock status, as well as in understanding the WNV transmission cycle as it continues to expand and establish itself in the New World. Transovarial transfer of maternal antibody in chickens can provide a model for other avian species, as the general mechanisms of the avian immune response are believed to apply to all bird species (Sharma 1999).

The objectives of this study were to 1. Determine the variability in transfer of passive immunity to WNV from hen to chick while quantifying and correlating antibody titers of hens, yolks, and chicks; 2. Characterize the decay of passively acquired WNV antibody in chicks; 3. Investigate the relationship between passively acquired antibody, development of viremia and clinical signs, and rate of seroconversion in chicks following WNV challenge at different time points post-hatch; 4. Examine viremia profiles, morbidity and mortality, and seroconversion among seronegative chicks inoculated with WNV at varying ages; and 5. Explore the possibility of vertical transmission of WNV from hen to egg and chick.

MATERIALS AND METHODS

Animals and animal care

Twelve 22-week PH white leghorn laying hens were acquired from Morning Fresh Farms in Platteville, Colorado and a 30-week PH barred rock cockerel was obtained locally. Upon arrival, all chickens were confirmed as seronegative for WNV by plaque reduction neutralization test (PRNT). The cockerel and hens were housed individually in a biosafety level-3 room, provided Family Farm® Egg Maker® 16 crumbles and fresh water *ad libitum*, and exposed to artificial lighting for 14 hr/d at

relatively constant temperature and humidity (approximately 70°C and 20%, respectively).

Semen was collected from the cockerel by digital manipulation and used to inseminate hens. Fertile eggs to be hatched were labeled with the date and hen number, then incubated and hatched within compartments; immediately following hatch, each chick was banded with a unique color combination to allow it to be traced back to its hen. Chicks were housed with same-age cohorts, provided fresh water *ad libitum* upon hatch, fed Family Farm® Chick starter/grower medicated crumbles at ≥ 24 hr PH, and given a supplemental heat source until > 1 week PH.

The care of all animals in this study was in compliance with National Institutes of Health guidelines for the humane use of laboratory animals. Birds were euthanized by pentobarbital overdose delivered intravenously.

Virus strain, virus detection, and virus neutralization assays.

A NY99 strain of WNV (isolate 4132, originally from a dead crow) was used for all animal inoculations and serologic testing. Sera, egg yolk and albumin, oral and cloacal swabs, and tissue homogenates were assayed for virus by Vero cell plaque assay as previously described (Bunning et al. 2002). Briefly, Vero cell monolayers in 6-well plates were inoculated in duplicate with 0.1 mL of sample per well. After one hr incubation at 37°C, the cells were overlaid with 3 mL/well of 0.5% agarose in MEM medium supplemented with 2% fetal bovine serum and antibiotics. Two days later, cells were overlaid with a second 3 mL overlay containing 0.004% neutral red dye. Viral plaques were counted on the third and fourth days of incubation. The minimum titers of WNV detection by virus isolation were as follows: approximately 50 PFU/mL or g for sera or

tissue (except sera of chicks for vertical transmission), 25 PFU/mL for egg parts, and approximately 5 PFU/mL for swabs and sera of chicks examined for vertical transmission.

Sera were heat inactivated (56°C for 30 min) and tested for neutralizing antibody to WNV by PRNT as previously described (Beaty et al. 1995), with different percentage reduction criteria used depending on the experiment (see Results). However, in most cases, 90% reduction at a dilution of 1:10 or greater was considered WNV-antibody positive. Serial two-fold dilutions were performed (starting at 1: 10) and tested in duplicate to determine endpoint titers of seropositive samples. Assays were grouped as much as possible to include all samples for a particular experiment and minimize interassay variability; the same positive control serum was used in all assays.

Passive transfer of antibody to yolk and chicks

Eight WNV-seronegative hens were inoculated subcutaneously with approximately 12,000 plaque forming units (PFU) of WNV, while four hens served as seronegative controls throughout the maternal antibody portion of the study. Infected hens were bled daily from 1--6 days post-inoculation (DPI) to assess viremia. A volume of 0.2 mL whole blood was added to 0.9 mL BA-1 medium (M199-Hank's salts, 1% bovine serum albumin, 350 mg/L sodium bicarbonate, 100 units/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL amphotericin B in 0.05 M Tris, pH 7.6), allowed to clot for 30 min at room temperature, centrifuged at 6000 x *g* for 5 min, and frozen to −80°C as the equivalent of 10% serum until assayed for virus.

On 20 DPI, sera from all hens were tested for WNV neutralizing antibody to confirm seroconversion in infected hens and continued seronegative status in uninfected

controls. Beginning on 36 DPI, hens were placed on a schedule of weekly insemination and were bled weekly (0.6 mL) for nine weeks, during which time eggs were collected for artificial incubation. The eight seropositive hens were euthanized at 5-months post-infection, while the four seronegative hens were later infected with WNV for evaluation of vertical transmission of virus.

Over the five weeks following the first weekly insemination, the first two eggs laid by each of the 12 hens were collected for yolk sampling, for a total of ten eggs per hen. Within several hours of eggs being laid, yolk samples were collected with a sterile syringe, diluted 1: 2.5 in phosphate buffered saline, vortexed, centrifuged at 14,000 x *g* for 10 min, and the resulting supernatants stored at −80°C until testing for antibody. All eggs not used for yolk sampling were incubated until hatching and between 9 and 11 chicks from each hen (130 chicks total; 81 from seropositive hens and 49 from seronegative hens) were bled within 24 hr PH to assay for antibody. For comparison of antibody titers between a given hen and her egg yolks and chicks, the date each egg was laid was correlated to the nearest weekly hen serum sample. Because hens were bled weekly, all yolk and chick serum samples were collected within 3.5 d of serum samples from each corresponding hen.

Decay of passively-acquired antibody in chicks

A group of maternal antibody positive chicks (n = 33) was used to characterize loss of maternally-acquired antibody over time. These birds were housed with same-age cohorts and bled weekly for up to 10 weeks PH. All of these chicks were bled weekly through 4 weeks PH, and at 6, 8, and 10 weeks PH, subsets (consisting of between 6 and 13 maternal antibody positive chicks) were challenged with WNV (see below), so that the

final 10-week group consisted of six chicks that were maternal antibody upon hatch (derived from immune hens). In-contact seronegative chicks were included among each of the aforementioned age groups and were bled following the same schedule. Sera were evaluated by PRNT to determine antibody titers.

Relationship between passively-acquired antibody and protection from WNV challenge

At various times PH, groups of chicks that hatched from immune hens were inoculated subcutaneously with approximately 1,000 PFU of WNV. Each of these challenge groups also contained chicks from seronegative hens to serve as susceptible controls and to characterize age-associated differences in WNV viremia and morbidity (see below). All chicks were bled immediately prior to WNV inoculation to assess serologic status at the time of challenge, and daily from 1-7 DPI to determine magnitude and duration of viremia by Vero cell plaque assay. Ages of challenge groups consisting of maternal antibody positive chicks (derived from immune hens) were from 1 day PH to 10 weeks PH. Following challenge, birds were euthanized on 10 DPI at which time a final serum sample was collected and assayed to determine neutralizing antibody titers by PRNT. In addition, for all seronegative chicks (derived from non-immune hens) that were inoculated at < 14 days PH, sera obtained on 5 and 7 DPI were screened for antibody to assess the timing of initial antibody detection following WNV infection in naïve chicks.

Clinical response and magnitude of viremia as a function of chick age

Groups of seronegative chicks originating from non-immune hens were challenged at various time points PH to evaluate age-based changes in magnitude of viremia and

clinical response to WNV challenge. Ages of these infection groups ranged from 1 day PH to 10 weeks PH. These birds were challenged in concert with the chicks from seropositive hens, and therefore used to evaluate and compare the protective effect of maternally-acquired antibody (see previous section).

Vertical transmission of WVN from viremic hens to eggs and chicks

Eggs were collected from six hens following inoculation of hens with approximately 12,000 PFU of WNV, and also from two uninfected control hens. Hens were bled daily from 1-6 DPI to evaluate viremia, and eggs from each hen were collected daily from 1-8 DPI. Eggs from two of the infected and one uninfected control hen were sampled immediately after laying to test egg parts for virus. For these eggs, yolk and albumin were diluted 1: 5 with BA-1 with 20% fetal bovine serum, and stored at −80°C until testing for virus by Vero cell plaque assay.

Eggs from the remaining four inoculated hens (and one seronegative control hen) were collected from 1-8 DPI and incubated until hatch. Within 12 hr PH of each chick, blood was collected via jugular venipuncture, after which chicks were immediately euthanized. Following euthanasia, oral and cloacal swabs and tissue samples were collected. Tissue samples included heart, brain, spleen, kidney, liver, lung, intestine, muscle, eye, and yolk sac. Cotton-tipped applicators were used to swab the oropharyngeal and cloacal cavities and were placed in 0.5 mL BA-1 with 20% fetal bovine serum (FBS). Tissues were placed in 1 mL BA-1 with 20% FBS as a 10% tissue suspension with a single steel 4.5 mm BB added to each tissue sample, which was then homogenized in a mixer mill (Retsch GmbH, Haan, Germany) for 5 min at 25 cycles/sec, clarified by centrifugation for 4 min at 16,000 x *g*, and frozen to −80°C until testing.

RESULTS

Passive transfer of antibody to yolk and chicks

All hens and the cockerel were WNV-seronegative prior to experimental infection. Of the 12 hens inoculated with WNV, 10 developed viremia. In two of these hens, viremia was detected by 1 DPI, and viremia was detected as late as 5 DPI in one hen. The average duration of detectable viremia was two days with peak titers ranging from $10^{2.0\text{-}3.7}$ PFU/mL serum, except for the two hens that failed to reach detectable viremia titers. None of the hens showed any clinical signs during the study. On 20 DPI, eight experimentally infected hens had endpoint 90% neutralization titers ($PRNT_{90}$ titers) ranging from 80-1280, and these titers remained relatively constant (within two-fold difference) until 100 DPI when hen antibody titers were last measured.

All egg yolks (n = 80) and 1 day PH chick sera (n = 81) originating from seropositive hens tested positive for WNV antibody. Alternatively, all yolks (n = 40) and 1 day PH chick sera (n = 49) originating from seronegative hens tested negative for WNV antibody. All ten egg yolk samples from each of eight hens yielded antibody titers equal to or within two-fold difference to titers of corresponding hen sera, and were therefore not considered significantly different. However, chick hatch-day antibody titers exhibited a greater range as compared to their corresponding hens' serum antibody titer. Chick serum antibody titers were usually at least four-fold (and up to 32-fold) below those of their hens (Table 3.1).

Decay of passively-acquired antibody in chicks

Thirty-one of 33 chicks (93.9%) had $PRNT_{90}$ titers of < 10 by 28 days PH, and were considered negative for WNV maternal antibody; $PRNT_{90}$ titers of all chicks were <

10 by 35 days PH (Table 3.2). With a less stringent criteria of $PRNT_{50}$, 8/33 (24.2%) of chicks had titers < 10 at 28 days PH ($PRNT_{50}$ range 10-80), but by 35 days PH, all 33 chicks had $PRNT_{50}$ titers of < 10. Chicks that still had detectable $PRNT_{90}$ titers at 28 days PH originated from the same two hens, both of which had the highest $PRNT_{90}$ titers of all hens ($PRNT_{90}$ = 1280). However, $PRNT_{90}$ titers of three other chicks from hens with this high titer had dropped to < 10 by 28 DPI. In-contact seronegative chicks (n = 14) bled weekly remained seronegative for the duration of the study.

Relationship between passively-acquired antibody and protection from WNV challenge

None of the six maternal antibody positive chicks challenged with WNV on 1 day PH demonstrated signs of morbidity, while all four of their seronegative counterparts succumbed to infection by approximately 5 DPI. Morbidity was not observed in any chick inoculated at > 1 day PH regardless of maternal antibody status. However, all WNV-inoculated chicks that were seronegative upon hatch (derived from seronegative hens) became detectably viremic between 1-5 DPI, while all maternal antibody positive chicks inoculated at ≤ 28 days PH failed to become detectably viremic. At 42 days PH, none of seven chicks that hatched from immune hens had detectable serum antibody (< 1: 10 $PRNT_{50}$), but three of these seven chicks failed to become viremic following challenge. Viremia titers of the remaining four chicks were later in onset and of lower magnitude than those of their seronegative counterparts. All chicks from immune hens inoculated on 56 and 70 days PH became viremic, with profiles similar to those of chicks from seronegative hens (Table 3.3).

The serologic responses of chicks that were maternal antibody positive upon hatch (derived from WNV immune hens) also differed according to age PH of WNV challenge. Following challenge, some chicks of immune hens that were $\leq$ 28 days PH when challenged had declining (two- to eight-fold) antibody titers evident on 10 DPI as compared to inoculation day titers; these low antibody titers detected on 10 DPI were presumed to be declining titers of maternal antibody. Two of the chicks derived from immune hens that were seronegative when challenged on 28 days PH subsequently seroconverted by 10 DPI, while the remaining 11 chicks did not show evidence of seroconversion by 10 DPI. The range of hatch day $PRNT_{90}$ titers of the 11 chicks that failed to seroconvert (20-320) encompassed titers of the chicks that did seroconvert following challenge (40, 160). Of the chicks derived from immune hens that were challenged on 42, 56, and 70 days PH, 6 of 7, 6 of 7, and 6 of 6 seroconverted by 10 DPI, respectively (Table 3.3).

For the evaluation of seroconversion in chicks derived from seronegative hens, a detectable antibody response was defined as $\geq$ 90% neutralization at a 1: 20 dilution, as these blood samples were diluted immediately upon collection as the equivalent of 10% serum so that the lowest dilution possible for neutralization assays (in which serum is added to an equal volume of virus solution) was 1: 20. Few seronegative chicks (3/36; 8.3%) inoculated at < 14 days PH had formed a detectable antibody response by 5 DPI, while 5/32 (15.6%) met this criteria by 7 DPI. However, by 10 DPI the majority of these chicks (21/32; 65.6%) had detectable $PRNT_{90}$ titers, ranging from 10-160. The four seronegative chicks that died following inoculation on 1 day PH had not mounted a

detectable antibody response by the time of death. All seronegative chicks (n=22) inoculated at ≥ 14 days PH had detectable $PRNT_{90}$ titers (range 10-320) on 10 DPI.

Clinical response and magnitude of viremia as a function of chick age

Age and magnitude of viremia titers were significantly negatively correlated (Spearman rank order correlation coefficient, $r_s = -0.9542$; n = 58; one-tailed p-value <0.0001) (Figure 3.1). The mean peak viremia titers of unprotected chicks inoculated on 1, 7, and 21 days PH were $10^{7.3}$ PFU/mL serum, $10^{5.2}$ PFU/mL serum, and $10^{3.8}$ PFU/mL serum, respectively; the mean peak viremia among the 12 hens (aged ≥ 6 months when inoculated) was $10^{2.9}$ PFU/mL serum. All four of the chicks inoculated at 1 day PH were evidently succumbing to WNV infection, and on 5 DPI, three of these chicks were euthanized and one died. These chicks developed detectable viremia of five days duration prior to death, unlike almost all other chicks, in which detectable viremia lasted from 1-4 days (in one 2-day PH unprotected chick, viremia was also detectable for 5 days). Clinical signs in these birds included intermittent somnolence and reluctance to move. No morbidity was observed in chicks inoculated at > 1 day PH.

Vertical transmission to eggs and chicks

Fourteen eggs (two of which were laid during detectable viremia of hens) and 21 chicks (five of which hatched from eggs laid during detectable viremia of hens) were collected from 1-8 DPI of hens. During the time of egg collection, hens were viremic from 2-3 DPI, with peak viremia levels ranging from $10^{<1.7-2.9}$ PFU/mL serum. Virus was not detected in any egg parts, chick tissues, sera, or swabs.

DISCUSSION

Adult chickens have not been reported to experience morbidity due to WNV infection, though chicken carcasses have tested positive for WNV (Bernard et al. 2001). In addition, WNV infection of chickens elicits significant antibody titers (Langevin et al. 2001), artificial insemination is relatively easy to perform, and hens provide a constant supply of eggs under defined conditions, making domestic chickens an excellent candidate for evaluation of passive transfer of WNV immunity from hen to chick. Immunoglobulins (Ig) are transferred from hen to egg and embryo via various routes. Immunoglobulins A and M in oviduct secretions are transferred to the egg as it passes down the oviduct, while IgG is transferred from hen serum via yolk into the embryo's circulation. Immunoglobulins are also passed to the chick via swallowed amniotic fluid (Rose et al. 1974). To our knowledge, maternal transfer of antibody to WNV in birds has not been explored experimentally, although other viral agents have been associated with the passive transfer of maternal antibodies to neonatal birds in an experimental setting (Kissling et al. 1954, Reeves et al. 1954, van Eck 1982, Powell 1987, Al-Natour et al. 2004).

A strong correlation between chicken hen serum and yolk IgG levels has been previously demonstrated (Schmittle 1950, Bollen and Hau 1997). In the present study, the correlation between hen, yolk, and chick WNV antibody titers suggests that levels of antibody circulating in hen sera are fully transferred to their eggs, but that titers begin to decline prior to hatch (Table 3.1). Subsequent to hatch, WNV maternal antibody underwent relatively rapid decay in chicks, as most had undetectable antibody titers by 28 days PH (Table 3.2). These results are consistent with the negative correlation observed

between maternal antibody titers in sera of Eastern screech owl chicks and the time of sampling post-hatch (Hahn et al. 2006). Additionally, maternal Ig titers in eggs of black-headed gulls (*Larus ridibundus* L.) decreased in eggs laid later within a clutch (Muller et al. 2004). While we did not find evidence of the latter in chickens, hens in our study were commercial quality, continuous egg-layers, which may have affected the pattern of passive transfer. In addition, siblings from eggs laid over a 100-day span did not exhibit a decline in 1 day PH antibody titers during this time period. Our results suggest that as hen WNV antibody titers remain constant, the passive transfer of antibody to their eggs behaves similarly, and while sibling chicks exhibit a range of hatch-day antibody titers, these titers do not appear to decline over time.

In our study, maternal antibody appeared to decay relatively rapidly, and was undetectable in most chicks by 28 days PH. However, undetectable titers of maternal antibody remained protective against the development of viremia in some chicks for up to 42 days PH (Table 3.3). Further, most chicks that were inoculated at ≤ 28 days PH failed to seroconvert upon WNV challenge, suggesting that maternal antibody persisted and therefore chicks failed to recognize or respond immunologically to infection. However, nearly all chicks derived from immune hens that were inoculated at 42, 56 and 70 days PH seroconverted following WNV challenge (Table 3.3), indicating that at these later times PH there was a lack of protective, circulating maternal antibody. We did not observe that antibody titers of hens or subsequent maternal antibody titers of chicks at time of hatch or upon challenge had an effect on rate of decay or level of protectiveness at later time points, though these data were relatively limited and not quantified.

The effect of age on the pathogenesis of WNV infection in birds is poorly studied, although some information has been obtained from domestic geese (Austin et al. 2004). Differential effects of age with other flavivirus infections have also been observed for Murray Valley, St. Louis encephalitis, and Japanese encephalitis viruses in domestic chickens (McLean 1953, Buescher et al. 1959, Sudia and Chamberlain 1959), and SLEV in mourning doves (*Zenaida macroura*) and house finches (*Caropodacus mexicanus*) (Mahmood et al. 2004). Experimental WNV infections in chickens demonstrated that adults reach relatively low peak viremia titers ($10^{2.4-5.0}$ PFU/mL serum) with no clinical signs of illness (Langevin et al. 2001), whereas 1-3 day old chicks undergo higher peak viremia titers ($10^{6.5-7.5}$ PFU/mL serum) and exhibit significant morbidity (Turell et al. 2000, 2001, 2002a). In our study, magnitude of viremia in unprotected chicks had a strong negative correlation with age (Figure 3.1), and no chicks inoculated at > 14 days PH reached viremia titers considered efficiently infectious to mosquitoes ($> 10^5$ PFU/ml) (Turell et al. 2002b). Further, no chicks infected at > 1 days PH showed signs of illness.

The phenomenon of passive transfer of maternal WNV antibody has important implications on transmission dynamics, as well as on the differential survival of young birds. First, existing data indicate that antibody produced in response to infection may persist (Gibbs et al. 2005) and remain protective for $\geq$ 1 year in some species of birds (Nemeth NM and others, unpublished data), and our data suggest that all offspring of seropositive female birds will benefit from protective maternal antibody. If exposed to WNV in the wild, maternal antibody positive chicks at $\leq$ 42 days PH would potentially be partially or fully protected from the effects of infection, and therefore less likely to play role in transmission. Chicks that resist WNV infection due to the presence of maternal

antibody are susceptible at a later age after maternal antibody wanes, but at this time they are less likely to experience morbidity and higher viremia titers, possibly contributing to decreased rates of mosquito WNV transmission (Reisen et al. 2005) and increased rates of survival for some bird species. However, unprotected chicks infected early in life have a lesser chance of survival because they are more susceptible to higher viremia titers and the ill-effects of WNV on their health. The potentially devastating effects of WNV on younger, naïve chicks, as well as the occurrence and persistence of maternal antibody, are important considerations in management and conservation of endangered avian species within endemic areas. Understanding these dynamics is especially important because the occurrence of neonatal and juvenile birds corresponds temporally to the WNV transmission season in many regions of the United States (Hayes et al. 2005).

The interpretation of serologic results from young birds can be confused by the presence of maternal antibody, whether from a diagnostic or surveillance perspective. From a diagnostic standpoint, a maternal antibody positive chick might lead to mistaken consideration of WNV as a possible differential diagnosis. Further, a chick derived from an immune hen that tests positive for WNV antibody at a young age represents a false positive from a surveillance standpoint, as it does not represent a recent WNV exposure. In turn, if a natural exposure were to occur in a bird protected by maternal antibody, this chick would likely fail to seroconvert in response to this exposure and after waning of maternal antibody would be considered a false negative. We concluded that antibody formation in response to WNV infection in the majority of young, naïve chicken chicks likely occurs between 7-10 DPI, so that there is a reasonable likelihood that WNV antibody detected in chicks at < 7 days PH is maternally-derived. Further confusion may

arise if a maternal antibody positive chick is retested at a later date, at which time, barring a subsequent natural exposure event, it will then test seronegative. In addition, within the timeframe that chicks from immune hens no longer have detectable antibody but remain partially protected from WNV infection (approximately 28-42 days PH), a negative PRNT result would be misleading because these chicks may not be susceptible to viremia and morbidity following a natural exposure event.

An additional application regarding passive transfer of WNV antibody involves vaccination of chicks, whether within a zoological collection, involved in wildlife rehabilitation or education programs, or part of endangered species programs. Based on results from the present study, we recommend that initial vaccination of chicks from mothers that are likely seropositive be delayed until after eight weeks PH to circumvent potential interference of maternal antibody with vaccination success. However, if vaccination at a younger age is deemed necessary, a booster vaccination at approximately eight weeks PH would be advisable. More research is needed in examining the efficacy of currently available WNV vaccines in birds, as well as future vaccines that might show promise.

Vertical transmission of WNV has been reported in mice (Julandera et al. 2005) but has not been described in birds (Komar 2003). While various modes of WNV transmission (e.g., mosquito, oral) have been documented in birds (McLean et al. 2002, Komar 2003), the potential for transmission of WNV from mother to egg and chick remains unexplored. While we had small sample sizes of eggs and chicks, our data suggest a lack of vertical WNV transmission in chickens. However, this does not rule out the possibility in other avian species, especially those that reach relatively high peak

viremia titers but survive infection, such as the American kestrel (*Falco sparverius*), American robin (*Turdus migratorius*), common grackle (*Quiscalus quiscula*), fish crow (*Corvus ossifragus*), great horned owl (*Bubo virginianus*), and house sparrow (Komar et al. 2003, Nemeth et al. 2006). The hens in our study had very low viremia titers of short duration, thereby decreasing the potential for virus transmission to eggs or chicks. More data are needed from a wider representation of avian species to better assess the potential for vertical WNV transmission in birds, though achievement of fertile eggs laid within the viremic phase of captive, experimentally manipulated adult female birds represents a challenge. Additionally, sampling of eggs during various phases of embryonic development prior to hatch may be necessary to fully explore viral transmission from mother to chick, as embryos of susceptible species may not survive to hatching if infected *in ovo*.

In summary, despite potential variances in WNV maternal antibody transfer among avian species, the results from this study will aid in the interpretation of wild bird WNV serosurveys, as well as epidemiological data involving the distribution of WNV antibodies of birds of varying age groups. In addition, these results should be considered in concert with avian management and conservation schemes, especially those involving endangered species propagation within WNV endemic areas. While chicks with maternal antibody are protected for only a limited period of time after hatching, this period likely includes much of the nestling stage for many altricial and semialtricial bird species, at which time these chicks are relatively immobile, sparsely feathered, and seemingly more vulnerable to mosquito feeding. This also corresponds to the time in a bird's life when they are likely most susceptible to high-titered viremia and morbidity if infected with

WNV. The dynamics of maternal antibody decay and subsequent immunological naïveté of previously maternal antibody positive birds are additional factors that affect WNV transmission and population health of birds. The role of maternally-derived WNV antibody, age-related differences in viremia and morbidity, and the possibility of vertical WNV transmission in free-ranging avian species must be explored to better understand their effects on WNV transmission and implications on the health of free-ranging avian populations.

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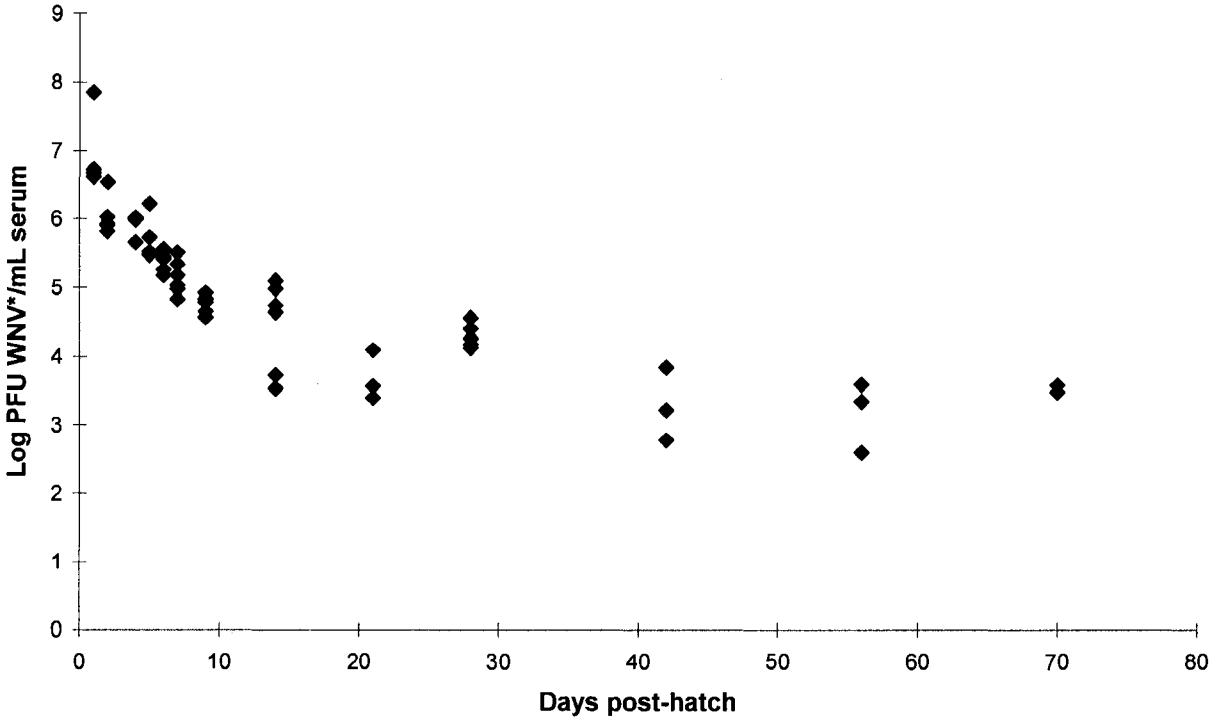

Plaque forming units (PFU) of West Nile virus.

FIGURE 3.1. Peak viremia for West Nile virus-seronegative chicken (*Gallus gallus domesticus*) chicks inoculated at various time points post-hatch (n = 58; age range 1-70 days post-hatch).

TABLE 3.1. Range of antibody titers measured from West Nile virus-seropositive chicken (*Gallus gallus domesticus*) hens with corresponding maternal antibody titer ranges of their egg yolks and chicks at < 1 day post-hatch.

Hen	Hen $PRNT_{90}$ range*	Yolk $PRNT_{90}$ range†	Chick $PRNT_{90}$ range‡
1	640—1,280	320—1,280	20—160
2	160—320	160—320	20—160
3	640—1,280	640—2,560	160—640
4	320—640	160—320	20—160
5	80—160	80—160	20—160
6	320—640	320—640	40—320
7	80—320	80—320	20—160
8	640	320—640	40—160

* Hens were bled over a ten week period.
† Ten yolks per hen were sampled over a ten week period.
‡ Between 9 and 11 chicks per hen were sampled at < 1 day post-hatch over a five week period.

TABLE 3.2. Decay of maternally-derived West Nile virus antibodies in chicken (*Gallus gallus domesticus*) chicks (n = 33) as measured by reciprocal endpoint 90% neutralization titers ($PRNT_{90}$) from 1-4 weeks post-hatch (WPH).

Chick ID	1 WPH	2 WPH	3 WPH	4 WPH
1	80	20	10	<10
2	80	40	10	<10
5	40	10	<10	<10
8	40	20	<10	<10
9	80	20	<10	<10
11	320	80	20	<10
19	320	80	40	10
21	20	10	<10	<10
23	80	20	<10	<10
24	40	20	10	<10
25	80	40	10	<10
26	40	20	<10	<10
29	20	10	<10	<10
33	160	80	20	<10
34	40	20	<10	<10
35	80	20	10	<10
37	80	20	10	<10
38	160	80	10	<10
42	80	40	10	<10
45	80	20	10	<10
46	160	40	10	<10
47	80	40	10	<10
50	40	20	10	<10
51	40	20	<10	<10
53	20	10	<10	<10
54	80	20	10	<10
59	160	80	20	10
60	160	40	20	<10
62	80	20	<10	<10
63	80	20	<10	<10
64	40	10	<10	<10
66	10	<10	<10	<10
70	80	20	10	<10

TABLE 3.3. Serologic responses of West Nile virus maternal antibody positive and seronegative chicken (*Gallus gallus domesticus*) chicks of ≥ 21 days post-hatch when inoculated.*

Days post-hatch when inoculated	Hen WNV immune status	$PRNT_{90}$† on inoculation day	Peak viremia (log PFU‡/mL serum)	Viremia duration (DPI)§	$PRNT_{90}$ on 10 DPI
21	Immune	10	< 1.7	—	< 10
	Immune	10	< 1.7	—	< 10
	Immune	40	< 1.7	—	10
	Immune	10	< 1.7	—	< 10
	Immune	< 10	< 1.7	—	< 10
	Immune	10	< 1.7	—	< 10
	Immune	20	< 1.7	—	< 10
	Non-immune	SN¶	4.1	1-2	10
	Non-immune	SN	3.6	1-2	20
	Non-immune	SN	3.4	1-2	10
28	Immune	< 10	< 1.7	—	40
	Immune	< 10	< 1.7	—	< 10
	Immune	< 10	< 1.7	—	10
	Immune	< 10	< 1.7	—	< 10
	Immune	< 10	< 1.7	—	< 10
	Immune	10	< 1.7	—	< 10
	Immune	10	< 1.7	—	< 10
	Immune	< 10	< 1.7	—	< 10
	Immune	< 10	< 1.7	—	< 10
	Immune	< 10	< 1.7	—	< 10
	Immune	< 10	< 1.7	—	< 10
	Immune	< 10	< 1.7	—	< 10
	Immune	< 10	< 1.7	—	< 10
	Non-immune	SN	3.0	1-3	10
	Non-immune	SN	4.1	1-3	10
	Non-immune	SN	4.6	2-3	160
	Non-immune	SN	4.4	2-3	10
	Non-immune	SN	4.2	2-3	40
42	Immune	< 5	< 1.7	—	20
	Immune	< 5	2.0	3	20
	Immune	< 5	2.7	2-3	80
	Immune	< 5	< 1.7	—	40
	Immune	< 5	2.6	2-3	160
	Immune	< 5	< 1.7	—	40
	Immune	< 5	< 1.7	—	< 10
	Non-immune	SN	3.9	1-2	320
	Non-immune	SN	3.2	1-3	40
	Non-immune	SN	2.8	1-2	160

Days post-hatch when inoculated	Hen WNV immune status	$PRNT_{90}$† on inoculation day	Peak viremia (log PFU‡/mL serum)	Viremia duration (DPI)§	$PRNT_{90}$ on 10 DPI
56	Immune	< 5	2.3	2-3	10
	Immune	< 5	2.2	3-4	80
	Immune	< 5	2.5	2-3	40
	Immune	< 5	2.7	2-3	40
	Immune	< 5	2.5	2-3	< 10‖
	Immune	< 5	2.7	2-3	10
	Immune	< 5	2.2	3	40
	Non-immune	SN	2.6	1-3	40
	Non-immune	SN	3.6	2	80
	Non-immune	SN	3.4	2-3	80
70	Immune	< 5	3.0	2-4	20
	Immune	< 5	3.6	1-3	40
	Immune	< 5	2.5	2-3	20
	Immune	< 5	3.7	2-3	40
	Immune	< 5	3.6	1-3	80
	Immune	< 5	2.5	2-3	10
	Non-immune	SN	3.5	2-3	40
	Non-immune	SN	3.6	1-2	40

* Age groups of < 21 days post-hatch (PH) when inoculated with WNV included: 1 day PH (n = 4 seronegative, 6 maternal antibody positive), 2 day PH (n = 5 seronegative, 7 maternal antibody positive), 4 day PH (n = 4 seronegative, 4 maternal antibody positive), 5 day PH (n = 4 seronegative, 1 maternal antibody positive), 7 day PH (n = 6 seronegative, 9 maternal antibody positive), 14 day PH (n = 6 seronegative, 6 maternal antibody positive). Additional seronegative chicks in the age-associated viremia and morbidity analysis included 6 day PH (n = 7), and 9 day PH (n = 6).

† Endpoint 90% neutralization antibody titer ($PRNT_{90}$).

‡ Plaque forming units (PFU) of West Nile virus.

§ Days post-inoculation (DPI); duration indicates which days post-infection on which viremia was detected.

¶ Seronegative (SN) indicates chicks that hatched from seronegative hens and were seronegative upon hatch.

‖ This chick showed some evidence of seroconversion, with $PRNT_{80}$ = 10 on 10 DPI.

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PASSIVE IMMUNITY TO WEST NILE VIRUS PROVIDES LIMITED PROTECTION IN A COMMON PASSERINE SPECIES

ABSTRACT

Passerine birds have played an important role in the establishment, maintenance, and spread of West Nile virus (WNV) in North America, and some are susceptible to WNV-associated mortality. Characterization of passive transfer of anti-WNV antibodies in passerines is important to understanding transmission and demographic effects of WNV on wild birds. We demonstrated passively acquired maternal antibodies to WNV in the house sparrow (*Passer domesticus*). While all seropositive females (n = 18) produced antibody positive egg yolks, only 20% of seropositive mothers (3/15) produced seropositive chicks. The estimated average half-life of maternal antibodies in chick sera was 3 days, and no antibodies were detected after 9 days post-hatch (DPH). Maternal antibodies failed to provide protection against viremia in chicks at 21-25 DPH. While the observed duration of persistence of passively inherited anti-WNV antibodies in house sparrows differs from some non-passerine birds, it remains unknown whether similar patterns occur in other passerines.

INTRODUCTION

Hatchling birds are exposed to an array of pathogens, thereby necessitating temporary immunological assistance (Tizard 2002, Pihlaja et al. 2006). Maternally-derived antibodies circulate within blood of newly hatched chicks, and provide this temporary protection while the immune system matures (Tizard 2002). Passive transfer of immunity to West Nile virus (WNV; family *Flaviviridae*, genus *Flavivirus*) has been

documented in captive rock doves (*Columba livia*), eastern screech owls (*Megascops asio*), and domestic chickens (*Gallus gallus domesticus*) (Gibbs et al. 2005, Hahn et al. 2006, Nemeth and Bowen 2007). In addition, young birds of some species are more susceptible to higher West Nile viremia titers and associated morbidity and mortality than older individuals (Austin et al. 2004, Nemeth and Bowen 2007), and nestling birds may be susceptible to higher mosquito feeding rates due to their lack of defensive behavior (Scott et al. 1988, 1990). Therefore, early-age immune protection against WNV infection should mitigate transmission and disease in birds (Nemeth and Bowen 2007).

Passive transfer of anti-WNV antibodies has yet to be documented in Passeriformes, the largest and most diverse order of birds. Passerines have been implicated as reservoir hosts of WNV, and were likely important in its spread across North America (Komar et al. 2001, Rappole and Hubálek 2003, Kilpatrick et al. 2006). The house sparrow (*Passer domesticus*) is of interest because it is a highly competent amplifying host of WNV, is abundant within many habitat types, and has a broad geographic range that includes much of North America (Lowther and Cink 1992, Komar et al. 2001, 2003). The extent of passive transfer of antibodies to WNV in passerines has important consequences on WNV transmission in nature, as well as the health of free-ranging birds (Nemeth and Bowen 2007). There may be differences in patterns of passive transfer of anti-WNV antibodies among avian individuals and species, and these differences could be associated with varying immune investment in offspring due to physiological trade-offs associated with life history traits of both adults and young (Lochmiller and Deerenberg 2000, Grindstaff et al. 2005, Pihlaja et al. 2006).

The hypothesis of the present study was that transovarial transfer of antibodies to WNV occurs in the house sparrow, and maternal antibodies are detectable in both eggs and chicks of seropositive mothers. In addition, we predicted that as in chickens, maternal antibodies in house sparrow chicks would be protective against viremia and clinical disease (Nemeth and Bowen 2007. The objectives of this study were to 1) demonstrate maternal antibodies to WNV in eggs and chicks derived from known seropositive female sparrows; 2) document the decay of maternal antibodies in chicks over time; and 3) evaluate viremia, oral shedding, and viral titers in tissues following WNV inoculation of chicks from both seropositive and seronegative mothers.

MATERIALS AND METHODS

Capture, husbandry, and inoculation of adult sparrows

A captive flock of house sparrows, captured via mist nets in Weld County, Colorado, was established in early 2005. The maintenance and care of the sparrow colony was in compliance with the National Institutes of Health guidelines for the humane use of laboratory animals, and the research was conducted under Institutional Animal Care and Use approval. Birds were housed free-flight in an indoor, mosquito-proof aviary (3.24m wide x 2.57m high x 12.12m long), and provided with tree branches (with leaves and buds depending upon season) and trunks, and sand baths. Environmental conditions within the aviary were maintained at approximately 10—20°C and 15-25% humidity, with photoperiod determined by natural sunlight.

Sparrow diet consisted of a mix of millet, milo, cracked corn, cracked sunflower seed and oats (in equal parts), supplemented 1-2 times a week with live mealworms. Cuttlefish bone was provided as an additional source of calcium and to prevent beak and

nail overgrowth. During the breeding season, adult sparrows were also provided with cooked egg, mealworm pupae, waxworms, soaked kitten chow, Family Farm® Egg Maker® 16 crumbles, and ground oyster shell.

West Nile virus serostatus of all individuals was determined upon arrival, and a portion of seronegative sparrows was experimentally inoculated via subcutaneous injection with approximately 1,000-2,000 plaque forming units (PFU) of WNV strain NY99-4132, which was originally isolated from an infected crow and passaged once in Vero cells, once in C6/36 mosquito cells, and once in baby hamster kidney-21 cells.

During the 2007 breeding season (April-August), approximately 25 months following WNV inoculation, some sparrows began nest building using provided materials (grass, cotton, and shredded paper) and structures (1-gallon plastic cartons with cut holes for nest openings). At this time, the flock consisted of 30 adult females (25 WNV seropositive and 5 seronegative) and 25 males (20 WNV seropositive and 5 seronegative).

Nest observations

Observations of breeding behavior, including identification of individuals that were mating, nest building, incubating eggs, and brooding and feeding chicks, were performed from within a blind inside the aviary. All observations were confirmed through video recordings prepared daily throughout the breeding season. The identities of the male and female attending a given nest were reassessed following fledging of each clutch of chicks to detect any change in parents. Females incubating, brooding and feeding young were assumed to be the producers of the eggs in their respective nests. Males observed to have copulated with the attending female, and also incubating,

brooding, and feeding young were identified; however, extra-pair copulations could not be ruled out, so definitive identities of fathers remained unknown.

Sample collection and preparation for assessment of maternal antibodies

Sparrow adults were bled just prior to initiation of the breeding season (April 1, 2007) and following its conclusion (October 1, 2007) to determine anti-WNV antibody titers.

From late April through July, nests were checked every other day; for the remainder of the nesting season, nests were checked daily to determine numbers of eggs and chicks present. For the time period when nests were checked every other day, chicks' hatch date was considered the earlier of the two possible dates.

Early in the breeding season (May-June), eggs that were laid on the ground or ejected from the nest were opportunistically collected if deemed fresh by the appearance of the yolk. Later in the season (July-August), 1-3 eggs were collected directly from active nests. Ova were collected at necropsy from several females that died with broken eggs and/or developing ova within the reproductive tract. In addition, yolk sacs (residual yolk supply contained within chicks' abdomen at the time of hatch) and blood samples were collected post-mortem when possible from chicks $\leq$ 1 day post-hatch (DPH) that were found dead or were injured and humanely euthanized. In some cases, the mothers of recovered eggs or chicks were unknown.

Ova, yolks, and yolk sacs were aseptically collected into cryovials, diluted 1:2.5 or 1:5 in BA1 medium (M199-Hank's salts, 1% bovine serum albumin, 350 mg/L sodium bicarbonate, 100 units/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL amphotericin

B in 0.05 M Tris, pH 7.6), vortexed until homogenized (approximately 5-10 seconds), and clarified by centrifugation. Supernatants were stored at −20°C until testing.

Some sparrow chicks were bled serially over time, beginning between 1-9 DPH, and then sampled every 3-5 days until fledging or soon thereafter (up to 26 DPH). Just prior to collection of blood samples, nestlings were weighed with a Pesola® spring balance (Pesola AG, Baar, Switzerland) of accuracy to 1 gram. Blood was collected via jugular venipuncture (≤ 1% of body weight), placed into serum separator tubes, maintained at room temperature for approximately 30 min, centrifuged for separation of serum, and stored at −20°C until testing.

Husbandry, inoculation, and sample collection from chicks

Chicks of two different age groups were separated from the flock for experimental inoculation with WNV. Environmental conditions for chicks were maintained at 24-27°C and 40-50% humidity. Chicks were either placed into an artificial nest cavity or a cage, depending upon age. The first group of chicks was removed from the nest between 4-5 DPH, and consisted of one chick from a seronegative female, and three chicks from two different seropositive females. These chicks were hand-fed approximately 65% body weight daily (divided into feedings every 1-1 ½ hours), consisting of mealworms, mealworm pupae, cooked egg, fruit cocktail, waxworms, and cricket abdomens. The second group of chicks was removed from the aviary between 19-23 DPH, and consisted of two siblings derived from a seronegative female and four chicks from three different seropositive females. This group was provided water, seed mix (millet, milo, cracked corn, cracked sunflower seed, and oats), cooked egg, soaked kitten chow, mealworm pupae, chicken crumble feed, and waxworms *ad libitum*.

Both cohorts of chicks were challenged with between 3,000-5,000 PFU WNV via subcutaneous injection, the younger cohort at 7-8 DPH and the older at 21-25 DPH. Blood samples (0.1 mL) were collected from the jugular vein of chicks (all weighing > 20 g) from 1-6 days post-infection (DPI), and sera were recovered following centrifugation. Oropharyngeal cavities were sampled with cotton-tipped swabs from 1-10 DPI (or until death/euthanasia), after which the swabs were placed into 1 mL BA1. Chicks demonstrating signs of morbidity (lethargy, fluffed feathers, and/or poor body condition) were euthanized by sodium pentobarbital overdose. Survivors were bled and euthanized on 10 DPI. Following death or euthanasia, chicks were necropsied, and the following samples collected for virus titration: oral and cloacal swab, heart, liver, spleen, kidney, small intestine, skeletal muscle, and cerebrum. Sera, swabs, and tissue homogenates (approximately 10% tissue suspension in BA1, as previously described in Nemeth et al. 2006) were stored at −80°C until testing.

Laboratory testing

Neutralizing antibody titers of yolk and yolk sac supernatant and sera were determined by plaque reduction neutralization test (PRNT) on Vero cell monolayers in six-well plates as previously described (Beaty et al. 1995). The same WNV strain used for inoculation of adults and chicks was used for PRNT. Serum samples that demonstrated ≥ 80% neutralization at a 1:10 dilution were considered positive for anti-WNV antibodies, and these samples were serially diluted (2-fold) to determine reciprocal endpoint 80% neutralization ($PRNT_{80}$) titers.

Viral titers of sera, oral swabs, and tissue homogenates of WNV-inoculated chicks were determined by Vero cell plaque assay as previously described (Bunning et al.

2002). Minimum levels of WNV detection were $10^{1.7}$ PFU/mL for sera, $10^{0.7}$ PFU/swab, and $10^{1.7}$ PFU/g for tissues.

Statistics and calculations

Chicks included in the calculation of estimated average half-life of passive antibodies (n = 10) had at least two serum samples collected over time, with the first demonstrating between 70-99% neutralization of WNV at a 1:10 dilution, and > 50% neutralization by a subsequent serum sample. Serial samples were collected from 2-12 days apart (with chick ages upon collection ranging from 1-16 DPH). To calculate half-life, the percent neutralization at a 1:10 dilution (50-99%) was rescaled to a value between 0 and ≈ 1.0 (the rescaled value is referred to as "neutralizing activity" or NtA) by converting the percentage to a proportion, subtracting 0.50 from the proportion, and multiplying the result by two (e.g., 75% neutralization would be adjusted to: [0.75 – 0.50] × 2 = 0.50 NtA). This adjustment considered 50% neutralization as having zero NtA, and 99% neutralization as having virtually complete NtA. Samples with 100% neutralization were excluded because an accurate relative quantification of this level cannot be achieved. An estimate of half-life was calculated by using the following equation, adapted from an equation for radioactive decay (Wang et al. 1975).

$$half-life = \frac{(days\ elapsed) \times \log_{10} 2}{\log_{10}\left(initial\ NtA / final\ NtA\right)}$$

Reservoir competence indices (RCI) were calculated for sparrow nestlings and juveniles inoculated with WNV, and were based on susceptibility (s), infectiousness (i), and duration of infectiousness (d), so that RCI = s * i * d (Komar et al. 2003, 2005,

Kilpatrick et al. 2007). Susceptibility for all sparrows was 1.0, because 100% were considered susceptible to WNV infection (Komar et al. 2005). Percent infectiousness was calculated as (Kilpatrick et al. 2007):

$$\%\ Infectiousness = 0.1349 \times \log_{10}(viremia) - 0.6235$$

The threshold for zero infectiousness was $10^{4.62}$ PFU/mL (based on data from *Culex pipiens*), and when an individual's daily viremia value was below this titer, infectiousness was considered zero for that day (Tiawsirisup et al. 2005, Kilpatrick et al. 2007). Duration of infectiousness was the number of days that viremia titers were above the threshold of infectiousness. These values were averaged for each individual to calculate mean values for the following experimentally inoculated groups: younger cohort (8-9 DPH) with seropositive mothers, younger cohort with seronegative mothers, older cohort (21-25 DPH) with seropositive mothers, and older cohort with seronegative mothers.

SAS/STAT MULTTEST software, version 9.1, (SAS Institute, Inc., Cary, North Carolina 27513, USA) was used to perform a paired t-test to compare mean peak viremia titers for chicks from seropositive versus seronegative mothers in the older cohort using $\alpha = 0.05$.

The MIXED procedure with repeated measures was used for data analysis and Akaike's Information Criteria (AIC) were used for model selection (Burnham and Anderson 2002) to test which sets of factors best predicted protection by mothers' WNV immunity status in the older cohort of chicks. Five models were used for the analysis of

the challenge experiment of the older cohort. Model 1 (intercept only) represented a scenario in which viremia profiles were independent of all other parameters included within the model set; model 2 examined the effect of DPI on chicks' viremia profiles; model 3 indicated the effect of mothers' serostatus on chicks' viremia profiles; model 4 evaluated the additive effects of DPI and mothers' serostatus; and, model 5 was fully interactive for DPI and mothers' serostatus. Individual AIC weights were calculated for each model.

Statistical analyses were not performed for the younger cohort of chicks because of the limited sample size.

RESULTS

Antibody titers of breeding females

Prior to experimental inoculation, all adult sparrows were negative for anti-WNV antibodies. Of the 15 seropositive sparrow females that produced chicks, $PRNT_{80}$ antibody titers ranged from 20-1,280. Titers were typically the same or declined 2-fold from April 1, 2007 to October 1, 2007. However, in two females, antibody titers dropped 4-fold during this time period (Table 3.4).

Maternal antibodies in ova, yolks, and yolk sacs

Ova (n = 5) were removed from two breeding females that died. The females had serum $PRNT_{80}$ titers of 160 and 640, and those of the yolk within their ova had $PRNT_{80}$ titers between 80-160.

All seropositive mothers (18/18) produced eggs with anti-WNV antibodies, with the majority of egg yolks produced by these females having detectable antibodies to WNV

(91.4%; 32/35). Adult females with $PRNT_{80}$ titers between 20-1,280 produced yolks with titers between < 10-320. In general, yolk antibody titers were 4- to 8-fold lower than those of the females' serum antibody titers. Three individual females produced both antibody positive and negative yolks; $PRNT_{80}$ titers of these females ranged from 20-80. Sixty-four percent (16/25) of yolks from unidentified females had anti-WNV antibodies ($PRNT_{80}$ titers of 10-80). Yolks (9/9) from four seronegative females were negative for anti-WNV antibodies, having < 60% neutralization at a dilution of 1:10 (Table 3.4).

Fifteen yolk sacs were collected from neonatal chicks, seven of which were from seropositive females having $PRNT_{80}$ antibody titers from 40-1,280; yolk sac $PRNT_{80}$ titers were from 10-320. Three of the six yolk sacs from unknown females were positive for antibodies to WNV, with $PRNT_{80}$ titers from 20-80. Two yolk sacs of chicks from seronegative females were negative for WNV antibodies, exhibiting < 60% neutralization at a dilution of 1:10.

Maternal antibodies in chicks

Fifty-eight chicks were bled on or before 7 DPH (20 of these were bled on 1 DPH). Fifty of these chicks were from 15 seropositive mothers, which had WNV $PRNT_{80}$ titers ranging from 20-1,280. Three of the 15 mothers (20%) produced seropositive chicks, and $PRNT_{80}$ titers of these mothers were 160 and 1,280. Alternatively, 24% (12/50) of the chicks from seropositive mothers had detectable WNV neutralizing antibodies, with $PRNT_{80}$ titers of chick sera ranging from 10-40. All of seven chicks from a female with a $PRNT_{80}$ of 1,280 had detectable maternal antibodies; these chicks were from three separate clutches, each approximately one month apart. No chicks were positive for maternal antibodies to WNV after 9 DPH. The estimated average half-life of

anti-WNV antibodies in chicks (n = 10) was approximately 71.92 hours or 3 days (range 1.43-5.91 days). Sera from all eight chicks from seronegative mothers exhibited < 60% neutralization at a dilution of 1:10.

Challenge of chicks from seropositive and seronegative females

All 11 of the chicks challenged with WNV by subcutaneous injection developed detectable WNV viremia titers of variable duration between 1-6 DPI (Table 3.5).

Within the younger cohort (8-9 DPH), peak viremia titers were higher in the two chicks that had undetectable antibodies upon inoculation (average $10^{9.0}$ PFU/mL serum); one of these chicks was from a seropositive mother (Table 3.5). The average peak viremia of the two chicks that had detectable maternal antibodies upon hatch (and also upon inoculation) was $10^{4.9}$ PFU/mL serum, while viremia in all four chicks was undetectable by 6 DPI. Based on these viremia data, the mean % infectiousness value for the younger cohort that had seropositive mothers was 0.30, versus 1.19 for the chick with a seronegative mother. Reservoir competence indices for these groups were 0.40 and 4.76, respectively (Table 3.6). All four chicks in the younger cohort shed virus from the oral cavity. The chick from the seronegative mother had higher viral tissue loads, and more virus-positive tissues than the chicks from seropositive mothers. While several chicks in the younger cohort were euthanized due to lethargy and weight loss, it was difficult to distinguish clinical signs attributable to WNV infection versus those resulting from the stress of frequent handling for feeding. All chicks in the younger cohort seroconverted by 6-10 DPI (Table 3.5).

None of the seven chicks in the older cohort had detectable anti-WNV antibodies at the time of inoculation on 21-25 DPH, though five had seropositive mothers. Viremia

was first detected in all chicks on 1 DPI, and lasted between 4 and ≥ 6 days (Table 3.5). There was no significant difference in the mean peak viremia titers of chicks from seropositive versus seronegative mothers in the older cohort ($10^{7.8}$ PFU versus $10^{7.4}$ PFU, respectively; t-test, P = 0.27). In addition, model selection based on AIC weight indicated that mothers' serostatus (model 3) had a minimal effect on chick viremia profiles (Table 3.7). Based on viremia data, the mean % infectiousness for the older cohort with seropositive mothers was 0.64, versus 0.55 for the chicks with seronegative mothers. Reservoir competence indices for these groups were 1.80 and 1.36, respectively (Table 3.6). Low WNV titers were detected at 10 DPI in spleen and/or kidney of chicks with seronegative mothers, while chicks with seropositive mothers had WNV in up to six different tissues. One chick, which was from a seropositive mother, experienced clinical signs attributed to WNV infection (older chicks were only handled for daily sample collection because they fed independently), including lethargy and weight loss first observed on 7 DPI. However, this chick continued eating and remained relatively alert and active, and was euthanized on 10 DPI with the others. All chicks in the older cohort seroconverted by 10 DPI (Table 3.5).

DISCUSSION

Maternal immunoglobulin G (IgG, or IgY in birds) is passively transferred from the female's serum to embryonic circulation via the yolk while eggs are in the ovary (Rose et al. 1974). Maternal antibodies to WNV have been documented in three bird species through captive studies, including pigeons, screech owls, and chickens, with additional evidence for maternal antibodies in free-ranging hawks, owls, and various aquatic species such as herons, egrets, ibis, flamingoes, storks, gulls, terns, and pochards

(Gibbs et al. 2005, Reisen et al. 2005, Stout et al. 2005, Hahn et al. 2006, Figuerola et al. 2007, Nemeth and Bowen 2007). More data are needed to better compare the relative abilities of a variety of avian species to produce and transfer anti-WNV antibodies to their young.

Maternal antibodies to West Nile virus have yet to be documented in a passerine species, some of which are highly susceptible to WNV infection and are probable amplifying reservoir hosts (Komar et al. 2003). The house sparrow is a common and ubiquitous passerine and is regarded as an important reservoir host of WNV, with a potential role in its spread throughout North America (Komar et al. 2001, Rappole and Hubálek 2003, Komar et al. 2005, Langevin et al. 2005). House sparrow nestlings are altricial, remaining sparsely feathered for 7-10 days and relatively inactive within the nest for approximately 14 days (Lowther and Cink 1992), and are thereby vulnerable to mosquito blood feeding. Mosquitoes appear to be attracted to bird nests, and may increase feeding rates on older nestlings, in part because brooding times decrease, leaving nestlings more vulnerable (Scott et al. 1990, Griffing et al. 2007, Savage et al. 2007). Further, numerous mosquito species in North America are ornithophilic and feed at heights that coincide with the presence of house sparrows (Darbro and Harrington 2006). House sparrows usually nest within enclosed spaces (Lowther and Cink 1992), and while the effects of nest structure on mosquito feeding rates are not well known, evidence suggests that house sparrow nestlings are exposed to arboviruses (Milby and Reeves 1990). Passive transfer in passerines could lead to dampened WNV transmission while increasing survival rates of exposed chicks.

The present study documented passive transfer of antibodies to WNV in the house sparrow; maternal antibodies were apparent in ova, egg yolks, yolk sacs, and chick sera. High proportions of ova, egg yolks, and yolk sacs derived from WNV seropositive sparrow females had detectable antibodies, while antibodies were present in a lower proportion of sparrow chick sera post-hatch. Chicks that did have detectable maternal antibodies also had mothers with relatively high anti-WNV antibody titers, and only 20% of seropositive mothers produced chicks with detectable antibodies. In contrast, all seropositive mothers produced eggs with detectable antibodies within the yolk (Table 3.4). Detectable maternal antibodies to WNV were relatively short-lived in sparrows (≤ 9 DPH) as compared to other bird species in which maternal antibodies were detected for up to approximately 28 DPH (Gibbs et al. 2005, Hahn et al. 2006, Nemeth and Bowen 2007). However, 44—50% of screech owl chicks from WNV seropositive mothers lacked detectable antibodies between 4—16 DPH (Hahn et al. 2006). Maternal antibodies to avian polyomavirus (APV) and Newcastle disease virus (NDV) have also been reported as short-lived, with antibodies observed in the majority (78—83%) of budgerigar (*Melopsittacus undulatus*) yolks derived from seropositive mothers (n = 14 for APV, n = 12 for NDV), but not in chick sera on 5 DPH (n = 35 for APV, n = 13 for NDV) (Phalen et al. 1995).

Numerous factors may affect the transovarial transfer of antibodies from mother to offspring. Differences within and across species may be based on variation in evolutionary pressures that in turn affect life history traits, and vice versa (Lee et al. 2006, Pihlaja et al. 2006). Animals must balance the costs (e.g., energetic, nutritional, developmental) and benefits (e.g., protection) of an immune response, a process driven

by selective pressures from pathogens and life history traits of the host (Moret 2003). Differences among individual mothers (e.g., genetics, age, hormone levels, stress, mate choice, stage in ovulation), as well as environmental factors (e.g., protein availability, time in season), also likely affect passive transfer (Tizard 2002, Grindstaff et al. 2003). The persistence of maternal antibodies in chick circulation is also a function of the chicks' body size upon hatch, and developmental and metabolic rates; maternal antibodies wane from circulation when the chick begins to produce its own antibodies (Rose and Orlans 1981, Grindstaff et al. 2003).

Catabolism of maternal antibodies begins once the antibodies reach the chick's circulation. High rates of metabolism in birds may contribute to the rapid decay of serum antibodies (Patterson et al. 1962), while high growth rates of house sparrow nestlings (Weaver 1942) correspond to increases in blood volume and dilution of circulating maternal antibodies. Antibodies in chicken chick sera fell from between 75-100% to < 20% of maternal levels during the first 14 DPH (Rose and Orlans 1981). In mallard (*Anas platyrhynchos*) ducklings, maximum serum levels of maternal IgY occurred from 3-7 DPH (Liu and Higgins 1990). The half-life of non-specific serum gamma globulin injected into chicken chicks from 1-7 DPH was approximately 72 hours, while the half-life of serum albumin was 42 hours (Patterson et al. 1962). In blue-and-gold macaw (*Ara ararauna*) chicks, antibodies against bovine serum albumin decreased exponentially, with an average serum IgY half-life of approximately 92 hours (3.85 days; range: 2.37-5.11 days), though antibodies were detected in the sera of some chicks until 42 days PH (Lung et al. 1996). The estimated average half-life of maternal antibodies to WNV in sparrow chicks in the present study was approximately 72 hours (3 days), and no antibodies were

detected beyond 9 DPH. This half-life is based on a limited sample of chicks with variable blood collection time points. When the criteria and equation for half-life from the current study were applied to previously published data from chickens (Nemeth and Bowen 2007), the estimated average half-life of anti-WNV-specific maternal antibodies in chicken chick sera (n = 26) was 20.7 days (range 7.9-101.8 days), with evidence of circulating maternal antibodies at 42 DPH. These chicken chicks were from hens with a range of $PRNT_{90}$ titers of 80-1,280.

Questions remain as to why patterns of passive transfer of maternal antibodies to WNV were different in the house sparrow versus other avian species studied thus far, and whether patterns in the house sparrow are similar to those of other passerine species. The energetic costs of immune investment in offspring are high (Lochmiller and Deerenberg 2000, Grindstaff et al. 2005), and the relatively rapid decay of maternal antibodies in the house sparrow may reflect a low investment by the mother. While life history traits of sparrows and many other passerines are consistent with lower immune investment in offspring (e.g., short lifespan, high reproductive output, rapid development, and small body size) (Lowther and Cink 1992), many of these species also have a relatively short incubation phase. Shorter incubation periods often correlate with longer periods of development of acquired immunity in hatchlings, and this would suggest a higher dependence on passive transfer and innate immunity. If the protection provided by maternal antibodies is short-lived in these species, they may be more vulnerable to infection early in life prior to complete development of the immune system. In addition, altricial birds (e.g., passerines) have a lower yolk supply, which contains maternal IgY, as compared to precocial birds (e.g., chickens), again suggesting that altricial chicks may be

more vulnerable to infection during the period when maternal antibodies have waned but acquired immunity has yet to fully develop (Klasing and Leshchinsky 1999). Young birds would be more susceptible to WNV infection during this period, having a potentially greater role in transmission, and possibly decreased probability of survival.

Challenge experiments of sparrow chicks in the present study suggest that maternal antibodies may provide some early (8-9 DPH) protection, but apparently no protection by 21-25 DPH, at which time sparrow chicks have fledged from the nest (Lowther and Cink 1992) (Tables 2,4). In contrast, chicken chicks were protected by maternal antibodies to WNV for up to 42 DPH, though protection against viremia was less complete at 42 DPH than at 28 DPH (Nemeth and Bowen 2007). Maternal antibodies also protected chicken chicks from hemorrhagic enteritis-associated clinical disease for up to 56 DPH (Fadly and Nazerian 1989), and delayed avian leukosis virus infection with reduced levels of viremia and shedding (Fadly and Smith 1991). Maternal antibodies against turkey rhinotracheitis virus did not completely protect against disease in turkey poults, though clinical signs were less severe than in poults without maternal antibodies at 1 DPH (Naylor et al. 1997). Ludwig and others suggested that house sparrow nestlings with mothers immune to St. Louis encephalitis virus (SLEV) experienced "viremic enhancement" following SLEV challenge at specific time points post-hatch, meaning that viremia levels were of greater duration and magnitude than in chicks from seronegative females (Ludwig et al. 1986). While West Nile viremia profiles of sparrow chicks in the present study do not appear to be consistent with viremic enhancement (Table 3.5), chicks in the older cohort having seropositive mothers had higher % infectiousness and RCI than chicks with seronegative mothers (Table 3.6). In contrast, chicks in the younger

cohort with seropositive mothers were less likely to be infectious to *Culex pipiens* mosquitoes than the single chick from a seronegative mother, and the RCI of the latter was higher than previously calculated for all other avian species. Further, only chicks in the younger cohort with seropositive mothers had a lower RCI than previously reported for house sparrows (RCI $\approx$ 0.49-1.25) (Komar et al. 2003, 2005, Kilpatrick et al. 2007), indicating partial protection by maternal antibodies in these younger chicks. These comparisons should be considered in the context of the limited sample sizes of chicks included. Also, frequent handling of the younger cohort of chicks for hand-feeding likely caused elevated stress levels that may have affected viremia profiles; effects of stress were evident through the relatively poor weight gain over time in these chicks versus those that remained with the parents (data not shown).

The ability of nestling birds to disperse and transmit pathogens may be partially dependent upon the immune status of their mothers, along with the dynamics of passive transfer and protection provided by maternal antibodies. If the behavior of maternal antibodies to WNV observed in captive house sparrows in the present study is similar to that of free-ranging sparrows, then maternal antibodies would offer limited protection among chicks from a portion of seropositive mothers. After this short-term protection wanes, these young chicks would be competent WNV amplifying hosts and susceptible to associated morbidity and mortality. The consequences of passive transfer of anti-WNV antibodies in passerines on transmission in nature are unknown, and studies of additional passerine species would greatly contribute to understanding the complex ecological picture.

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TABLE 3.4. West Nile virus (WNV) antibody titers in house sparrow mothers and their corresponding ova, egg yolks and/or chicks.

Adult females		Yolks/ova*		Chicks		
ID	$PRNT_{80}$ range†	$PRNT_{80}$ range‡	N	$PRNT_{80}$ range	N	DPH§ first bled
1	20-40	NT¶	—	< 10	2	5-6
2	20-40	< 10-10	2	< 10	3	1-6
3	20-40	< 10-40	4	< 10	3	3-5
4	20-80	< 10-10	2	< 10	3	1
5	80	NT	—	< 10	3	2-7
6	80	20-40	3	< 10	1	1
7	80	NT	—	< 10	3	1
8	80	40	1	NT	—	—
9	160	80-160	2	NT	—	—
10	160	160	1	NT	—	—
11	80-160	20	4	< 10	8	1-5
12	80-160	20	1	NT	—	—
13	160	NT	—	10	4	4-6
14	160	40-80	3	< 10	2	3-6
15	160	20	1	< 10-10	2	2-4
16	80-320	20	2	< 10	5	1-5
17	320	80	2	NT	—	—
18	160-320	20-40	3	< 10	1	4-5
19	160-320	20	1	NT	—	—
20	160-320	NT	—	< 10	3	5-9
21	640	40-80	4	NT	—	—
22	320-640	80-160	2	NT	—	—
23	1,280	160-320	2	10-40	7	1-6
24	< 10	< 10	1	NT	—	—
25	< 10	< 10	5	NT	—	—
26	< 10	< 10	2	< 10	1	1
27	< 10	NT	—	< 10	4	1-6
28	< 10	< 10	1	< 10	3	3-7

* Ova were collected from two females: female 9 (with no yolks and 2 ova) and female 21 (with 1 yolk and 3 ova).

† Reciprocal endpoint 80% WNV neutralization titer ($PRNT_{80}$); $PRNT_{80}$ ranges for adult females reflect serum titers measured prior (April 1) and following (Oct. 1) the 2007 breeding season. Females 24-28 were seronegative.

‡ Yolks, ova, and chick sera with $PRNT_{80}$ titers < 10 were considered negative. Some mothers produced either antibody positive and negative yolks or chicks within the same clutch.

§ Day(s) post-hatch (DPH) when first bled (presented as a range when there are multiple chicks from one female).

¶ None tested (NT).

TABLE 3.5. Peak West Nile virus (WNV) titers and duration in sera, oral swabs, and tissues of experimentally inoculated house sparrow chicks originating from WNV seropositive and seronegative mothers.

		Chick challenge		Days post-inoculation						Oral shedding		Mortality		Tissues*	
Chick ID	Mother $PRNT_{80}$ Range†	Age (DPH)‡	$PRNT_{80}$ upon challenge§	1	2	3	4	5	6	Peak (log PFU/ swab)	DPI**	Euthanasia/ Death†† (DPI)	$PRNT_{80}$ at death‡‡	No. positive/ total	Peak (log PFU/g)
				Viremia (log PFU/mL)¶; % Infectiousness‖											
1	20-40	8-9	NEG	3.3; 0.00	6.3; 0.23	8.2; 0.48	5.5; 0.12	4.4; 0.00	<1.7; 0.00	1.7	3-5	7	80	6/7	4.0 B,K,H
5	1,280	8-9	10	<1.7; 0.00	<1.7; 0.00	1.7; 0.00	3.3; 0.00	3.0; 0.00	<1.7; 0.00	1.2	6	10	80	2/7	3.5 K
6	1,280	8-9	10	<1.7; 0.00	2.0; 0.00	4.2; 0.00	5.2; 0.08	2.7; 0.00	<1.7; 0.00	1.7	3-6	9	640	5/7	7.2 B
7	< 10	8-9	NEG	5.2; 0.08	7.9; 0.44	9.3; 0.63	4.9; 0.04	3.0; 0.00	<1.7; 0.00	2.2	2-7	7	≥ 1,280	7/7	8.5 B
17	160	24-25	NEG	3.4; 0.00	6.7; 0.28	7.8; 0.43	5.9; 0.17	3.2; 0.00	1.2; 0.00	2.0	2-7	10	1,280	5/7	3.9 B
20	20-40	24-25	NEG	3.4; 0.00	6.1; 0.20	7.6; 0.40	6.4; 0.24	5.2; 0.08	2.8; 0.00	3.1	3-7	10	1,280	6/7	3.2 B, S
22	80-320	24	NEG	3.5; 0.00	6.2; 0.21	5.5; 0.12	2.7; 0.00	<1.7; 0.00	<1.7; 0.00	1.3	3-4	10	≥ 1,280	2/7	2.0 S
23	80	21-22	NEG	2.7; .00	4.9; 0.04	5.6; 0.13	2.3; 0.00	<1.7; 0.00	<1.7; 0.00	1.7	4-6	10	160	0/7	< 1.7 S
24	80	21-22	NEG	4.2; 0.00	6.8; 0.30	8.3; 0.50	5.6; 0.13	4.4; 0.00	<1.7; 0.00	4.6	2-7	10	≥ 1,280	2/7	3.4 S
27	< 10	21-22	NEG	5.1; 0.06	7.3; 0.36	6.5; 0.25	3.4; 0.00	<1.7; 0.00	<1.7; 0.00	1.8	2-5	10	≥ 1,280	1/7	2.5 S
28	< 10	21-22	NEG	3.9; 0.00	6.8; 0.29	5.5; 0.12	3.1; 0.00	<1.7; 0.00	<1.7; 0.00	1.3	2,4	10	640	2/7	2.8 S

* Seven tissues were tested per bird; tissues with peak WNV titers included kidney (K), brain (B), spleen (S), and heart (H).
† Reciprocal endpoint 80% WNV neutralization titer ($PRNT_{80}$); $PRNT_{80}$ ranges for adult females reflect serum titers measured prior (April 1) and following (Oct. 1) the 2007 breeding season. Chicks 7, 27, and 28 had seronegative mothers.
‡ Days post-hatch (DPH).
§ Chicks with sera reported as 'NEG' had 1:10 WNV PRNT antibody titers of $\leq 60\%$ neutralization and were considered seronegative.
¶ Plaque forming units (PFU) of WNV.
‖ % Infectiousness represents the percent of mosquitoes (*Culex pipiens*) that become infectious after feeding on a host as a function of host viremia, and is calculated by: $0.1349 \times \log_{10}(\text{viremia}) - 0.6235$ (Kilpatrick et al. 2007).
** Day(s) post-inoculation (DPI); the number(s) indicate which day(s) post-inoculation on which oral shedding was detected; swab samples were collected from 1-7 DPI.
†† Chicks that died or were euthanized due to morbidity included 1, 6, 7, and 20; the remainder was clinically normal and euthanized on 10 DPI.
‡‡ $PRNT_{80}$ titers were determined on the day of death or euthanasia, except for chicks 1 and 7, for which $PRNT_{80}$ titers were determined for the last serum samples collected from each bird, which was on 6 DPI.

TABLE 3.6. Viremia parameters and reservoir competence indices (RCI) for house sparrow chicks from females with and without antibodies to West Nile virus (WNV).

Mother antibody status	Age (DPH)* at inoculation	Susceptibility	Mean % infectiousness†	Mean duration of infection‡	RCI§
Positive	8-9	1.0	0.30	1.33	0.40
Negative	8-9	1.0	1.19¶	4.00	4.76
Positive	21-25	1.0	0.64	2.80	1.80
Negative	21-25	1.0	0.55	2.50	1.36

* DPH (days post-hatch) when inoculated with West Nile virus.
† Mean infectiousness is based on a viremia–infectiousness relationship for *Culex pipiens* (Kilpatrick et al. 2007).
‡ Mean duration of infection represents the average number of days that viremia titers were above the zero threshold of infection for *Culex pipiens* ($10^{4.62}$ PFU/mL) (Tiawsirisup et al. 2005).
§ RCI = susceptibility × mean infectiousness × duration of infectiousness (in days) (Komar et al. 2003, 2005).
¶ % Mean infectiousness represents only one individual's viremia.

TABLE 3.7. The model set testing the relationship between factors [mothers' West Nile virus (WNV) immunity status, and time post-inoculation] and chicks' viremia profiles following WNV challenge at 21-25 days post-hatch.

	Model	K*	-2logL†	AIC‡	AICc§	ΔAICc¶	AIC weight‖
1	Intercept-only	2	198.80	202.80	203.11	16.88	0.000
2	Time (days post-inoculation)	3	179.60	185.60	186.23	0.00	0.763
3	Mothers' WNV serostatus	3	198.30	204.30	204.93	18.70	0.000
4	Time + serostatus	4	178.80	186.80	187.88	1.65	0.147
5	Time + serostatus + (Time × serostatus)	5	176.70	186.70	188.37	2.14	0.090

* K = number of parameters in each model.
† -2logL = -2 × log likelihood.
‡ AIC = Akaike's Information Criteria.
§ AICc = AIC with a small sample size correction factor.
¶ ΔAICc = standardized AICc values (most supported model = 0).
‖ Akaike weight = the weight of evidence for each model.

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CHAPTER 4 WEST NILE VIRUS PERSISTENCE IN HOUSE SPARROWS

PERSISTENT WEST NILE VIRUS INFECTION IN THE HOUSE SPARROW (*PASSER DOMESTICUS*)

ABSTRACT

Long-term viral persistence within vertebrate hosts is a potential mechanism for over-wintering of arboviruses at temperate latitudes. It is also possible that chronic infection of tissues may allow reactivation under certain circumstances, thereby re-initiating transmission cycles after periods of unfavorable conditions to mosquitoes. Juvenile house sparrows experimentally inoculated with West Nile virus (WNV) were serially sampled until 30 or 65 days post-inoculation (PI), and adults at 1-, 6-, 12-, 18-, and 24-months following experimental WNV inoculation. Persistent shedding of infectious WNV via oral or cloacal secretions was detected in some birds at 12, 15, and 30 days PI and low titers of infectious virus were present in tissues (spleen and kidney) at 30-43 days PI, but not from sera after 6 days PI. Viral RNA was detected in tissues by RT-PCR in 92.3% (12/13) of individuals tested at 30 days PI and in 14.3% (2/14) of individuals at 65 days PI, although immunohistochemical labeling for viral antigen was negative. Documentation of infectious virus or viral RNA within tissues of numerous sparrows at 30, 43, and 65 days PI supports the possibility of viral recrudescence. In addition, RT-PCR positive tissues at 65 days PI and oral swabs at 27 and 44 days PI have implications

for the interpretation of avian carcass surveillance data, as well as the etiologic diagnosis of mortality among birds.

INTRODUCTION

Viral persistence, here defined as the continued presence of infectious virus beyond the acute viremic stage, within a broad range of vertebrate hosts has been documented for most families of arboviruses. This phenomenon may have implications for the maintenance and re-initiation of virus transmission cycles in nature (Kuno 2001). In temperate regions, extended periods of mosquito inactivity interfere with the continuous transmission of mosquito-borne viruses (Reisen et al. 2003). Over-wintering strategies for these viruses include hibernation of infected adult female mosquitoes, transovarial transmission from female mosquitoes to their offspring, and reintroduction from warmer climates (Reeves 1990).

The over-wintering mechanisms of West Nile virus (WNV; Family *Flavivirus*, Genus *Flaviviridae*) are still not fully understood, though WNV has been detected in over-wintering mosquitoes in New York, Connecticut, and Utah (Nasci et al. 2001, Anderson and Main 2006, Phillips and Christensen 2006). However, WNV infection in over-wintering mosquitoes is rare in nature (Taylor et al. 1956, Peiris and Amerasinghe 1994, Farajollahi et al. 2005, Reisen et al. 2006, Bolling et al. 2007). The recovery of infectious WNV from the brain of a hawk in New York in February, a period of mosquito inactivity, raised questions as to potential persistent infection within the hawk, or alternatively, oral transmission to the hawk via consumption of persistently infected prey (Garmendia et al. 2000). Further evidence of a non-mosquito source of transmission during cold periods in a temperate region, again New York, was the detection of lethal

infections among communally roosting crows (Dawson et al. 2007). The principal mechanism for annual spring emergence and re-initiation of transmission remains unknown.

The house sparrow (*Passer domesticus*) has been implicated in the epizootic cycles of numerous arboviruses in the United States, e.g., Eastern equine encephalitis, St. Louis encephalitis (SLEV), Venezuelan equine encephalitis, and Western equine encephalitis viruses (WEEV; Kruszewicz 1995), and may be important for the maintenance and local spread of WNV. House sparrows are amplifying hosts of WNV and are widespread and abundant throughout North America, where they have demonstrated high seroprevalence rates in numerous regions (Komar et al. 2001, 2003, 2005, Godsey et al. 2005). Because viral persistence has been demonstrated for numerous arboviruses (including WNV) in a variety of vertebrate hosts, we hypothesized that such infections in house sparrows could be important for overwintering of WNV. To characterize WNV persistence in this host, we inoculated sparrows and monitored their infection status for up to 65 days.

MATERIALS AND METHODS

Husbandry and initial sampling

Adult house sparrows were collected by mist net in northern Colorado in January and February of 2005. Birds were bled via jugular venipuncture upon arrival to assess WNV serostatus, and then housed free-flight in a 12.12m long (L) x 3.24m wide (W) x 2.57m high (H) room provided with sand baths, and branches and ropes for perching. Cuttlefish bone, fresh water, and food were available at all times; food consisted of a dry

mix with equal parts of millet, milo, cracked corn, cracked sunflower seed, and oats. Dry food was supplemented with live mealworms 1-2 times a week.

These captive sparrows bred in 2007, after which 36 offspring were separated from the flock at approximately 2-4 months of age and housed within cages (0.61m L x 0.38m H x 0.41m W or 0.76m L x 0.43m H x 0.46m W; 2-6 birds per cage). Juveniles were independent and fully developed when separated, and were fed the adult diet plus cooked egg and mealworm pupae *ad libitum*. Sparrows were acclimated to captivity and/or caged housing for days to weeks prior to experimental inoculation.

Inoculation and sampling scheme of sparrows

All sparrows were bled just prior to WNV inoculation for confirmation of WNV seronegative status. Both seronegative adult (n = 115) and juveniles (n = 35) were needle-inoculated subcutaneously over the chest with between 1,000-4,000 plaque forming units (PFU) of WNV strain NY99-4132 administered in 0.1 ml BA1 (M199-Hank's salts, 1% bovine serum albumin, 350 mg/L sodium bicarbonate, 100 units/mL penicillin, 100 µg/mL streptomycin, 2.5 µg/mL amphotericin B in 0.05 M Tris, pH 7.6). Adults were inoculated in April 2005, and juveniles in November 2007. Twenty seronegative adults and one seronegative juvenile were not inoculated. Anti-WNV antibody status was confirmed in all sparrows surviving to 1-month post-inoculation (PI), including non-inoculated control sparrows.

Sparrows exhibiting signs of morbidity, such as lethargy, anorexia, and/or fluffed feathers were humanely euthanized via sodium pentobarbital overdose administered intravenously. Carcasses of sparrows that died or were euthanized within 8 days of inoculation (n = 11 adults and 8 juveniles) due to morbidity were immediately necropsied

or refrigerated and necropsied within 12 hours. Tissue and swab samples were also collected opportunistically from adult sparrows that died or were euthanized due to clinical illness (n = 10) between 30-354 days PI. At necropsy, oral and cloacal swabs and tissues were collected, including skin (from breast), pectoral muscle, heart, liver, lung, spleen, small intestine, kidney, and cerebrum from all birds, plus breast feathers from juveniles.

The post-inoculation sampling scheme of adult sparrows that survived acute infection (> 8 days PI) included collection of oral swabs from 104 adult sparrows at 1-month PI (plus 20 non-inoculated controls), 98 adults (plus 19 non-inoculated controls) at 6-months PI, and 20 previously inoculated birds at each time point of 12-, 18-, and 24-months PI. In addition, all adults were bled at 1-, 6-, 12-, 18-, and 24-months PI to confirm WNV seropositive status. Three birds were sacrificed at each time point followed by necropsy and sample collection as previously described.

The post-inoculation sampling scheme of the 27 juvenile sparrows that survived acute infection plus one negative control was as follows: sera, and oral and cloacal swabs were collected every three days from 3-30 days PI, at which time 14 birds were euthanized (including the negative control). Thereafter, the 14 remaining sparrows were bled and swabbed weekly, and euthanized on 65 days PI.

Sample processing and storage

For juvenile sparrows, 0.1 ml of blood was placed into a cryovial containing 0.45 ml of BA1 medium for an approximate 1:10 serum dilution, held at room temperature for approximately 20-30 minutes for coagulation, and centrifuged at 2,000 x G for 10 min. A portion of each sample was stored at 4°C and tested within 24 hours by Vero cell plaque

assay to avoid a freeze-thaw cycle prior to testing, while the remainder was frozen to -80°C for testing by reverse transcriptase-polymerase chain reaction (RT-PCR) within one month.

For adult sparrows, blood samples were placed undiluted into serum separator tubes, held at room temperature for approximately one hour, and centrifuged at 16,000 x G for 3 min and sera stored at -20°C.

Oral and cloacal cavities were sampled by passing cotton-tipped swabs across mucosal surfaces, after which swabs were placed into 1 ml BA1 medium. Swabs from juveniles were aliquoted and stored as for diluted serum samples, while those from adults were stored at -80°C until testing.

Upon necropsy, tissue samples were weighed and placed into cryovials containing 1 ml of BA1 medium with 20% FBS as a 10% tissue suspension (except for spleen, which was at an approximate 5% tissue suspension). A single copper-coated steel 4.5 mm ball bearing ("BB") was added to each cryovial, and tissue samples were homogenized in a mixer mill (Retsch GmbH, Haan, Germany) for 5 min at 25 cycles/sec and homogenates were clarified by centrifugation (16,000 x G for 3 min). Swab and tissue supernatants from adults were stored at -80°C, while swabs from juveniles were handled as described for live bird samples. Tissue homogenates from juveniles were pooled into three aliquots for testing by RT-PCR as follows: kidney, spleen, small intestine and liver; skin, feather and muscle; and heart, lung and brain. Tissues from positive pools were tested individually. Tissues collected from juveniles at 30- and 65-days PI time points (gastrointestinal tract, brain, liver, heart, lung, kidneys, and breast skin) were placed into 10% neutral buffered formalin for 24 hours, and then into 70% ethanol.

Virus isolation and quantification

Plaque assays were performed to assess and quantify infectious WNV on Vero cell monolayers as previously described (Bunning et al. 2002). Samples tested by plaque assay from juveniles included serially collected oral and cloacal swab and serum samples, as well as oral and cloacal swab, heart, kidney, spleen, brain, feathers, skin, skeletal muscle, liver, lung, and intestine collected at necropsy. Samples from adults included oral swabs collected at serial time points, as well as oral swab, heart, kidney, spleen, and brain collected at necropsy. Minimum levels of WNV detection by plaque assay were $10^{1.7}$ log PFU/ml for serum, $10^{0.7}$ log PFU/swab, and $10^{1.7}$ PFU/g for tissues. Viral plaques detected by Vero plaque assay were confirmed by reisolation from the original sample, and identified as WNV by VecTest WNV Antigen Assay (VecTest; Medical Analysis Systems, Camarillo, CA as in Nemeth et al. 2007) or by RT-PCR.

Serology

Sera collected from all birds just prior to inoculation, as well as from adults and juveniles at approximately 1-month PI and subsequent time points (6-, 12-, 18- and/or 24-months PI for adults and 65 days PI for juveniles) were assessed for WNV-neutralizing antibodies by plaque reduction neutralization test (PRNT) as previously described (Beaty et al. 1995). Samples with $\geq$ 90% neutralization at a 1:10 dilution were considered anti-WNV antibody positive, while samples with $\leq$ 60% neutralization were antibody negative (no neutralization results fell between these criteria of 60-90%).

RT-PCR

Serum, swab, and tissue samples collected from juveniles at ≥ 9 days PI were tested by RT-PCR. RT-PCR methods for detection of WNV RNA followed those previously described (Lanciotti et al. 2000) except for use of the Viral RNA Minikit (QIAGEN Inc., Valencia, CA) for RNA extraction and use of the Bio-Rad Icycler IQ™ Real-time Detection system (Bio-Rad, Hercules, CA) for cDNA amplification. A Ct value of 36.5 or less was considered positive for target sequence amplification, while values between of 36.5 and 37.5 were re-tested. Samples were screened with one set of primers specific for the envelope protein of WNV (genome positions were 1160 for forward primer, 1229 for reverse primer, and 1186 for probe; Lanciotti et al. 2000).

Immunohistochemistry

Tissues (heart, lung, kidney, spleen, intestine, and cerebrum) from convalescent juveniles were embedded in paraffin, sectioned at 6 microns and stained. Negative and positive control tissues for immunohistochemistry (IHC) were obtained from a seronegative juvenile that was not inoculated and from a juvenile that died within the acute phase of infection (4 days PI), respectively. The methodology for IHC was adapted from a published protocol with minor modifications (Miura et al. 2008). The primary antibody used was WNV B-956 diluted 1: 500 in blocking solution. To minimize non-specific staining, tissues were incubated for 30-minute with 0.15 M glycine in phosphate buffered saline, following the 1% H_2O_2 incubation step. Endogenous biotin binding was blocked using a kit from DAKO (Carpintaria, CA), and the blocking solution contained 0.2% Tween-80 in addition to Tween-20 and normal serum.

RESULTS

Morbidity and mortality

The majority of adult (90%; 104/115) and juvenile (77%; 27/35) sparrows inoculated with WNV survived beyond the acute phase of infection.

Mortality after 8 days PI among inoculated adults occurred on or near 30, 43, 105 (two individuals), 150, 160, 247, 280, 319, and 354 days PI. In nearly all cases, sparrows were found dead; however, one sparrow was euthanized on 43 days PI due to open mouth breathing and lethargy; upon necropsy, an extensive space occupying lesion was evident within the coelomic cavity that histologically revealed a ruptured spleen. Several sparrows had gross evidence of blunt head trauma, another had hemorrhage within the gastrointestinal tract, and several had no gross lesions. The carcasses of sparrows found dead on 160 and 343 days PI were desiccated and not subjected to necropsy or sample collection. None of these convalescent deaths were attributed to WNV infection, or appeared in any way to reflect recrudescence of acute infection. No juveniles died after 8 days PI.

WNV shedding, viremia, and detection in tissues among acute and fatal infections

Most juveniles (31/35; 88.6%) had detectable oral shedding on 3 days PI with a mean concentration of $10^{3.5}$ PFU/swab (samples with undetectable levels were considered zero in calculating the mean). Forty percent of juveniles (14/35) had detectable cloacal shedding on 3 days PI, with an average of $10^{2.9}$ PFU/swab. Of the sparrows that died within 8 days PI, all swabs were positive for infectious WNV with titers ranging from $10^{2.7\text{-}7.0}$ log PFU/oral swab (includes juveniles and adults) and $10^{2.6\text{-}5.7}$ log PFU/cloacal

swab (juveniles only). Acute oral and cloacal shedding was not evident in surviving juveniles after 6 days PI.

The mean peak viremia on 3 days PI among juveniles that died within 8 days of inoculation (n = 8) was $10^{9.4}$ PFU/ml serum, while the mean peak viremia among survivors was $10^{6.0}$ PFU/ml serum. West Nile virus was not detected in serum beyond 6 days PI.

All tissues from adults that died within 8 days of inoculation (n = 8) tested positive by plaque assay with the exception of the spleen and brain of one bird on 7 days PI, and spleen of another on 8 days PI. All tissues from juveniles that died within 8 days of virus inoculation were positive except for feathers of one bird. The highest WNV titers in any juvenile sparrow were in kidney ($10^{9.2}$ PFU/g), spleen ($10^{9.8}$ PFU/g), brain ($10^{9.6}$ PFU/g), and liver ($10^{9.0}$ PFU/g). Of birds that died acutely mean peak titers were generally higher in juveniles than adults (n = 11) (oral swab: $10^{6.2}$ versus $10^{5.8}$ PFU/swab; heart: $10^{7.6}$ versus $10^{6.7}$ PFU/g; kidney: $10^{8.8}$ versus $10^{7.5}$ PFU/g; spleen: $10^{9.1}$ versus $10^{6.9}$ PFU/g; brain: $10^{8.9}$ versus $10^{7.3}$ PFU/g, respectively).

WNV persistent shedding and detection in tissues

At one month PI, one of 104 (1.0%) adult sparrows had a low titer of infectious virus ($10^{0.7}$ PFU/swab) isolated from the oral swab; this same sparrow was euthanized on 43 days PI and WNV was isolated from the spleen ($10^{2.7}$ PFU/g). Virus was not isolated from oral swabs of 98 sparrows sampled at 6-months PI, nor from any of 20 sparrows sampled at 12-, 18-, and 24-months PI. West Nile virus was not isolated from swabs or tissues of any of the sparrows that died between 30-354 days PI, nor from the three birds sacrificed at each time point of 6-, 12-, 18-, or 24-months PI.

Oral and cloacal shedding was detected during the convalescent phase (> 8 days PI) in four juveniles and one adult up to 44 days PI (Table 4.1). Only two juveniles and one adult had detectable oral or cloacal shedding of infectious WNV during this phase, with low titers of $10^{0.7\text{-}2.0}$ PFU/swab.

WNV detection in tissues by RT-PCR occurred from most (12/13; 92.3%) juvenile sparrows euthanized on 30 days PI, with skin (10/13; 76.9%), spleen, and kidney (9/13 each; 69.2%) having most frequent detections. Low titers of WNV ($10^{1.7}$ PFU/g) were detected by plaque assay in spleen and kidney of two different juveniles euthanized on 30 days PI. At 65 days PI, spleen and kidney of one individual and kidney from another were positive by RT-PCR (Table 4.1). Contact transmission from infected to negative control sparrows was not detected during the study, as determined by lack of viral shedding and lack of seroconversion among these birds at all subsequent sampling time points.

None of the tissues from sparrows euthanized at 30 or 65 days PI tested positive by IHC staining, and no lesions were observed by microscopic examination.

Anti-WNV antibodies

All inoculated sparrows had anti-WNV antibodies by 1 month PI, and antibodies remained detectable at all subsequent sampling time points. No non-inoculated sparrows demonstrated evidence for anti-WNV antibodies during the study.

DISCUSSION

West Nile virus is an emerging zoonotic pathogen that has rapidly spread across much of the North American continent. WNV has become endemic within some

temperate latitudes despite extended periods of unfavorable conditions for its major vector, the mosquito (Dauphin et al. 2004). Enzootic transmission of numerous arboviruses, including WNV, is maintained in nature through mosquito-bird cycles, which re-initiate each season with the virus reaching established as well as new foci (Reisen et al. 2003, Komar et al. 2005). Both laboratory and field data suggest that WNV is transmitted vertically among mosquitoes, a potential mechanism for virus over-wintering that would allow progeny infected in the fall to re-initiate transmission when they emerge in the spring (Baqar et al. 1993, Miller et al. 2000, Turell et al. 2001, Goddard et al. 2003). However, vertical transmission among mosquitoes in nature may be rare, with rates generally between 0.1 and 1.0% (Taylor et al. 1956, Peiris and Amerasinghe 1994, Farajollahi et al. 2005, Reisen et al. 2006). The over-wintering of viruses in persistently infected hosts may be a more plausible explanation for the survival of mosquito-borne arboviruses through seasons of cold temperatures and shortened photoperiods, with annual recurrence when conditions become favorable. In the latter case, persistent infection of tissues may lead to relapsing viremias in hosts and subsequently, infection of vectors (Reeves 1990).

While it is difficult to demonstrate the phenomenon of persistent viral infection of hosts in nature, field studies of wild birds have revealed supporting evidence. For example, a free-ranging catbird (*Dumetella carolinensis*) that was seropositive for Eastern equine encephalitis virus (EEEV) had viremia early in the next transmission season (Crans et al. 1994), and early season seroconversion was documented in a free-ranging bird prior to detection of mosquito or epizootic horse activity (Emord and Morris 1984). Free-ranging house finches in California exhibited reversions of St. Louis

encephalitis virus (SLEV) serostatus, from seronegative to seropositive to seronegative, potentially representing viral reinfection by mosquitoes or recrudescence of virus. These "seroreconversions" occurred mostly during cooler months when mosquito activity was minimal and lacked SLEV positive pools (Gruwell et al. 2000).

Arboviral persistence has been documented experimentally within a variety of vertebrate hosts, including bats, snakes, primates, and birds. Kuno defines a minimum of 21 days following infection to consider virus within cells, tissues or other bodily fluids, such as feces, saliva, semen, or urine as persistent (2001). Persistent infections with Japanese encephalitis (JEV) and SLEV have been documented in birds, with JEV detection (via inoculation of tissue suspension into suckling mouse brain) in liver and kidney of a needle-inoculated pigeon at 39 days PI (Chunikhin and Takahashi 1971) and SLEV in gizzard of a cowbird at 38 days PI (Chamberlain et al. 1957). St. Louis encephalitis virus was detected by RT-PCR (but undetectable by Vero plaque assay) in the lung and spleen of a needle-inoculated house finch at 1 year PI, though weekly blood samples collected from 8-12 months PI were negative for SLEV (Reisen et al. 2001).

Controlled studies have revealed the possibility of persistent WNV in vertebrate hosts, including both birds and mammals. Experimental studies in Russia in the 1970s led to successful isolation of WNV from the blood of experimentally-inoculated ducks (Fedrova and Stavskiy 1972 in Kuno 2001) and pigeons at approximately 100 days PI; the pigeons had intermittent viremia and seroconverted (Semenov et al. 1973 in Kuno 2001). More recently, North American strains of WNV have been used in various controlled experiments. Relatively high titers of infectious WNV (approximately $10^{2.6-4.3}$ PFU/0.5 cm^3) were detected in skin of several experimentally inoculated birds at 10-11

days post-viremia; the authors suggested that skin infection may permit WNV transmission to vectors during feeding in the absence of host viremia (Komar et al. 2003). West Nile virus was frequently detected by RT-PCR in tissues (spleen, kidney, and lung) of experimentally-inoculated Passeriformes and Columbiformes (e.g., house sparrow, house finch, mourning dove) at > 6 weeks PI; detection in sera was rare but occurred from one house sparrow (Reisen et al. 2006). On the contrary, there was no serological evidence of recrudescence of WNV in naturally infected rock pigeons (*Columba livia*), in which anti-WNV antibodies were observed to remain at consistent levels for at least 15 months (Gibbs et al. 2005). West Nile virus persisted for up to 167 days PI in brains of experimentally inoculated rhesus macaques (*Macacus rhesus*), though virus lacked cytopathogenicity (Pogodina et al. 1983). Infectious WNV was detected in the urine of experimentally inoculated hamsters (*Mesocricetus auratus*) for up to 247 days PI, with antigen visible in kidneys by IHC (Tesh et al. 2005), while persistent WNV RNA was detected in the kidney of an experimentally inoculated fox squirrel (*Sciurus niger*) at 29 days PI (Platt et al. 2008). Finally, persistent WNV infection (35 days PI) was documented in mice that were deficient in CD8+ T cells, but not in wild-type mice (Shrestha and Diamond 2004), suggesting that immuno-compromised individuals may be more susceptible to persistent WNV infection.

The possibility of persistent or relapsing WNV infection within avian hosts in nature has been raised (Garmendia et al. 2000, Yaremych et al. 2004, Lopes et al. 2007). Avian carcasses have tested positive for WNV RNA during winter periods in Texas and California (Tesh et al. 2004, Reisen et al. 2006). In addition, multiple WNV infections within a single roost of American crows (*Corvus brachyrhynchos*) occurred in New York

during winter; transmission among these crows was hypothesized to be horizontal due to the finding of WNV-positive feces. However, the initiation of this transmission focus was unknown but unlikely to be associated with mosquitoes; chronic WNV infection in crows was considered improbable due to high fatality rates of American crows experimentally inoculated with WNV (Dawson et al. 2007). However, the carcass of a free-ranging American crow that was seropositive for anti-WNV antibodies 56 days earlier tested positive for WNV by RT-PCR and/or IHC (Yaremych et al. 2004), leaving the possibility that crows in the latter study could have been persistently infected, or in turn, became infected after consumption of persistently infected prey.

Persistent WNV infection was detected in several needle-inoculated sparrows in the present study, confirming an earlier observation by Reisen et al. (2006), in which 41.5% (34/82) of needle-inoculated individuals of six avian species had detectable WNV RNA at > 6 weeks PI. The house sparrow was among these species, of which 33.3% (3/9) of individuals had positive tissues (spleen, lung, and/or kidney), and one of these sparrows had RT-PCR positive sera. Infectious WNV was recovered from a portion of RT-PCR tissue samples; tissue samples were passaged through C6/36 *Aedes albopictus* (Skuse) cell culture prior to Vero cell plaque assay to maximize infectious virus recovery. Our results are in accordance with those of Reisen et al. (2006), and support the notion that detection of viral RNA at chronic time points following initial infection is not uncommon, especially in kidney, while the detection of circulating WNV or RNA in sera appears much less frequent. More rigorous detection methods such as those employed by Reisen et al. (2006), and others (e.g., cocultivation of kidney tissue; Tesh et al. 2005) increase the likelihood of recovering infectious virus. On the other hand, RT-PCR is a

common method used in surveillance and diagnostics. The significance of detection of WNV RNA toward chronic infection in these birds is not entirely clear, but could potentially signify latent or chronic infection.

The potential effects of low levels of WNV in sparrow tissues on the health of these birds remain unknown. The observation of low levels of WNV or viral RNA in a tissue or swab sample may not provide an accurate diagnosis in a diseased bird, as observed in the present study. Further, an avian carcass that demonstrates low titers of persistent virus or viral RNA in tissues or swabs may represent mortality due to a primary cause unrelated to WNV infection. Such carcasses would not reflect current WNV transmission, an important consideration for surveillance (Reisen et al. 2006). In Colorado, low levels of infectious WNV were detected in avian carcasses of species not commonly believed to undergo WNV-associated morbidity or mortality, such as rock pigeons (*Columba livia*) and mourning doves (*Zenaida macroura*) (Gerhold et al. 2007, Nemeth et al. 2007).

While it remains an unsubstantiated theory, persistent WNV infection could lead to relapse and viral recrudescence in birds due to intrinsic factors within the bird (e.g., reproduction, molt, migration) or extrinsic factors in the environment (e.g., poor food or habitat availability, adverse environmental conditions). Relapse from persistent infection may be associated with decay of humoral immunity below a certain protection threshold, allowing recurrence of viremia to titers infectious to vectors (Reeves 1990, Reisen et al. 2003, 2004). Relapse of chronic SLEV infections were potentially attributable to rapid decay of anti-SLEV antibodies in house finches (*Carpodacus mexicanus*; Reisen et al. 2001). While antibodies to WNV are protective and long-lasting in some birds (Nemeth

et al. 2008, Nemeth and Bowen, unpub. data), their presence does not exclude the possibility of persistence of WNV within tissues (Reisen et al. 2001, Diamond et al. 2003). In addition, the potential for viral recrudescence following immunosuppression was studied by inoculating cyclophosphamide-treated house finches with either WEEV or SLEV. Immunosuppression did not lead to relapses in infectious viremia (Reisen et al. 2001), and increased viral doses did not alter viremic responses (Reisen et al. 2004). Migration is a natural activity for many birds and may lead to immunosuppression (Owen and Moore 2006); the effect of WNV infection during migratory activity has been examined. Two migratory bird species, the Swainson's thrush (*Catharus ustulatus*) and gray catbird (*Dumetella carolinensis*), were experimentally inoculated with WNV and exhibited migratory behavior during acute infection. Resulting viremia titers were not significantly elevated in individuals exhibiting migratory behavior versus those that did not, suggesting that immunosuppression did not occur or did not affect viremia during migratory activity (Owen et al. 2006). Finally, persistent WNV in prey animals, including birds, may lead to oral transmission to predatory birds and other animals during times of interrupted mosquito activity (Garmendia et al. 2000, Komar et al. 2003, Austgen et al. 2004, Klenk et al. 2004, Nemeth et al. 2006). Efforts should continue to characterize the occurrence of persistent viral infections in birds and other vertebrates and to understand the significance of this phenomenon in nature.

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TABLE 4.1. Detection of West Nile virus in swabs or tissues of house sparrows as detected by RT-PCR or Vero plaque assay following experimental inoculation.

			Detection method*	
ID	Age	Days PI	Plaque assay†	RT-PCR
30	Juvenile	12	Oral swab	Oral swab
45	Juvenile	12	—	Cloacal swab
58	Juvenile	15	Cloacal swab	—
10	Juvenile	27	—	Oral swab
3	Juvenile	30	—	Kid, Spl, Liv, Ski, Mus, Hrt, Brn‡
9	Juvenile	30	Spl	Kid, Spl, Liv, Ski
19	Juvenile	30	—	Kid, Ski, Lun
30	Juvenile	30	—	Kid, Ski, Mus
31	Juvenile	30	—	Spl
33	Juvenile	30	—	Kid, Liv, Ski, Mus
40	Juvenile	30	Kid	Kid, Spl, Liv, Ski, Mus, Brn
45	Juvenile	30	—	Kid, Spl, Liv, Ski, Mus, Hrt, Brn, Fea
48	Juvenile	30	—	Spl, Ski, Brn, Fea
57	Juvenile	30	—	Kid, Spl, Ski, Mus, Hrt
58	Juvenile	30	—	Kid, Spl, Ski, Mus, Hrt, Brn
60	Juvenile	30	—	Spl, Liv, Lun
41§	Adult	30	Oral swab	—
41	Adult	43	Spl	—
35	Juvenile	44	—	Oral swab
10	Juvenile	65	—	Kid, Spl
34	Juvenile	65	—	Kid

* Either West Nile viral RNA or infectious PFU (plaque forming units) were detected.
† The detection threshold for swabs and tissue homogenates was $10^{0.7}$ PFU/swab or per ml, respectively.
‡ Positive tissues included: Kidney (Kid), Spleen (Spl), Liver (Liv), Skin (Ski), Muscle (Mus), Heart (Hrt), Brain (Brn), Lung (Lun), and Feather (Fea).
§ Samples from sparrow 41 were not tested by RT-PCR.

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