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The Effects of West Nile Virus on the Reproductive Success and Overwinter Survival of Eastern Bluebirds in Alabama

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Abstract

We tested for negative effects of West Nile virus (WNV) on a breeding population of eastern bluebirds in Alabama by comparing fecundity and reproductive success in years before and after the arrival of WNV and by comparing fecundity, reproductive success, and overwinter survival of seropositive and seronegative individuals within the same population in the same years. We found that female bluebirds were more likely to be seropositive than male bluebirds. Age and individual condition did not affect likelihood of being seropositive. Being seropositive for WNV was not associated with any negative effects on reproduction or survival. However, female fecundity was higher in years after WNV compared to years before the arrival of WNV. The reproductive success of males who tested positive for WNV exposure was higher than that of males that were seronegative. Overall, we found no negative effects on reproduction or survival after exposure to WNV.

Key Words: Aedes—Arbovirus(es)—Birds—Culex—Mosquito(es)—Vector-borne.

Introduction

Since it was first detected in the New York City area in 1999 (Nash et al. 2001), West Nile virus (WNV) spread rapidly through bird populations throughout North America (Kilpatrick et al. 2007). Birds are the primary hosts of WNV (Bernard et al. 2001), but it also causes illness and death in humans and equines (Nash et al. 2001), making it a focus of research in an effort to minimize human impacts. Because WNV is primarily an avian pathogen, it is necessary to understand the effects of the virus on native birds in North America (Kilpatrick et al. 2007) if we are to understand the distribution, prevalence, and persistence of the virus.

WNV induce fatal illness in a number of bird species across a wide range of taxonomic groups, but seem to be especially pathogenic to American crows, *Corvus brachyrhynchos* (Nash et al. 2001, Nemeth et al. 2007); blue jays, *Cyanocitta cristata* (Komar et al. 2005); and yellow-billed magpies, *Pica nuttalli* (Crosbie et al. 2008), all in family Corvidae. Much of the information on the impacts of WNV on bird species comes from analyses of the carcasses of dead birds, which is biased toward detection of large species that are likely to die where they can be found by a human (Ward et al. 2006). Another means to assess the impact of WNV on populations of birds is to look at regional and range-wide population trends. Analyses based on winter bird censuses (Caffrey 2003, Hochachka et al. 2004) and feeder-watch data provided by amateurs (Bonter and Hochachka 2003) found limited evidence for regional declines, even in American crows, which carcass data showed to be heavily impacted. An analysis of breeding bird data, however, found clear patterns of decline for 7 out of 20 bird species that were studied, and the patterns of decline were consistent with a negative effect of WNV (LaDeau et al. 2007).

One of the seven bird species in the LaDeau et al. (2007) study that showed range-wide decline after the spread of WNV was the eastern bluebird (*Sialia sialis*), a small species of thrush (family Turdidae) that nests in cavities in field-edge habitat across much of eastern North America. WNV have been directly implicated in the death of wild eastern bluebirds. In a carcass study in Kentucky in 2002, 86% of the 21 dead eastern bluebirds tested positive for WNV (Roberts et al. 2003). In June 2002, an eastern bluebird died in an outdoor aviary on the campus of Auburn University in Alabama, and this bird tested positive for WNV (unpublished data).

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To date, no study has attempted to assess sublethal effects of WNV on a population of wild birds. We have monitored the breeding biology of eastern bluebirds in Auburn, Alabama, since 1999, 3 years before WNV became widely circulating in Alabama in 2002. Across four breeding seasons, 2005–2008, we tested birds in this population for antibodies to WNV. Here we report two comparisons testing for the effects of WNV on the reproductive output and survival of eastern bluebirds. First, we compared fecundity in the same population of eastern bluebirds before (1999–2001) and after (2005–2007) the arrival of WNV. Second, within years, we compared the fecundity, reproductive success, and survival of individual bluebirds that had antibodies for WNV (and hence had been exposed to the virus) versus individuals with no WNV antibodies and presumably no exposure. Our goal was to test the hypothesis that WNV cause sublethal negative effects on eastern bluebirds.

Materials and Methods

Field studies

We monitored a population of eastern bluebirds in Lee County, Alabama (32°35′ N, 82°28′ W), across 10 breeding seasons (1999–2008). We captured birds during the breeding season and marked them with unique combinations of three color bands and one numbered metal band. We estimated the age of all newly banded birds as either second year (in their first year of life) or older based on the shape of the 10th primary feather (Pitts 1985). Further, we knew the exact age of a subset of birds that were banded as nestlings on our field site. One hundred and fifty nest boxes were checked at least once every 3 days throughout the breeding season to document the date at which the first egg was laid. Bluebirds in Alabama raise up to three broods per season, and we estimated annual reproductive success as the total number of offspring fledged by each pair in the season.

Upon capture, we measured mass of the bird and the length of its tarsus, wing, tail, and bill. We used the residuals of a mass-to-tarsus regression as an index of body condition (Jakob et al. 1996). We estimated body size using a principal components analysis of mass, and the length of the tarsus, wing, tail, and bill. Principal components 1, 2, and 3 explained 73.5% of the variation in morphology (32.8%, 20.7%, and 20.0%, respectively). Principal component 1 was primarily influenced by wing and tail length (loading factors: tail = 0.90, wing = 0.89). Principal component 2 was primarily influenced by bill length and mass (loading factors: mass = 0.74, bill = 0.68), while principal component 3 was primarily influenced by tarsus length (loading factor = 0.91).

Comparison of birds breeding before and after WNV occurrence

Statewide screening of bird carcasses for WNV in Alabama began in early 2001, but the first WNV-positive bird was not found in Alabama until August 2001. We therefore consider the 2001 bluebird nesting season in Auburn, Alabama (March–July), to fall in a pre-WNV period. By 2002, WNV was being detected from numerous bird carcasses throughout the state. We used 1999, 2000, and 2001 as our pre-WNV years and 2005, 2006, and 2007 as our post-WNV years in comparisons of reproduction. We excluded data collected in 2002 to 2004 because various nest manipulations were conducted in those years, and the data on reproductive performance from those years are not comparable to reproductive data from other years.

Two circumstances potentially confound comparisons between populations of bluebirds before and after WNV was present. First, between the early years of our bluebird studies, which were the pre-WNV years, and later years, which are post-WNV years, we improved our ability to exclude snakes from nest boxes. Thus, the annual reproductive success of birds in later years was better than early years, at least in part because of reduced nest predation. Second, a few nests were part of a brood manipulation study in 2000 and 2001. Because of these confounding factors, we excluded all nests that were depredated and all manipulated nests. Moreover, we focused on average clutch size, brood size, and number of fledglings and excluded annual reproductive success (total number of fledging from all nests of the year) in our pre-WNV versus post-WNV comparisons. Our within-season comparisons avoided these problems, and we compared average clutch size, brood size, and number of fledglings as well as total reproductive success and survival among antibody-positive and antibody-negative individuals.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assays to detect antibodies in bluebird sera were performed essentially as previously described (Ebel et al. 2002). In brief, the wells of Immulon 1B 96 plates (Dynatek Laboratories, Winooski, VT) were coated with 50 μL of WNV antigen (a gift of Dr. Robert Tesh) diluted 1:100 in fresh coating buffer (0.015 M Na_2CO_3 and 0.035 M $NaHCO_3$, pH 9.6). The plate was incubated at 4°C overnight. The solution containing the antigen was discarded, and the plate was washed eight times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST). A total of 100 μL of blocking buffer (PBST plus 2.0% casein) was added to each well, and the plates were incubated at 37°C for 1 h. After incubation, the blocking was discarded, and the test serum samples, diluted 1:100 in PBST containing 0.5% bovine albumin (PBST-BA), were applied to sample wells. Plates were incubated at 37°C for 1 h and washed as above, and 100 μL of horseradish peroxidase–conjugated goat anti-bird immunoglobulin (Bethyl Laboratories, Montgomery, TX), diluted 1:1000 in PBST-BA, was applied to each well. After incubation for 30 min at 37°C and washing as above, plates were developed with the addition of 100 μL of tetramethylbenzidine-peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) for 10 min. The reactions were stopped with 50 μL of 0.3 M H_2SO_4, and the optical density of each well was read at 450 nm. A set of 10 negative controls were included on each plate, and the cutoff was set as the mean plus three standard deviations of the negative control wells. Any sample with an optical density value greater than the cutoff was deemed putatively positive and re-tested in triplicate. If all of the triplicate wells in the second test were above the cutoff, the sample was scored as positive for antibodies against WNV.

Comparison of birds testing positive or negative for WNV

We preformed statistical analyses using SPSS 15.0. Data conformed to normality (Shapiro-Wilk tests). Over three

breeding seasons before WNV reached in the population (1999–2001), we monitored the reproductive success of 250 pairs of eastern bluebirds. During three breeding seasons in which WN virus was prevalent in the population (2005–2007), we tested 254 female and 225 male eastern bluebirds for antibodies to WNV. In 2008, we captured birds to determine whether individuals from 2007 had returned. Because some birds were captured in multiple years, we randomly chose 1 year (1999–2001 data: 186 pairs; 2005–2008 data: 226 females and 194 males); no birds were included in both pre-WNV and post-WNV datasets. Further, we did not know the age of all adults. We found significant variation due to year of capture date and reproductive parameters (analysis of variance: all $F > 4.0$, all $p < 0.02$); therefore, for the analyses that compare birds that tested positive with birds that tested negative for WNV, we standardized the data for each year to a mean of zero and a standard deviation of one. Because the likelihood of testing positive for WNV varied with sex (Fisher's exact test), for all future analyses, we analyzed males and females separately.

Additionally, because birds are more likely to become infected with WNV later in the season, we analyzed the data in two ways. First, we included data from all birds captured during the field season, and second, we included only birds that had been captured and tested after June 1. Using chi-squared tests, we tested whether males or females are more likely to test positive for WNV, and whether young (second year) or older (after second year) individuals are more likely to test positive for WNV. Also using a chi-squared test, we asked whether birds that tested positive in year one were less likely to return to breed at the field site in the following year. Using a backward stepwise logistic regression, we asked whether body condition or body size was related to WNV status. Using another backward stepwise logistic regression, we then asked whether first egg date or total reproductive success was related to WNV status. Because we found a relationship between total reproductive success and WNV status, we further explored the dataset to determine whetherthe variation in reproductive success was influenced by clutch size, brood size, or fledging success (Student's *t*-tests).

Results

Comparison of birds breeding before and after WNV occurrence

We compared the average clutch size, brood size, and number of fledglings of birds breeding before (1999–2001) and after (2005–2007) WNV occurred in the population. Population sizes did not differ before and after WNV were circulating in the population: in all years approximately 70% of the nest boxes had breeding pairs (Fisher's exact, $p = 0.81$, $n = 6$). Females laid larger clutches in years' when WNV was present than in pre-WNV years; however, the number of nestling and fledglings did not differ (Table 1). To further explore these trends, we ran a one-way analysis of variance with year as the fixed factor and clutch size as the dependent variable. Clutch size varied with year ($F_{5,\ 454} = 3.15$, $p < 0.01$). Tukey's *post hoc* tests revealed that in years 2000 and 2001 females laid significantly fewer eggs than in years 2006 and 2007 (all $p < 0.05$). All other year-by-year comparisons were not significant ($p > 0.10$).

Table 1. Reproductive Parameters [Mean ± Standard Deviation (*n*)] of Female Eastern Bluebirds Before (1999–2001) and After a West Nile Virus Entered the Bluebird Population (2005–2007) Using a Two-Tailed Student's *t*-Test

Trait	*Pre-WNV*	*Post-WNV*	t	p
Clutch size	4.36 + 0.75 (186)	4.58 + 0.68 (274)	3.21	0.001
Brood size	2.67 + 1.77 (186)	2.89 + 1.76 (274)	1.31	0.19
Offspring fledged	2.52 + 1.72 (186)	2.38 + 1.86 (274)	0.81	0.42

Data are not standardized by year.
WNT, West Nile virus.

Comparison of birds testing positive or negative for WNV

In the 2005–2007 breeding seasons, females were more likely than males to become infected with WNV (Fisher's exact, $p = 0.015$). Ninety-four of 226 or 41.6%, of females were infected, while only 58 of 194, or 29.9%, of males tested positive for the virus. Because males and females were not equally likely to contract the virus, we analyzed all other data separately by sex.

Among second year (1 year old) and older birds, age was not related to the likelihood of testing positive for WNV for females or males (16.0% second-year females vs. 26.3% of after-second-year-females positive: Fisher's exact, $p = 1.0$, $n = 66$, 109; 9.9% second-year males vs. 18.6% of after-second-year-males positive: Fisher's exact, $p = 0.71$, $n = 51$, 110). Testing positive for WNV in year 1 did not influence the likelihood that females or males would return to breed at the field site in the following year: 41.8% of positive females returned, while 58.2% of negative females returned (Fisher's exact, $p = 1.0$, $n = 225$); 34.1% of positive males returned, while 65.9% of negative males returned (Fisher's exact, $p = 0.27$, $n = 191$). When we repeated these tests using only individuals captured after June 1, the comparisons remained nonsignificant.

We used a backward stepwise logistic regression to determine whether body size (PC1, PC2, and PC3) or body condition was related to WNV. When we included all birds captured throughout the breeding season, the female body condition predicted WNV status (female full model: $R^2 = 0.02$, $X^1_{203} = 4.46$, $p = 0.03$). Females that were relatively lighter in mass were more likely to test positive for WNV. Overall, the model correctly classified 60% of the females as either positive or negative for WNV according to body condition (Wald = 4.25, $p = 0.039$). The male model, however, was not significant (male full model: $R^2 = 0.016$, $X^4_{174} = 2.81$, $p = 0.59$), indicating that none of the variables predicted WNV response. When we re-ran the above models with the smaller data set (only individuals captured after June 1), neither the female nor the male model was significant (female full model: $R^2 = 0.018$, $X^4_{54} = 1.00$, $p = 0.91$; male full model: $R^2 = 0.028$, $X^2_{48} = 1.36$, $p = 0.85$). Thus, the initial relationship between female body condition and WNV status was likely caused by a decrease in female body condition with season and a concurrent increase in likelihood of females testing positive for WNV later in the season.

Next, we used another backward logistic regression to determine whether measures of reproductive success (first

egg date and annual reproductive success) were related to WNV status. When we included all females, our model was not significant (full model: $R^2 = 0.015$, $X^2_{210} = 3.22$, $p = 0.20$) and the model remained nonsignificant when we included only females captured after June 1 (full model: $R^2 = 0.004$, $X^2_{67} = 0.27$, $p = 0.88$). In the male model that included all males captured, the model was not significant (full model: $R^2 = 0.007$, $X^2_{173} = 1.14$, $p = 0.57$). However, when we included only males that were capture after June 1, reproductive success was significantly related to WNV status (full model: $R^2 = 0.13$, $X^2_{58} = 8.06$, $p = 0.018$). Overall, the model correctly classified 67% of the males as either positive or negative for WNV according to annual reproductive success. Although both first egg date and annual reproductive success contributed to the model, annual reproductive success (Wald = 6.49, $p = 0.01$; Fig. 1) was more important than first egg date (Wald = 2.72, $p = 0.10$).

To explore which reproductive parameters contributed to positive males attaining greater reproductive success, we used Student's *t*-tests to compare the clutch size, brood size, and number fledged of males that tested positive versus negative for WNV. Although males that were positive and those that were negative for WNV did not differ in clutch size and brood size, males that were positive reared nests with greater fledging success (Table 2), suggesting that a smaller number of nestlings died before independence.

Table 2. Comparison of the Reproductive Parameters [Mean ± Standard Deviation (*n*)] of Male Eastern Bluebirds in Alabama Sampled in 2005–2007 That Tested Positive or Negative for West Nile Virus Antibodies Using a Two-Tailed Student's *t*-Test

Trait	*Positive*	*Negative*	t	p
Clutch size (*z*)	−0.02 ± 0.88 (21)	0.41 ± 1.02 (36)	1.29	0.20
Brood size (*z*)	0.56 ± 0.62 (21)	0.21 ± 0.83 (36)	1.61	0.11
Offspring fledged (*z*)	0.81 ± 0.56 (21)	0.25 ± 0.86 (36)	2.99	0.004

Data are standardized by year and measured as *z* scores.

Discussion

The eastern bluebird is one of seven North American bird species that showed range-wide population declines that coincided with the spread of WNV (LaDeau et al. 2007). The eastern bluebird is also a species for which carcasses tested positive for WNV (Roberts et al. 2003). These observations indicate that exposure to WNV can cause death of eastern bluebirds. Many eastern bluebirds nest in periurban environments where incidence of WNV can be high in local mosquito populations (Andreadis et al. 2004) and in which many individuals are likely to be exposed. There is a potential for WNV to not just kill some exposed individuals but to also impair individuals who contract the virus but survive (Kilpatrick et al. 2007). We looked for sublethal effects of the virus on adult bluebirds in an Alabama population.

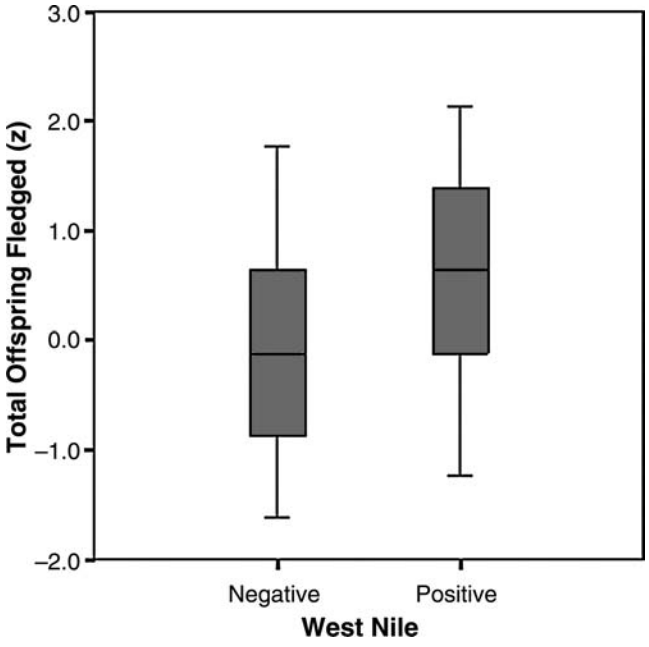

FIG. 1. Annual reproductive success of male eastern bluebirds in Alabama sampled in 2005–2007 that tested positive or negative for West Nile virus antibodies (data are standardized by year [*z*]). The line within each box represents the median reproductive success, the upper and lower borders of each box are the 25th and 75th percentiles, and the lower and upper bars are the 10th and 90th percentiles.

We found that female eastern bluebirds were significantly more likely to carry antibodies for WNV compared to males. Despite the apparent susceptibility of female bluebirds to WNV, however, we detected few effects of the virus on the fitness of females. We found no difference in fecundity, reproductive success, or overwinter survival between antibody-positive and -negative female eastern bluebirds sampled in the same years. In these analyses our sample sizes were large, and we had a high probability of detecting even a modest effect of the virus. We did find, however, that in the period after the virus had spread to Alabama, the mean clutch size of female bluebirds, which we use as a measure of fecundity (Badyaev et al. 2000), increased compared to the period before WNV were present. This effect is in the direction opposite to our prediction of the effect of the virus on reproduction—we expected reduced fecundity to coincide with the exposure of the population to WNV. It is possible that exposure of the population to the virus could have caused females to shift to greater investment in reproduction (Gustafsson et al. 1994). However, we feel that this explanation is unlikely as we found no differences in the clutch sizes of antibody-positive and -negative female eastern bluebirds sampled in the postexposure years. A more likely explanation is that no causal link exists between exposure to WNV and increased clutch size, but that some unmeasured environmental factor such as food availability could have created the difference in clutch size between years. However, as 2000, 2001, 2006, and 2007 were drought years in Alabama (www.noaa.gov), lower precipitation is not likely the cause of smaller clutch sizes in pre-WNV years.

We also found few effects of WNV on male bluebirds; again, the effects that we did observe are somewhat puzzling. We found no differences in overwinter survival between males that were antibody positive or negative. Surprisingly, however, we found higher reproductive output among virus-positive males compared to virus-negative males. This effect

is in the direction opposite to our prediction of the effect of the virus on reproduction—we expected exposure to WNV to be associated with reduced reproductive output. We suggest two explanations for this observation. First, males that have been exposed to the virus may shift to greater investment in reproduction (Gustafsson et al. 1994). There was no effect of male exposure to WNV on clutch size, so the difference in reproductive success resulted from a higher proportion of eggs that fledged young. Greater male investment in offspring could have caused this result. Alternatively, there may be no causal link between exposure to WNV and reproductive success, but some unmeasured factor such as proximity to water or soil moisture may have affected both exposure to virus and food resources, thus creating an apparent effect of virus on reproductive success.

It is unknown how long birds maintain measureable antibody titers after exposure to WNV (Kilpatrick et al. 2007). In this sense our within-season comparisons were conservative. All of the birds that were seropositive for WNV infection had been exposed to the virus at some previous time, but some birds that were seronegative could have also been exposed to and cleared the virus. Other birds could have contracted the virus after we sampled them. Given our large sample sizes, however, we should have detected a modest or large effect of WNV on reproduction or survival even if our comparison was between WNV-exposed birds and random birds (both exposed and unexposed) in the population. Failure to find such an effect suggests that there are no significant negative long-term effects of the virus on reproductive output or overwinter survival. If an eastern bluebird is not killed by exposure to WNV, it appears to make a full recovery. The observation that there was no drop in fecundity in the population after appearance of the WNV further supports this conclusion. The lack of sublethal effects of WNV on Eastern Bluebirds suggests that the observed population decline (LaDeau et al. 2007) is likely due to immediate lethal effects.

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Disclosure Statement

No competing financial interests exist of any authors.

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