Rapid Amplification of West Nile Virus: The Role of Hatch-Year Birds

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ABSTRACT

Epizootic transmission of West Nile virus (WNV) often intensifies rapidly leading to increasing risk of human infection, but the processes underlying amplification remain poorly understood. We quantified epizootic WNV transmission in communities of mosquitoes and birds in the Chicago, Illinois (USA) region during 2005 and 2006. Using quantitative polymerase chain reaction (PCR) methods, we detected WNV in 227 of 1195 mosquito pools (19%) in 2005 and 205 of 1685 (12%) in 2006; nearly all were Culex pipiens. In both years, mosquito infection rates increased rapidly in the second half of July to a peak of 59/1000 mosquitoes in 2005 and 33/1000 in 2006, and then declined slowly. Viral RNA was detected in 11 of 998 bird sera (1.1%) in 2005 and 3 of 1285 bird sera (<1%) in 2006; 11 of the 14 virus-positive birds were hatch-year birds. Of 540 hatch-year birds, 100 (18.5%) were seropositive in 2005, but only 2.8% (14/493) tested seropositive in 2006 for WNV antibodies using inhibition enzyme-linked immunosorbent assay (ELISA). We observed significant time series cross-correlations between mosquito infection rate and proportion of virus-positive birds, proportion of hatch-year birds captured in mist nets (significant in 2006 only), seroprevalence of hatch-year birds, and number of human cases in both seasons. These associations, coupled with the predominance of WNV infection and seropositivity in hatch-year birds, indicate a key role for hatch-year birds in the amplification of epizootic transmission of WNV, and in increasing human infection risk by facilitating local viral amplification. Key Words: West Nile virus—Hatch-year birds—Culex infection—Rapid amplification.

INTRODUCTION

Since the appearance of West Nile virus (WNV) in New York in 1999 (Lanciotti et al. 1999), the virus has spread rapidly westward across North America, and southward into the Caribbean Basin, Mexico, and Central and South America (Komar and Clark 2006). In the 7 years since its establishment, WNV has been responsible for over 20,000 human cases of disease and nearly 1000 human fatalities in the United States (CDC 2007). Illinois led the nation in human cases (884) and deaths (64) in 2002, was second to California in 2005 (252 human cases and 13 deaths), and ranked sixth in 2006 (211 human cases and 9 deaths). Most human cases in Illinois have been reported from the Chicago region (Gu et al. 2006, IDPH 2007), where infection rates in Culex mosquitoes of 60/1000 mosquitoes during peak transmission were observed; this rate is much higher than those observed elsewhere (Andreadis et al. 2004, Gu et al. 2004, Ezenwa et al. 2006, Reisen et al. 2006).

The incidence of human cases of WNV infection, when mapped by home address, is
highly clustered within urban environments (Ruiz et al. 2004, Watson et al. 2004). In the Chicago metropolitan area, Ruiz et al. (2004, 2007) showed that human WNV incidence was highest in urban areas characterized by medium-density housing, housing constructed in the 1950s, moderate income, and high proportion of white people. Annual variation in incidence has been principally attributed to weather patterns (Andreadis et al. 2004, Shaman et al. 2005); specifically, drought and high temperatures consistently coincide with years of increased transmission. High temperatures also increase the rate of WNV dissemination in *Cx. pipiens*, contributing to amplification (Dohm and Turell 2001). Kilpatrick et al. (2006) suggested that bird community structure, diversity, and presence of particular species such as the American robin (*Turdus migratorius*) were the main determinants of mosquito infection rate in Maryland and Washington, D.C. Thus, presence of avian hosts, competent *Culex* mosquitoes, and suitable climate provide the general conditions for epidemic transmission of WNV (Day 2001, Andreadis et al. 2004, Gu et al. 2004, Reisen et al. 2004).

The specific associations between hosts and vector that may influence sudden, seasonal WNV amplification remain unknown. Scott and Edman (1991) postulated that avian age structure modulates intensity of arbovirus transmission. Specifically, they speculated that nestling and fledgling birds make especially important contributions to amplification, not just because of the influx of nonimmune susceptibles, but also because nestling and fledgling birds are more prone to mosquito bites. We therefore initiated an intensive investigation of WNV epizootic transmission and local viral amplification in a 25-km² study area in suburban Chicago, Illinois. This area is known for historic St. Louis encephalitis (SLE) and WNV activity and for a high incidence of human cases of SLE (Zweighaft et al. 1979) and WNV (Ruiz et al. 2004, 2007). We sought to quantify WNV amplification at a local scale, with explicit attention to possible associations among seasonal trends, the local abundances of hatch-year birds, and timing of infection in birds, mosquitoes, and humans.

**MATERIALS AND METHODS**

**Study sites**

Our study areas were located in southwest suburban Chicago, IL (Cook County; 87° 44' W, 41° 42' N; Fig. 1). Specific residential and natural sites for mosquito and bird sampling were selected by a stratified random design to represent a range of environmental and demographic features. The residential sites were selected to include various levels of income, housing density, and distance to nearest natural area. Using these criteria, in 2005 we established 11 residential sites that encompassed seven municipalities. The four “green spaces” in the region included three cemeteries and a wildlife refuge. In 2006 we sampled an additional 10 residential sites selected with the same design and one additional green space. Precipitation and temperature conditions from 1873 to 2006 for Chicago were obtained from the National Oceanic and Atmospheric Administration (http://www.crh.noaa.gov/lot/?n=climate) and daily temperature and precipitation were obtained from the National Climatic Data Center (http://cdo.ncdc.noaa.gov/qclcd/QCLCD). Data for human WNV date of onset and spatial occurrence were made available by the Illinois Department of Public Health. Human cases considered in this paper occurred within a 5-km buffer around the 15 field sites in 2005 and 26 field sites in 2006. Spatial data were processed using the ArcGIS 9.0 software (ESRI, Redland, CA).

**Field sampling**

Mosquitoes were collected from each of the field sites in 2005 and in 2006 once every 2 weeks from mid-May through mid-October. A mosquito trapping session at each site in 2005 consisted of four CO₂-baited CDC miniature light traps (two elevated into tree canopy and two at ground level), four CDC gravid traps baited with rabbit pellet infusion (Lampman and Novak 1996), and battery-powered backpack aspirators (Meyer et al. 1983). In 2005, elevated light traps captured more *Culex* spp. mosquitoes; therefore, our 2006 sampling consisted of two elevated light traps, two gravid traps, and aspirators. Mosquitoes were identi-
fied (Andreadis et al. 2005) and pooled into groups of 25 or less, grouped by species, sex, collection site, and date, and placed in 2-mL microcentrifuge tubes. Culex individuals that could not be identified to species were grouped as Culex complex. The cold chain was maintained while processing and pools were stored at −20°C or −80°C prior to testing.

Wild birds were captured with 36-mm mesh nylon mist nets (Avinet, Inc.) from mid-May to mid-October in both years. In 2005, five of the residential sites (Sites 1, 5, 7, 10, 11) and all four natural areas were sampled on 3-week rotations (slightly longer rotations late in the season) resulting in six visits to each study site. We sampled eight additional residential sites in 2006. Captured birds were identified, weighed, measured, sexed, aged, and then released. Age was based on plumage characteristics and yellow gape on base of bill and allowed classification of “hatch year” or “after hatch year” (i.e., adults; see Pyle 1997). For a few species, some or all individuals were classified as unknown age and/or sex. Birds were marked with numbered USFWS leg bands (U.S. Department of Interior Bird Banding Laboratory), as authorized by Federal Bird Banding Permit 06507. Blood was sampled by jugular or brachial venipuncture using a 25-gauge tuberculin syringe or a 28-gauge insulin syringe. The volume of blood collected varied by bird size but did not exceed 1% of the bird’s body weight or 0.2 mL. Blood was added to 0.8 mL of BA-1 diluent in a microcentrifuge tube. Blood was stored on ice packs in the field and centrifuged within 5 hours. Serum and BA-1 was pipetted and placed in a 2.0-ml cryovial; clots and the serum were stored at −20°C or −80°C. All fieldwork was carried out under appropriate collecting permits with approvals from the Institutional Animal Care and Use Committee at Michigan State University, Animal Use Form 12/03-152-00 and UIUC Animal Use Protocol 03034.
Laboratory analyses

Mosquitoes were homogenized by adding 1 mL of a 50:50 mixture of phosphate-buffered saline (PBS) and 2× lysis buffer (Applied Biosystems, Foster City, CA) and three #7 steel shots using a high-speed mechanical homogenizer (Retsch MM 300) for 4 minutes at 20 cycles/second. Each homogenized pool was centrifuged for 2 minutes at 13,000 rpm. RNA was extracted from mosquito pools using an ABI Prism 6100 Nucleic Acid Prep Station following the Tissue RNA Isolation Protocol (Applied Biosystems; P/N 4330252). RNA was eluted in a final volume of 60 μL of elution solution. A region of the WNV RNA envelope gene was detected using real-time, reverse transcription-polymerase chain reaction (RT-PCR) (Lanciotti et al. 2000). The thermocycling was performed on an ABI Prism 9700HT sequence detector at the Research Technology Support Facility at Michigan State University, following the TaqMan One-Step RT-PCR Master Mix Protocol (Applied Biosystems; P/N 04310299).

We used blocking enzyme-linked immunosorbent assay (ELISA) for detection of WNV antibodies in bird serum samples (Blitvich et al. 2003). The inner 60 wells of a 96-well EIA/RIA medium binding microtiter plate (Corning Incorporated 3591) were loaded with a 1:12,000 dilution of 4G2 capturing antibody and coating buffer, and incubated overnight (all incubations at 37°C, humidified with wet paper towel). Plates were washed six times with PBS-Tween 20, pH 7.4, and then wells were blocked with a milk-PBS solution (BIO RAD nonfat dry milk) and incubated for 2 hours at 37°C. Plates were washed and a 1:50 dilution of WNV antigen and PBS was loaded into wells and incubated for 2 hours. Plates were washed and 100 μL of field collected serum (1:20 dilution with BA-1) was loaded along with positive and negative controls. The plate was incubated for 2 hours, washed, and wells were loaded with a 1:4000 dilution of 6B6C-1 monoclonal antibody (MAb) labeled with horseradish peroxidase and milk-PBS. After another 2-hour incubation and washing, 100 μL of tetramethylbenzidine (Sigma Aldrich, Inc.) was added and then incubated and stopped with 50 μL of sulfuric acid. The reduction in optical density was determined with plate blanks subtracted at a wavelength of 450 nm on an automated plate reader (Molecular Devices). Percent inhibition was calculated as 

\[
\frac{1}{100} \left( \frac{TS}{CS} - 1 \right)
\]

where TS is the optical density of the test serum and CS is the mean optical density of the negative control serum. Two different positive controls and four negative controls were used on each plate. Samples testing positive on the first screen were serially diluted and tested to find the endpoint titer.

We tested bird serum samples for the presence of WNV RNA using similar methods described for mosquito pools. We extracted RNA from 100 μL of bird serum in a 1:20 dilution with BA-1 using a protocol developed for the isolation of viral RNA from noncellular samples on the ABI 6100 Nucleic Acid Prep Station (Felton 2003). WNV was detected using RT-PCR as described above.

Data analysis

Maximum likelihood estimates and 95% confidence intervals (CI) for Culex spp. infection rates were calculated by week using the Pooled Infection Rate Version 3.0 Add-In (Biggerstaff 2006) and Excel (Microsoft 2005). Cross-correlation analyses were used to estimate the correlation between paired time series measured by week. We estimated the correlation of Culex spp. mosquito infection with the proportion of virus-positive birds, with hatch-year and adult bird seropositivity, with the proportion of hatch-year birds captured in mist nets, and with the date of onset of human cases of WNV in the study region. We identified the time lag at which the estimated correlation was maximized. Differences in proportions were compared on frequency data using Pearson’s chi-square test. Endpoint titers for adult and hatch-year birds were compared using the Kruskal-Wallis test. All statistical analyses were performed using the R software environment (R Development Core Team 2007; http://www.R-project.org).

RESULTS

Precipitation and temperature data are shown in Figure 2. In 2005, the mean precipi-
tation in the Chicago region during the months of June, July, and August was the third lowest since 1871, and temperatures were the 12th hottest summer on record. In 2006, the mean summer temperature was cooler (35th hottest summer on record), and the area received over twice the rainfall during the 3-month period compared with 2005 (Fig. 2).

We collected 21,285 individual mosquitoes which comprised 1195 pools consisting of 13 mosquito species in 2005, and 24,332 individual mosquitoes which comprised 1685 pools and 18 species in 2006. *Cx. pipiens* was the dominant mosquito in both years, and the *Culex* spp. (*Cx. pipiens*, *Cx. restuans*, *Cx. tarsalis*, *Cx. erraticus*) accounted for 79% of the pools in 2005 and for 64% in 2006. *Culex* abundance standardized by mosquitoes per gravid trap increased in mid-July 2005 and remained steady till late-September (Fig. 3). In 2006, *Culex* abundance increased earlier in mid-June, and steadily declined till September. We detected WNV in 227 pools (19%) in 2005 and in 205 pools (12%) in 2006, a significantly smaller proportion (*χ^2 = 21, df = 1, p < 0.001*). Of the 432 positive pools, all were *Culex* spp. except two in 2005 and five in 2006. The infection rate cal-

![FIG. 2. (A) Cumulative precipitation (cm) and (B) daily average temperature (°C) recorded at Chicago Midway International Airport from May–September in 2005 and 2006.](image)

![FIG. 3. Temporal patterns of (A) *Culex* spp. mosquito infection rate (IR), (B) *Culex* spp. mosquito abundance per gravid trap, and (C) date of onset of human WNV cases from late-May to mid-October in the southwest suburban Chicago, IL region in 2005 and 2006.](image)
culated only for *Culex* spp. mosquitoes reached a peak of 59 (95% CI 43.9–80.2) during week 30 (July 23–29) in 2005 and of 33 (95% CI 22.2–47.7) during week 32 (August 12–18) in 2006 (Fig. 3). We observed a rapid amplification during weeks 29 and 30 (mid–late July) in 2005 and a lesser amplification during the same time period in 2006.

We captured 1407 birds of 57 species using mist nets in 2005 and 1479 birds of 63 species in 2006. The most commonly captured species were the house sparrow (*Passer domesticus*; combined years *n* = 871), the American robin (*Turdus migratorius*; *n* = 479), the gray catbird (*Dumetella carolinensis*; *n* = 180), and the northern cardinal (*Cardinalis cardinalis*; *n* = 163). The proportion of hatch-year birds in the mist-net samples reached 60% by late-July and remained high (>50%) through mid-October in both years (Fig. 4). The proportion of hatch-year birds in the mist nets was significantly cross-correlated with *Culex* infection rate 1 week later in 2006 (*r* = 0.55, *p* < 0.05).

We collected 1062 avian blood samples in 2005, of which 225 (21%) tested seropositive for WNV antibodies. In 2005, adult bird seroprevalence was 24.4% (115 of *n* = 471), significantly higher than the hatch-year bird seroprevalence of 18.5% (100 of *n* = 540; *χ*² = 4.9, df = 1, *p* < 0.05). In 2006, adult bird seroprevalence of 4.2% (33 of *n* = 792) was not different (*χ*² = 1.17, df = 1, *p* > 0.2) from hatch-year bird seroprevalence of 2.8% (14 of *n* = 493). The most abundant seropositive hatch-year birds in 2005 were the house sparrow (21% seroprevalence), northern cardinal (71%), American robin (11%), and gray catbird (36%). In 2006, the most abundant seropositive hatch-year birds were the northern cardinal (14%) and American robin (4%), but not the house sparrow (<1%). In both years, seropositive hatch-year birds were captured between June

![Graphs A, B, C, D](image.png)

**FIG. 4.** Temporal patterns of (A) proportion of hatch-year birds in mist-net samples, (B) virus-positive birds, (C) seropositivity of hatch-year birds, and (D) adult bird seropositivity from late-May to mid-October in the southwest suburban Chicago, IL region in 2005 and 2006. Discontinuous data in A and D represent weeks when hatch-year or adult birds were not collected.
27 and October 16, with a steady increase in the proportion of seropositivity through mid-August. We found a significant cross-correlation between hatch year bird seropositivity and Culex infection 2 weeks later in 2005 and 2006 (Fig. 5; $r = 0.45, p < 0.05$, and $r = 0.55, p < 0.55$, respectively). One hatch-year song sparrow captured twice at Holy Sepulchre Cemetery tested seronegative for WNV antibodies on July 27, 2005, and then tested seropositive upon re-

FIG. 5. Statistically significant correlations between the Culex spp. infection rate in 2005 and virus-positive birds (A), hatch-year (HY) bird seropositivity (B), percent HY birds captured in mist nets (C), and date of onset of human cases (D) and between Culex infection in 2006 and virus-positive birds (E), HY bird seropositivity (F), percent HY birds captured in mist nets (G), and date of onset of human cases (H) in southwest suburban Chicago, IL.
capture on August 17. Hatch-year birds averaged significantly higher endpoint titers than adult birds in 2005 (Kruskal-Wallis test, \( p < 0.001 \)) but not in 2006 (Kruskal-Wallis test, \( p > 0.5 \)).

We detected 11 birds (1.1% of 998) in 2005 that were virus positive at the time of capture: seven house sparrows, two house finches \((Carpodacus mexicanus)\), a red-winged blackbird \((Agelaius phoeniceus)\), and a northern flicker \((Colaptes auratus)\). The proportion of virus-positive hatch-year birds (10 of 517) was statistically higher than adult birds (1 of 433; \( \chi^2 = 4.6, df = 1, p < 0.05 \)).

In 2006 we detected three virus-positive birds (0.3% of 1285): three house sparrows (two adults and one hatch year). The proportion of virus-positive hatch-year birds (1 of 495) was not statistically different from adult birds (2 of 791; \( \chi^2 = 0.17, df = 1, p > 0.6 \)). Most (13 of 14) virus-positive birds were captured between weeks 30 and 32 (early August) in both years. There was a significant cross-correlation between weekly proportion of virus-positive birds and \(Culex\) infection rate in 2005 (Fig. 5A; \( r = 0.89, p < 0.05 \)) in which virus-positive birds lagged 1 week behind mosquito infection. In 2006, this cross-correlation was also significant, but with no time lag (Fig. 5E; \( r = 0.59, p < 0.05 \)).

The Illinois Department of Public Health reported a total of 252 human cases of WNV infection during the 2005 season, and 211 cases in 2006 (IDPH 2007). In 2005, 20 of these cases occurred within a 5-km radius of our study sites between weeks 30 and 39, eight of them occurring during weeks 31 and 32 (July 30–August 12; Fig. 2). There was a significant cross-correlation between human case date of onset and \(Culex\) infection rate with no time lag (\( r = 0.85, p < 0.05 \); Fig. 5) in 2005. Our larger study region in 2006 contained 42 human cases of WNV, with a peak of 11 occurring in week 33 (August 6–12). The number of human cases in 2006 was significantly correlated with mosquito infection rate and lagged it by 3 weeks (\( r = 0.84, p < 0.05 \)).

**DISCUSSION**

Our results suggest that increases in local abundances of hatch-year birds facilitate rapid WNV amplification. Local increases in the relative abundance of hatch-year birds started just before the onset of peak WNV infection in mosquitoes, birds, and humans. We found that the proportion of seropositive hatch-year birds was significantly and positively correlated with \(Culex\) infection 2 weeks earlier. Bird and human virus infections were also significantly correlated with \(Culex\) infection with a lag time of 1–3 weeks.

Seroprevalence rates observed in samples of hatch-year birds in 2005 (18.5%) during an intense epizootic were greater than those reported elsewhere (Nasci et al. 2002 [0%–1.2%], Ringia et al. 2004 [4.1%], Beveroth et al. 2006 [5.5%], Gibbs et al. 2006 [1.5%–3.6%]). These seropositive hatch-year birds were exposed to WNV during the 2005 transmission season, as the presence of maternal antibodies is unlikely (Ludwig et al. 1986). Seropositive hatch-year birds represent only those that were infected and survived. We did not observe dead birds while in the field. Recovery of dead small bird species is known to be low owing to intensive scavenging on their carcasses and the difficulty of observing them (Wobeser and Wobeser 1992, Ward et al. 2006). Experimental studies show that mortality rates for passerines infected with WNV are high (Komar et al. 2003). This would suggest that many passerine birds, including hatch-year birds, are being exposed to WNV, die, and go undetected at our study sites. Seropositive hatch-year birds had higher endpoint titers than did adults in 2005, probably due to recent exposure of hatch-year birds and a waning of neutralizing antibodies (i.e., seroreversion) in adult birds (Main et al. 1988, Komar 2001). Most of the species of hatch-year birds captured are known to be local breeders. All but 1 of 77 and all 30 hatch-year migrants were seronegative in 2005 and 2006, respectively, indicating that very few of these northern-breeding species are exposed to WNV on their breeding grounds or en route to their stopover in northern Illinois, where they were captured.

Seropositivity rates do not reflect a bird population’s force of infection (Komar 2001), but experimental evidence shows that hatch-year mourning doves and house finches infected with SLE produce a viremic titer high enough
to be infectious (Mahmood et al. 2004). We documented a significantly higher WNV infection in hatch year birds \((n = 11)\) compared with adult birds \((n = 3)\) with cycle threshold values ranging from 18.9–37.3, but our passive capture methods probably underestimate the proportion of virus-positive birds because many may die. Also, levels of activity and flight habits of infected birds may lower capture probability in mist nets. The importance of young-of-the-year birds in eastern equine encephalitis (EEE) transmission prompted Unnasch et al. (2006) to develop a dynamic transmission model in which vector feeding success and host preference prior to the peak in transmission were shown to be responsible for driving the subsequent peak in EEE viral activity. Our results suggest a similar effect of hatch-year birds in WNV amplification. If hatch-year birds function as an inflow of new susceptible hosts, the WNV reproduction rate could increase, resulting in an epidemic stage of transmission (Heesterbeek and Roberts 1995, Anderson and May 1991). However, as the number of susceptible hosts are exposed and removed, WNV fades out. This speculation could explain our observation that Culex abundance continued to be high through September in 2005, but the Culex infection rate declined prior to September.

The temporal patterns of mosquito and bird WNV infection and seroprevalence of hatch-year birds observed in this study provide insight into the mechanisms of seasonal dynamics of transmission. The peak of virus-positive birds followed closely the peak in Culex infection in both years. Although the magnitude of these factors changed between the 2 years, the temporal patterns were remarkably similar as was the timing of events. The cooler and wetter weather in 2006 likely influenced the observed lower mosquito and bird infection rates, lower bird seroprevalence, and fewer statewide human cases. Above average temperatures and below average precipitation have been correlated with increased WNV transmission in North Dakota, Florida, Connecticut, California, and Russia (Andreadis et al. 2004, Reisen et al. 2004, Bell et al. 2005, Shaman et al. 2005). Arbovirus transmission increases with higher temperatures due to the increased dissemination rates in mosquitoes, shorter gonotrophic cycle resulting in female Culex refeeding more often, and shorter extrinsic incubation period (Meyer et al. 1990). Drought conditions are favorable for WNV transmission due to increased contact between mosquitoes and amplifying bird hosts at rare water sources (Shaman et al. 2005) or reduced flushing of Culex mosquitoes in catchbasins during drought events (Andreadis et al. 2004). The latter is the more likely mechanism operating at our study sites, as fewer mosquito larvae were found in catchbasins following rainfall events (unpublished data).

Intensive simultaneous collection of bird, mosquito, human, and environmental data across an entire transmission season and across several urban site types allowed for unprecedented accuracy in the quantification of longitudinal infection in mosquitoes, birds, and humans. The intensive nature of data collection allowed us to investigate the hypothesis that increases in hatch-year bird populations are related to seasonal peaks in WNV transmission occurring in a historical foci with above average levels of transmission. Our data suggest that young-of-the-year birds are important amplifying hosts, increasing the susceptible host population. This primed host community triggered by hot and dry conditions leads to rapid amplification resulting in epizootics and human epidemics. Continued work in the study area will further clarify the interactions between annual variation in climatic conditions and local transmission dynamics of WNV.

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