EcoHealth (© 2006)
DOI: 10.1007/s10393-006-0025-9

ECOHEALTH

Original Contributions

Migrating Birds as Dispersal Vehicles for West Nile Virus

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Abstract: Whereas migrating birds have been implicated in the spread of West Nile virus (WNV), there is no direct evidence of birds actively migrating while infectious. The role of birds in WNV dispersal is difficult to assess in the field. However, this role can be evaluated experimentally because birds in migratory disposition display increased locomotor activity or restlessness under captive conditions. We tested the following hypotheses: (1) migrating passerine birds continue to exhibit migratory activity while infectious with WNV and (2) the migratory state of the individual affects the magnitude of viremia. We examined the migratory activity of two neoarctic-neotropical passerine migrants, Swainson's thrush (*Catharus ustulatus*) and gray catbird (*Dumetella carolinensis*), during acute WNV infection. All gray catbirds and six of nine Swainson's thrushes exhibited migratory activity while infectious. Moreover, migratory status did not appear to influence viremia titers, as might be expected if individuals were immunosuppressed during migration. Therefore, we demonstrate that migrating passerine birds are potential dispersal vehicles for WNV.

Key words: West Nile virus, dispersal, migratory bird, Swainson's thrush, gray catbird, migratory disposition

INTRODUCTION

West Nile virus (WNV) is one of the most widespread mosquito-borne flaviviruses (Flaviviridae), recently having spread to South America, its sixth continent (Mattar et al., 2005). Its ability to spread quickly has been demonstrated in North America. Since 1999, the virus has spread from New York City throughout the continent, apparently dispersing in part along bird migration routes (Komar, 2003; Peterson et al., 2003). While migrating birds are thought to transport WNV, evidence for this role remains circumstantial (Rappole et al., 2000; Malkinson et al., 2002; Rappole and Hubálek, 2003). Certain birds are the primary vertebrate amplifying hosts of WNV (Komar et al., 2003). Once infected, these birds develop viremias of sufficient duration and magnitude to infect a biting mosquito, which then serves as a vector for transmitting the virus to other avian amplifying hosts or to incidental hosts such as other birds, horses, or humans. Experimental infection studies show birds, particularly passerines, may be infectious for a period of 1–6 days (Komar et al., 2003). While WNV has been isolated from birds during migration (Malkinson et al., 2002), there is no direct evidence of birds actively migrating while infectious. Isolating WNV from a migratory bird during migration does not necessarily establish birds as WNV dispersal vehicles. Such a bird may have become infected locally at the site of its capture and the infection may prevent it from migrating on subsequent days.

Whether a bird will migrate while infectious is virtually impossible to test in the field with free-ranging birds because we are precluded from releasing birds infected with a

Published online:

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human pathogen. However, this question can be addressed experimentally because the normal progression of migratory disposition is endogenously programmed (Gwinner, 1986; Berthold, 2001) and can be induced by artificially prolonged day length. Birds in migratory disposition dramatically increase their rate of food intake (*hyperphagia*), which results in hyperlipogenesis (Blem, 1980), and show increased locomotor activity or *zugunruhe* (Berthold, 1975) under captive conditions during the migratory season. This migratory restlessness in caged migrants corresponds to the daily and circannual patterns of migratory activity in free-ranging migrants (e.g., Berthold, 2001).

A migrating bird must satisfy at least two criteria if it is to contribute to the spread of WNV. One, the bird must be competent to infect mosquitoes (Komar, 2003). Second, it must migrate through at least part of the infectious period. In this study, we test the hypothesis that migrating passerine birds continue to exhibit migratory activity while infectious with WNV. Accordingly, we examined migratory activity during infection of two abundant migratory passerine species, Swainson's thrush (*Catharus ustulatus*) and gray catbird (*Dumetella carolinensis*). Both species are naturally exposed to WNV infection and survive to develop antibodies (Bernard et al., 2001; Ringia et al., 2004; Owen, unpublished data). Furthermore, Swainson's thrushes are phylogenetically related to the American robin (*Turdus migratorius*), a species which exhibits high viremia titers (Komar et al., 2003); and robins and gray catbirds are the preferred host of several species of *Culex* mosquitoes known to transmit WNV (Apperson et al., 2004; Kilpatrick et al., 2006; Molaei et al., 2006). Finally, the breeding ranges of Swainson's thrushes and catbirds span, longitudinally, the breadth of North America (Cimprich and Moore, 1995; Evans and Yong, 2000), which makes these species potentially important dispersal vehicles. In addition, we tested whether the migratory state of the individual affects the magnitude of viremia. There is evidence that birds are immunosuppressed during migration (Muñoz and Fuente, 2003; Owen, 2004), and an immunocompromised bird may experience higher virus titers.

Methods

Source of Birds

We captured hatching-year Swainson's thrushes, gray catbirds, and wood thrushes (*Hylocichla mustelina*; 2002 only) at the Fort Morgan Peninsula in Alabama (30°13′N, 88°10′W) during autumn of 2002 and 2003 (federal permit 21221, Institutional Animal Care and Use Committe (IACUC) protocol 21-022). Birds were captured using mist nets (12 × 2.6 m with 30 mm mesh). Birds were immediately transported to the University of Southern Mississippi Animal Research Facility, where they were housed individually and kept near room temperature (21–24°C). Birds were fed ad libitum a semisynthetic diet consisting of meal worms, blackberries, blueberries, wheat and malted barley cereal, moistened monkey biscuits, cottage cheese, freeze-dried crickets, and a vitamin supplement. Body condition was assessed by periodically weighing birds (nearest 0.01g) and quantifying visible subcutaneous fat stores (Helms and Drury, 1960). The birds had sufficient fat stores throughout the experiment. A blood sample (0.2 ml) was collected from each bird, and plasma was tested for WNV-neutralizing antibodies using the plaque-reduction neutralization test (Beaty et al., 1995). Each species is sexually monomorphic, and sex was not determined for this study.

Measurement of Bleeding Effect

To determine whether the act of bleeding a bird would negatively impact migratory activity, we conducted a pilot study with Swainson's thrushes (control, $n = 6$; treatment, $n = 7$), catbirds (control, $n = 2$; treatment, $n = 5$), and wood thrushes (control, $n = 8$; treatment, $n = 8$) captured in 2002. In the pilot study, both groups were migratory (see Measurement of Migratory Activity, below) but the control group did not receive the virus. Birds from both groups were bled daily between 0900 and 1100 hours for 6 days following subcutaneous inoculation (see Infection of Birds, below) of the treatment group with 1,000 pfu (Reisen et al., 2000) of WNV strain NY99-4132.

In 2003, we captured Swainson's thrushes ($n = 18$) and catbirds ($n = 17$) to conduct the principal experiment. One catbird tested positive for neutralizing antibody and was subsequently released. The remaining birds were randomly assigned to control (nonmigratory) and treatment (migratory) groups, which were housed in separate rooms.

Measurement of Migratory Activity

Each cage was equipped with an infrared motion detector, which records activity via a data logger (JoAC Electronik, Lund, Sweden). Birds were maintained on a nonmigratory 12:12 light:dark (L:D) photoperiod from time of capture until 11 March 2004, when the photoperiod of treatment birds (Swainson's thrush, $n = 9$; catbird, $n = 8$) was

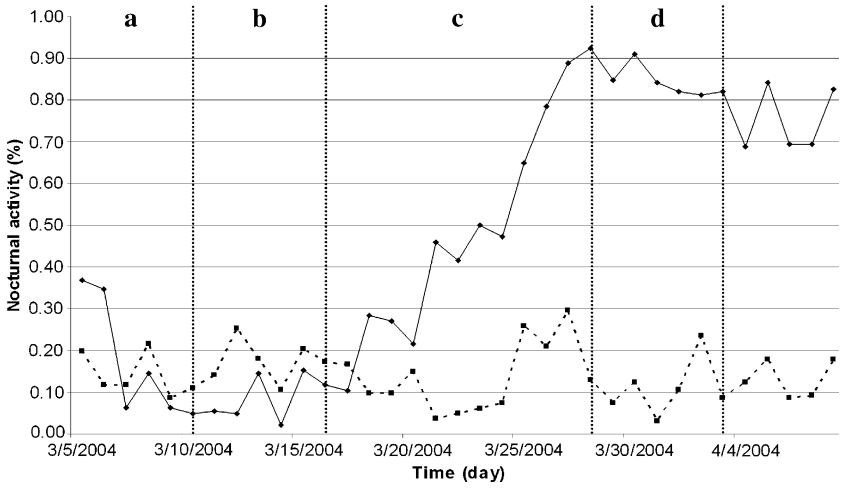

Figure 1. Percent (mean) of the nighttime that gray catbirds exhibited migratory activity. Solid line, experimental birds (n = 8); dotted line, control birds (n = 9). Time periods: **a**, when both groups were at a 12:12 L:D photoperiod; **b**, experimental birds were photoadvanced 1 hour each day to induce migratory disposition; **c**, experimental birds were at a 16:8 L:D and control birds were at a 12:12 L:D photoperiod; **d**, experimental period.

increased to a 16:8 L:D schedule over an 8-day period, after which lights were off between 2200 and 0600 hours. The artificially prolonged day lengths induced migratory disposition, which is characterized by an increase in nocturnal activity. Based on previous studies (Yong and Moore, 1993), we considered birds to be migrating when they displayed *zugunruhe* >40% of the night. Control birds (Swainson's thrush, n = 9; catbird, n = 8) were maintained on a 12:12 L:D schedule throughout the experiment.

Infection of Birds

To determine whether migratory restlessness of an already migrating individual would change due to WNV infection, we designed an A-B-A experiment. On 30 March 2004, after experimental birds had exhibited an increase in *zugunruhe* (Fig. 1), we injected both control and treatment birds subcutaneously with 10,000 pfu of WNV strain NY99-4132 (isolated from brain of American crow [*Corvus brachyrhynchos*] and passed once in Vero cells). The higher titer used here was to ensure we would have infectious birds for the experiment yet still reflect what a mosquito could transmit in the wild (Reisen et al., 2000). Blood (0.05 ml) was drawn via the brachial vein using a 26-gauge needle and heparinized microhematocrit capillary tubes to monitor viremia for the 6 days postinoculation (dpi). Whole blood was placed in cyrovials and immediately on dry ice, then stored at −70°C. Blood was diluted with BA-1 (composed of Hank's M-199 salts, 1% bovine serum albumin, 350 mg/l sodium bicarbonate, 100 units/ml penicillin, 100 mg/l streptomycin, 1 mg/l Fungizone in 0.05 M Tris, pH 7.6) for determination of viremia titers. Titers were determined in duplicate using Vero cell plaque assay in six-well plates with a double 0.5% agarose overlay (Beaty et al., 1995). At 14 dpi, we collected a blood sample (0.4 ml) and killed each bird by CO_2 asphyxiation.

Data Analysis

Activity Data

We monitored nighttime activity every day of the experimental period. We quantified amount of migratory activity by counting the number of 20-minute blocks in which each bird displayed at least five hops between the hours of 2300 and 0500 (18 20-minute blocks) for both groups. Birds showed elevated viremia for approximately 4 days. Therefore, we divided the activity data into three periods, each consisting of 4 days, separated by 2-day intervals: previremia, days 2–5 prior to inoculation; viremia, days 1–4 postinoculation; and postviremia, days 7–10 postinoculation. Our analyses used the mean number of active 20-minute blocks per night for each period.

Viremia Titer

Viremia titers at 3 dpi could not be determined for 11 of the 35 individuals (2–4 individuals/group) due to excessive thawing of the specimens collected from that day. In the analysis, we replaced these missing values by taking the mean of the virus titer at 2 and 4 dpi for that particular individual. In addition, due to a data entry error, we were unable to discern virus titer data between one Swainson's thrush and one catbird in the control group and, thus, excluded these two individuals from virus titer analyses. However, both birds exhibited elevated virus titers, so we

Table 1. Daily Mean (± Standard Error) WNV Viremia Titers (log pfu/ml Serum) for 16 Gray Catbirds (GRCAs) and 17 Swainson's Thrushes (SWTHs) Divided into Migratory ("Migrant") and Nonmigratory ("Control") Groups: Minimum and Maximum Viremia Titers Are Reported for All Birds with Detectable Levels on the Given Day

	Day postinoculation					
Species and group	1	2	3	4	5	6
GRCA control	2.2 ± 0.21	5.3 ± 0.36	4.1 ± 0.57	3.6 ± 0.84	2.2	–
min, max (*n*)	1.7, 2.7 (5)	3.2, 6.5 (8)	2.6, 5.4 (7)	1.7, 5.2 (4)	(1)	
GRCA migrant	2.5 ± 0.20	5.0 ± 0.39	2.6 ± 0.27	2.2 ± 0.06	1.7	–
min, max (*n*)	1.7, 3.3 (6)	3.6, 6.3 (8)	1.7, 3.3 (6)	1.7, 2.3 (2)	(1)	
SWTH control	3.1 ± 0.21	5.4 ± 0.38	2.3 ± 0.27	2.6 ± 0.71	2.2	1.7
min, max (*n*)	2.0, 3.6 (8)	3.7, 6.8 (8)	1.7, 3.0 (5)	1.7, 4.0 (3)	(1)	(1)
SWTH migrant	3.8 ± 0.28	5.7 ± 0.26	2.4 ± 0.17	2.1 ± 0.13	–	–
min, max (*n*)	2.3, 5.2 (8)	4.0, 6.6 (9)	1.7, 3.0 (9)	1.7, 2.9 (9)		

included them in the analysis of changes in migratory activity during the experimental period. All virus titers were log-transformed for the analyses.

All analyses were conducted using repeated-measures analysis of variance (ANOVA). When the sphericity assumption was violated, the Greenhouse-Geisser test was used. Paired *t*-tests were used for all post hoc comparisons. An alpha level of 0.05 was set for all analyses, and in each case, the derived *P* value refers to two-tailed tests. Analyses were performed with SPSS 12.0 (SPSS, Inc., Chicago, IL).

Results

Bleeding Effect

In the pilot study, bleeding birds did not negatively affect the intensity of their nighttime activity. We found no significant main effect of time on activity for the control and treatment groups combined (Swainson's thrush, $F_{2,22} = 0.51$, $P = 0.86$; catbird, $F_{2,10} = 0.12$, $P = 0.76$) and no main effect of group (Swainson's thrush, $F_{1,11} = 0.15$, $P = 0.70$; catbird, $F_{1,5} = 1.52$, $P = 0.27$). In addition, the analysis detected no interaction between group and time period (Swainson's thrush, $F_{2,22} = 2.93$, $P = 0.07$; catbird, $F_{2,10} = 0.50$, $P = 0.52$).

Viremia Titers

All birds ($n = 55$) in 2002 and 2003 survived inoculation with a North American strain of WNV. In 2002 ($n = 20$), two Swainson's thrushes and one wood thrush did not exhibit detectable levels of viremia (≥1.7 log pfu/ml serum), whereas in 2003 all birds ($n = 35$) became viremic. Peak viremia titers occurred at 2 dpi except for one bird that peaked at 3 dpi (Table 1, Fig. 2).

In 2003, daily virus titers did not differ between migrating and nonmigrating groups (Table 1; catbird, $F_{1,14} = 3.01$, $P = 0.105$; Swainson's thrush, $F_{1,15} = 1.60$, $P = 0.226$). Analyzing the same data while excluding day 3, we still found no difference (catbird, $F_{1,14} = 0.921$, $P = 0.354$; Swainson's thrush, $F_{1,15} = 2.48$, $P = 0.136$). Subsequent univariate *t*-tests, still excluding third day of infection, revealed no significant differences in viremias between the control and treatment groups. However, we may have been able to detect a difference given larger sample sizes.

We examined dose response by comparing the 2002 birds inoculated with 1,000 pfu (low dose) with the 2003 birds inoculated with 10,000 pfu (high dose, Table 2). The birds in 2003 were pooled because migrating and nonmigrating birds' viremia levels did not significantly differ from each other. In 2002, we only performed statistical analyses using viremia titers on 2 and 3 dpi because of small samples. A significant dose response was observed in catbirds (repeated-measures ANOVA, $F_{1,19} = 5.13$, $P = 0.04$) but not in Swainson's thrush ($F_{1,17} = 0.48$, $P = 0.50$). Post hoc analyses in catbirds revealed a significant difference in dose response at 2 dpi (*t*-test, $t = 2.86$, $df = 19$, $P = 0.01$) but not at 3 dpi ($t = 1.17$, $df = 19$, $P = 0.26$). Peak viremias were higher in individuals of both species inoculated with the 10,000 pfu compared with conspecifics inoculated with 1,000 pfu (Table 2). Furthermore, the data suggest that the duration of viremia was longer in the high-dose compared to the low-dose group (Table 2).

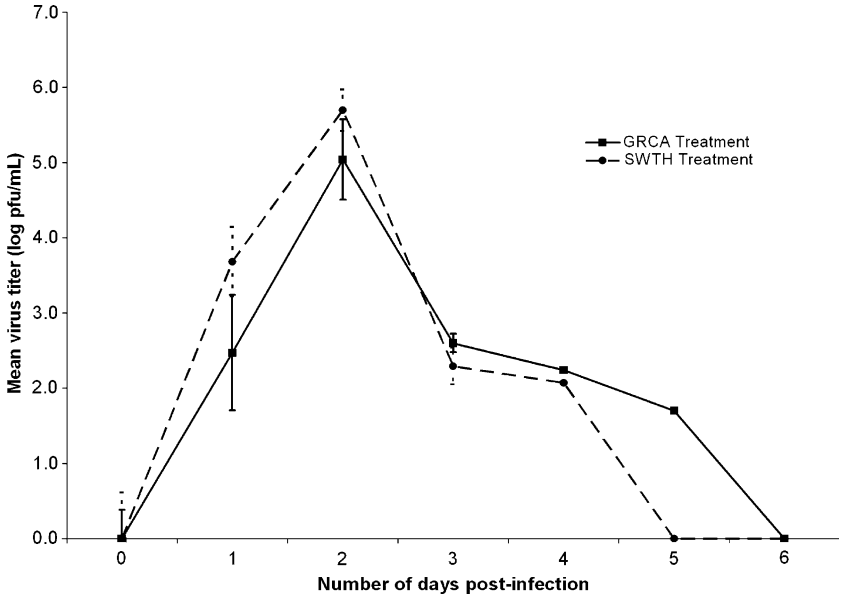

Figure 2. Mean daily viremia titers for treatment birds. Solid line, gray catbird (GRCA); dashed line, Swainson's thrush (SWTH). Limit of detection = 1.7 log pfu/ml serum. Error bars represent 95% confidence intervals.

Table 2. Daily Mean (± Standard Error) Viremia Titers (log pfu/ml Serum) for Birds Inoculated with a Low Dose (2002; 1,000 pfu) and a High Dose (2003;10,000 pfu) of WNV: Minimum and Maximum Viremia Titers Are Reported for All Birds with Detectable Levels on the Given Day

	Day postinoculation					
Species and group (*n*)	1	2	3	4	5	6
GRCA low dose (5)	1.7	3.8 ± 0.28	2.6 ± 0.34	2.0 ± 0.30	–	–
min, max (*n*)	(1)	2.7, 4.4, (5)	2.6, 5.4, (5)	1.7, 2.3, (2)		
GRCA high dose (16)	2.3 ± 0.14	5.2 ± 0.26	3.4 ± 0.40	3.1 ± 0.60	1.9 ± 0.24	–
min, max (*n*)	1.7, 3.3, (11)	3.2, 6.5, (16)	1.7, 5.4, (11)	1.7, 5.2, (6)	1.7, 2.2, (2)	
SWTH low dose (7)	3.5 ± 0.47	4.3 ± 0.26	3.2 ± 0.30	–	–	–
min, max (*n*)	2.3, 4.5, (5)	3.8, 5.3, (5)	2.9, 3.5, (2)			
SWTH high dose (17)	3.4 ± 0.21	5.5 ± 0.22	2.4 ± 0.13	2.2 ± 0.19	2.2	1.7
min, max (*n*)	2.0, 5.2, (16)	3.7, 6.8, (17)	1.7, 3.0, (11)	1.7, 4.0, (12)	(1)	(1)
WOTH low dose (7)[a]	3.1 ± 0.22	4.0 ± 0.20	2.7 ± 0.36	1.7	–	–
min, max (*n*)	2.2, 4.0, (7)	3.5, 5.0, (7)	1.7, 4.3, (6)	(1)		

GRCA, gray catbird; SWTH, Swainson's thrush; WOTH, wood thrush.

Migratory Restlessness

In 2004, all birds in the treatment group exhibited increased nocturnal restlessness in response to an increase in photoperiod (Fig. 1). The species differed in their response to the viral infection. Catbird nighttime activity did not differ between the three periods for the treatment group (preinoculation, 12.9 ± 1.9; viremia, 15.2 ± 1.1; postviremia, 13.8 ± 1.4; repeated-measures ANOVA, $F_{2,14} = 1.20$, $P = 0.33$) and control group (preinoculation, 4.2 ± 2.1; viremia, 2.5 ± 0.7; postviremia, 2.7 ± 1.1; repeated-measures ANOVA, $F_{2,14} = 0.81$, $P = 0.47$). Whereas Swainson's thrush nighttime activity did differ significantly between the different periods (preinoculation, 15.8 ± 0.76; viremia, 9.2 ± 2.10; postviremia, 12.7 ± 1.8; repeated-measures ANOVA, $F_{2,16} = 7.97$, $P = 0.004$). Post hoc comparisons revealed that Swainson's thrush nighttime activity was significantly lower during the viremic period compared to the preinoculation and postviremia periods (paired *t*-test, preinoculation vs. viremia, $t = 3.41$, $df = 8$, $P = 0.01$;

viremia vs. postviremia, $t = -3.05$, $df = 8$, $P = 0.02$). Swainson's thrush nighttime activity did differ across periods in the control group (preinoculation, 4.2 ± 2.1; viremia, 2.5 ± 0.7; postviremia, 2.7 ± 1.1, repeated-measures ANOVA, $F_{2,16} = 5.30$, $P = 0.02$). Thrushes were more active in the preinoculation period compared with the viremia (paired t-test, $t = 3.80$, $df = 8$, $P = 0.005$) and postviremia ($t = 2.62$, $df = 8$, $P = 0.03$) periods. However, activity did not differ between viremia and postviremia periods ($t = -0.26$, $df = 8$, $P = 0.98$).

Further inspection of Swainson's thrush nighttime activity in the treatment group showed that four of the nine individuals dropped below the migratory threshold during the viremic period. If we exclude those four individuals, we find no significant difference in nocturnal activity across the three periods (preinoculation 16.27 ± 1.10, viremia, 13.70 ± 3.78, postviremia, 16.20 ± 1.96; repeated-measures ANOVA, $F_{2,8} = 1.97$, $P = 0.20$). The daily viremia titers did not differ between the four nonactive individuals and the five active thrushes ($F_{1,5} = 1.84$, $P = 0.23$).

Discussion

Gray catbirds and Swainson's thrushes experimentally infected with WNV displayed migratory activity before, during, and after the period of infectious viremia. The pattern of that activity was consistent with that observed in other studies in which migratory activity was induced by manipulation of photoperiod and when conspecifics were captured during the migratory period and their migratory activity recorded (Yong and Moore, 1993; Owen, 2004). Migratory activity did differ between species during the infectious period. The intensity of migratory activity did not change while gray catbirds were infectious with WNV. However, while five of the nine infected Swainson's thrushes maintained their migratory activity, three individuals reduced their activity to nonmigratory levels while infectious with WNV (and a fourth reduced its activity shortly thereafter). We conclude that both Swainson's thrushes and gray catbirds are potential dispersal vehicles for WNV.

We did not detect a significant immunosuppressive effect of migration on the outcome of infection in the catbirds and thrushes. There is evidence that caged birds will suppress immune function while exhibiting migratory activity compared with nonmigratory individuals (Owen, 2004). However, in this study, the migratory status of individual birds did not affect viremia titers. If avian immunity is suppressed during migration, it did not manifest itself in higher viremias compared with nonmigrating individuals. Overall, the viremia titers observed in gray catbird and Swainson's thrush were lower, particularly in individuals inoculated with 1,000 pfu of WNV, than titers observed in other species of migratory passerine (Komar, 2003). Other migratory species investigated are shorter-distance migrants and were infected by mosquito bite rather than needle inoculation. However, viremia titers of birds infected with 10,000 pfu WNV did surpass 10^5/ml serum, which is sufficient to infect *Culex* mosquitoes throughout North America (Turell et al., 2001; Goddard et al., 2002).

Not all migratory birds infected during migration will necessarily spread WNV. First, the infected bird must fly to a new potential WNV transmission focus, where sufficient competent vectors (Turell et al., 2001, 2005; Apperson et al., 2002; Goddard et al., 2002) and amplifying hosts are present to support local WNV amplification. The successful introduction of WNV into a new amplification focus by a migrating bird is a stochastic process, for which probabilities are currently unknown. For instance, not only does the mosquito species need to be a competent vector, it must also bite an infectious bird with a high enough frequency to receive an infected blood meal and then bite another avian (or mammalian) host. If insufficient vectors were host-seeking at the moment of arrival of an infectious migrating bird, an alternative means of introducing WNV into the transmission focus could be a predator ingesting an infected bird and developing viremia that could potentially infect vector mosquitoes (Komar et al., 2003; Austgen et al., 2004; Klenk et al., 2004).

To understand how a migrating bird may spread WNV, consider a northward migrating Swainson's thrush in late April that receives an infectious mosquito bite while resting at a stopover site in Mississippi. This bird departs on its migration soon after sunset and flies anywhere from 200 to 600 km (see Cochran, 1987; Kerlinger and Moore, 1989), possibly for each of the next three nights. This infected bird arrives at a stopover site up to 1,800 km farther north (e.g., Michigan) from the point of infection. Moreover, this nocturnal migrant, infectious with WNV, arrives in the predawn hours, when mosquitoes are biting and viremia is maximal (48–60 hours postinoculation). The thrush may depart the following night or rest for several days, depending on a variety of factors including its energetic state and prevailing weather conditions (see Moore et al., 1995).

The precise mechanisms of WNV spread by migrating birds remain unknown. However, we have taken a large step in demonstrating experimentally that at least two species of passerine bird are competent to spread WNV from one transmission focus to another. Field studies are needed to determine the probability of such events and the potential role of alternative transmission modes in facilitating the spread of WNV by migrating birds.

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