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Theoretical Potential of Passerine Filariasis to Enhance the Enzootic Transmission of West Nile Virus

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Abstract

Vertebrate reservoirs of arboviruses are often infected with microfilariae (MF). Laboratory studies have shown that MF can enhance the infectivity of arboviruses to mosquitoes. Soon after being ingested, MF penetrate the mosquito midgut. If the host blood also contains virus (i.e., vertebrate is dually infected), penetrating MF may introduce virus into the hemocoel. This can transform otherwise virus-incompetent mosquito species into virus-competent species and simultaneously accelerate viral development, allowing mosquitoes to transmit virus sooner than normal. This phenomenon is termed microfilarial enhancement of arboviral transmission. The prevalence of MF is very high in many passerine populations in North America. Therefore, we investigated if microfilarial enhancement could have facilitated the establishment and rapid spread of West Nile virus (WNV) across the mid-western United States. Our investigations revealed that mosquitoes, WNV, and passerine MF do interact in nature because; 1) 17% of 54 common grackles (*Quiscalus quiscula* L.), 8% of 26 American robins (*Turdus migratorius* L.), and 33% of three eastern kingbirds (*Tyrannus tyrannus* L.) were concurrently microfilaremic and seropositive to WNV; 2) feeding activities of mosquitoes overlapped temporally with the appearance of MF in the blood of common grackles; 3) mosquitoes fed on common grackles and American robins in nature; and 4) mosquito ingestion of two taxonomically distant species of passerine MF (i.e., *Chandlerella quisicali* and *Eufilaria* spp.) resulted in penetration of mosquito midguts. To estimate the theoretical effect that MF enhancement could have on WNV transmission in areas of high MF prevalence, vectorial capacity values were calculated for *Culex* mosquitoes feeding on common grackles, whereby MF enhancement was either invoked or ignored. For *Cx. pipiens*, vectorial capacity increased over three-fold when potential effects of MF were included in the calculations. For *Cx. tarsalis*, the effect was less (i.e., 1.4-fold increase). Closer attention should be paid to the potential of MF to enhance mosquito transmission of arboviruses.

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Keywords

microfilarial enhancement of arboviral transmission; microfilariae; West Nile virus; mosquito; *Culex*

Defining vector competence is fundamental to understanding arboviral transmission cycles. Not every arthropod species can transmit every arboviral species, meaning there can be barriers to the infection process. The most important is the “midgut barrier” (Chamberlain and Sudia 1961). Ingested virus may either be unable to enter the arthropod midgut cells because of a receptor-ligand incompatibility (=midgut infection barrier) or, once inside, virus may be unable to disseminate from the midgut to the hemocoel (=midgut escape barrier) (Kramer et al., 1981, Hardy et al., 1983). In many cases, once the midgut barrier is overcome, viral infection of the salivary glands and subsequent transmission ensues. Thus, any mechanism that effectively bypasses midgut barriers will greatly increase the potential transmission of arboviruses by vectors.

The standard way to identify midgut barriers is to feed suspected vectors on viremic blood. Individuals are then sampled at various time intervals and their extremities (e.g., legs) are excised and assayed for virus to determine if ingested virus disseminated from the gut into the hemocoel. Vectors are fed using *in vitro* methods (e.g., membrane feeder) or by allowing them to feed on anesthetized or restrained vertebrates purposefully infected with the virus in question. Almost always, the vertebrates used are “clean,” laboratory-raised species (e.g., rodents, poultry). However, neither method accounts for the fact that the actual vertebrate reservoirs involved in arboviral transmission cycles are often infected with other blood-borne parasites. Particularly important are the filarioid nematodes.

Filarioid nematodes produce chronic infections among a wide variety of amphibians, reptiles, birds, and mammals, including humans. The prevalence of filarioid infections among wildlife can be very high (Higby 1943, Pung et al. 1966, Sousa et al. 1974, Weinmann et al. 1973). Indeed, blood surveys often underestimate the prevalence of filarioid infections (Homstad et al. 2003) because many filarioid species are nocturnally periodic, that is, the transmissible larval form (=microfilariae) only appear in the peripheral blood at night when field biologists are unlikely to be collecting blood samples. Many hematophagous arthropods (e.g., mosquitoes) feed at night and are likely to ingest nocturnally periodic microfilariae (MF). Laboratory studies have demonstrated that concurrent ingestion of MF and arboviruses by mosquitoes and biting midges can result in significantly greater infectivity of the arbovirus than when the same dose of arbovirus is ingested alone (Mellor and Boorman 1980; Turell et al. 1984, 1987; Zytoon et al. 1993; Vaughan and Turell 1996; Vaughan et al. 1999). As part of their developmental cycle, MF may penetrate the arthropod midgut after being ingested. If the bloodmeal also contains infectious virions, some of the virions can enter the hemocoel directly, circumventing the initial developmental events required for normal arboviral infection of arthropod vectors (i.e., viral attachment, invasion, replication, and release from within the mosquito midgut epithelium). This phenomenon, termed microfilarial enhancement of arboviral transmission, can simultaneously enhance two important transmission parameters. First, virus-incompetent

species that are normally refractory to infection because of midgut barriers, may now develop infections. Increasing the susceptibility of otherwise refractory vectors can increase the number of secondary vector species involved in an arboviral transmission cycle. Second, immediate dissemination of virus by MF can accelerate viral development within the vector, shortening the time required for infected vectors to become infectious vectors (=extrinsic incubation period [EIP]). Because EIP affects transmission in an exponential fashion, small reductions in EIP can lead to large increases in vectorial capacity (MacDonald 1952).

Theoretically, MF enhancement could apply to any hematophagous arthropod species (e.g., sand flies, ticks) as long as the following requirements are met. First, the vertebrate must be concurrently viremic and microfilaremic (i.e., dually infected). Second, the arthropod must ingest both virus and MF. Third, the MF must penetrate the arthropod midgut. Fourth, upon MF penetration sufficient virus must pass from the bloodmeal into the hemocoel to establish a disseminated viral infection. And finally, the arthropod must be able to transmit the virus by bite (i.e., there must be no salivary gland barriers to viral infection or secretion).

The phenomenon of MF enhancement was described over 30 yr ago and has been demonstrated by three different research groups for at least nine different vector-arbovirus-MF model systems (Mellor and Boorman 1980; Turell et al. 1984, 1987; Zytoun et al. 1993; Vaughan and Turell 1996; Vaughan et al. 1999). However, its true impact on arboviral transmission cycles remains uncertain because many of the model systems used to demonstrate the concept were artificial and do not occur in nature. To understand the likelihood of MF enhancement occurring in nature, we investigated the interaction of mosquitoes with co-indigenous songbirds and their filarioid parasites during the time when West Nile virus (WNV) was becoming established in the Red River Valley of eastern North Dakota. Specifically, we determined the applicability of requirements one (=dual infections) and three (=MF penetration of the midgut) as they related to the possible role of MF enhancement of WNV transmission. We then used our findings, together with reported, regional-wide prevalences of MF infections in common grackles, to compare vectorial capacities whereby we either invoked or did not invoke the scenario of MF enhancement into our calculations. In this way, we quantified the theoretical potential that MF enhancement, at least in common grackles, could have had in facilitating the rapid spread of WNV across the mid-western United States.

Materials and Methods

Mosquitoes

To determine the circadian habits of host-seeking mosquitoes, a Mosquito Magnet trap was operated in a residential neighborhood in south Grand Forks, ND (47° 52'36" N, 97° 01'51" W) from 11 July through 24 July 2005. Collections were made at sunrise (5–6:30 a.m.) and at sunset (9–10:00 p.m.) over nine 24 h cycles. Mosquitoes were sorted by species and counted. To investigate the feeding preferences of local mosquitoes, engorged mosquitoes were collected from natural resting places (e.g., inside barns, culverts, etc.) and from Mosquito Magnet traps placed in and around Grand Forks, ND, and in Steele Co., ND. Engorged mosquitoes were identified to species, abdomens were removed, and DNA was extracted with guanidine thiocyanate (Tkach and Pawlowski 1999). Most of the mosquito

species used in feeding trials (see below) were wild mosquitoes collected in eastern North Dakota using Mosquito Magnet traps. The exception was a recently colonized strain of *Culex pipiens* (L.) that originated from Larimer Co., CO (kind gift of K. Kobylinski and B. Foy, Colorado State University).

Birds and MF Parasites

Songbirds were collected in eastern North Dakota and western Minnesota under the authority of U.S. Fish & Wildlife scientific collection permit (MB072162), state collecting permits (North Dakota GNF02136294 and Minnesota 13083), and University of North Dakota IACUC protocol (0605–1). Because many MF species of songbirds are nocturnally periodic (Anderson 2000), two methods were used to examine birds for MF. For general surveys on MF prevalence and serology, birds were collected by gunshot and necropsied. Lungs were dabbed/smeared onto a microscope slide and the resultant “lung blood” was examined (40–100×) for live MF. Additional blood was collected in capillary tubes or on filter paper for detection of WNV antibodies (see below). For feeding trials requiring live birds, birds were captured by baited live-trap or by mist net and transported to the Biology Department where they were maintained in outdoor aviaries. At night, blood was collected from the brachial vein in heparinized capillary tubes. Tubes were centrifuged and motile MF were easily visualized (100×) at the interface of plasma and cell pack (Collins 1971). To determine the pattern of MF periodicity, seven grackles displaying varying intensities of MF infections were bled from the wing vein ($\approx 60 \mu\text{l}$) every 26 h, beginning at 8:00 p.m. and ending at 6:00 a.m. This regime allowed birds to recuperate between bleeds. Blood was immediately expelled into a tube containing 10 μl of liquid K_3EDTA and thoroughly mixed to prevent clotting. Total volumes were measured and the contents were then placed on a clean microscope slide, covered with a glass coverslip and the total number of MF were counted. All counts were expressed as MF per 20 μl .

Immunoassays

Bird sera and blood were assayed for the presence of antibodies against WNV using the methods of Blitvich et al. (2003). If blood was collected by capillary tube, the tubes were centrifuged and the sera stored at -80°C until assayed. If blood was collected on filter paper, a small punch was taken and blood eluted into $\approx 250 \mu\text{l}$ phosphate buffered saline (PBS). Standard flat-bottomed 96-well plates (Costar, Fisher, Pittsburg, PA) were incubated overnight at 4°C with 100 μl WNV antigen and diluted in casein blocking buffer (Wirtz et al. 1993). Plates were emptied and 200 μl of blocking buffer was added to each well. Plates were incubated for 1 h at 37°C , then washed four times with PBS + 0.1% Tween 20. Test samples (50 μl diluted 1:10 in casein blocking buffer) were added to appropriate wells and incubated at 37°C for 2 h. Plates were washed four times and 50 μl of dilute anti-WNV monoclonal antibodies (Mab 3.1112G = 1:2,000, Mab 6B6C-1 = 1:9,000) were added to appropriate wells and incubated for 37°C for 1 h. Plate were washed four times and 50 μl of dilute (1:2,000) horseradish peroxidase (HPR)-labeled rabbit antimouse IgG (KPL, Gaithersburg, MD) were added to appropriate wells and incubated for 37°C for 1 h. Plates were washed four times and 75 μl of ABTS substrate (KPL) were added to appropriate wells. Optical densities (OD) were read at regular intervals until the average OD for the control serum exceeded 0.3 (usually 10–20 min). The percentage of inhibition value of the

test serum was calculated as follows: % inhibition = $100 - [100 * (TS - B / CS - B)]$; where TS = OD of test serum, CS = OD of control serum, and B = background OD. Samples displaying a percent inhibition of 45% or more were interpreted as positive for anti-WNV antibodies.

Mosquito Bloodmeal Identification

The DNA samples extracted from bloodmeals of field-collected mosquitoes were coded and stored at -80°C until analyzed. Vertebrate cytochrome B gene sequences present in the extracted DNA were amplified by polymerase chain reaction (PCR) using vertebrate specific primers, as previously described (Hassan et al. 2003). The resulting amplicons were sequenced and aligned with published sequences to identify the vertebrate species composition of the bloodmeals.

MF Penetration of Mosquito Midguts

Feeding trials were conducted with different species of MF parasitizing the common grackle, American robin, and dark-eyed junco. Microfilaremic blood was administered to mosquitoes using either membrane feeders or live birds. For membrane feeding, blood from nonmicrofilaremic grackles were collected into citrated saline, centrifuged, and the erythrocytes were stored in sterile Alsevere's solution. Microfilaremic birds were euthanized and the lungs were removed, minced, suspended in buffered saline, vortexed, filtered through ultra-fine nylon mesh, and centrifuged. The MF densities of lung filtrates were determined by counting the MF in a small ($\approx 10 \mu\text{l}$) aliquot. Measured amounts of MF were then added to a 1:1 mixture of uninfected grackle erythrocytes and domestic goose sera. Two dilutions were prepared; one that approximated the normal MF density found in infected birds and one that was ≈ 10 -fold higher than a normal microfilaremia. Microfilaremic blood ($\approx 2 \text{ ml}$) was placed in water-jacketed membrane feeders maintained at 38°C via a circulating water bath and fitted with de-salted sausage casing (=pig intestine). Feeders were placed on the screen tops of cages containing mosquitoes for $\approx 1 \text{ h}$ in darkness. For live bird feedings, microfilaremic birds were anesthetized with a mixture of ketamine (20 mg/kg intramuscular [IM]) and xylazine (4 mg/kg IM) injected with a 27 gauge needle into the pectoral muscles. Our preliminary studies indicated that ketamine-xylazine anesthesia did not alter the nocturnal periodicity of grackle MF, as has been reported for *Brugia malayi* (Brug) MF in gerbils (Beerntsen et al. 1996). Birds were anesthetized at night ($\approx 2:00 \text{ a.m.}$) and placed on their backs inside a screen cage containing mosquitoes. Mosquitoes were allowed to feed in complete darkness for $\approx 1 \text{ h}$ after which the bird was removed and a blood sample taken from the wing vein to obtain an estimate of microfilaremia at the time of mosquito feeding. Most mosquitoes fed on the face and feet of anesthetized birds. Mosquitoes were maintained at 24°C until morning ($\approx 4 \text{ h}$ after feeding), whereupon engorged mosquitoes were sorted by species and dissected. Replete midguts were carefully excised, opened, and the contents were placed on a slide with coverslip and examined for MF. Likewise, eviscerated carcasses were carefully teased apart, compressed with a coverslip and examined microscopically for MF. MF found in mosquito carcasses were interpreted as having penetrated the midgut. The numbers of MF in a bloodmeal and in the corresponding carcass represented the total number of MF ingested by a mosquito.

Vectorial Capacity

To assess the magnitude by which MF enhancement could theoretically have contributed to the rapid spread of WNV across the midwestern United States, we calculated vectorial capacities for *Cx. pipiens* and *Cx. tarsalis* mosquitoes feeding on common grackle, whereby MF enhancement was either invoked (VC_{YES}) or not invoked (VC_{NO}). Vectorial capacity (VC) is defined as the daily rate at which infective mosquito bites arise from a single infective host within an otherwise uninfected population (MacDonald 1952). It can be expressed by the formula

$$VC = (ma)(a)(b) \left(P^T / -\log_e P \right)$$

where:

ma = mosquito biting rate, expressed as number of mosquito bites per bird per night.

a = "avian host preference" or the probability of a mosquito feeding on a bird during 1 d. This can be calculated by dividing the bird blood index (i.e., proportion of the vector population feeding on birds) divided by the daily biting frequency of a mosquito (i.e., the gonotrophic period).

b = infectiousness of birds to the vectors (=vector competence).

P = daily probability of survival for the vector.

T = extrinsic incubation period of the virus within the vector.

Data from the literature, as well as results reported here, provided the parameter estimates used to calculate vectorial capacities (Tables 1 and 2). Certain parameters were held constant. For example, the intensity and selective behavior of mosquito feeding on birds (i.e., 'ma' and 'a') were held constant because we know of no empirical studies or compelling reason to suggest that these parameters would be affected by the MF status of viremic birds. In addition, we showed that daily mosquito survival ('P') was unaffected by mosquito ingestion of *Ch. quiscalis* MF (see below). However, co-ingestion of virus and MF has been shown experimentally to alter both vector competence (b) and extrinsic incubation period (T). Thus, these were the two parameters that differed between calculations for VC_{YES} and VC_{NO} . For VC_{NO} , estimates of 'b' were based on transmission rates of mosquitoes that were orally exposed to WNV whereas for VC_{YES} , estimates of 'b' were based on transmission rates of mosquitoes that were intrathoracically inoculated and/or had disseminated infections of WNV. The VC_{YES} , estimates of 'T' were calculated as 42.4% reductions in the normal T reported in the literature, as determined experimentally for the dengue virus/*Brugia* MF/*Aedes aegypti* model system by Vaughan et al. (2009).

Because the point of this exercise was to obtain a quantitative estimate on how MF enhancement could affect the spread of WNV within naïve host populations, parameter values for vector competence, and extrinsic incubation periods were based on studies using the original progenitor strain of WNV in North America, NY99. The common grackle was

identified as the most appropriate avian species for these calculations because 1) grackles are synanthropic and very common throughout the Midwest, 2) grackles produce high viremias when infected with WNV (Komar et al. 2003), 3) grackles are known to be fed on by *Culex* mosquitoes (Savage et al. 2007), and 4) unlike most other passerine reservoirs of WNV (e.g., American robin, house sparrow, etc.), there are several robust estimates of MF prevalence for grackles that span a large geographic area (see Fig. 1).

Once a value for VC_{YES} was obtained, the value was further adjusted to reflect a realistic estimate of the proportion of mosquitoes that might be expected to experience midgut penetration in nature. This reflects the fact that not all hosts in a population are microfilaremic and not all mosquitoes feeding on microfilaremic hosts may have their midguts penetrated by ingested MF. For example, if 50% of the hosts in a particular area were microfilaremic and 30% of the mosquitoes that ingested MF had their midguts penetrated, then the scaling factor would be 0.5×0.3 or 0.15. In such a scenario, only 15% of the mosquitoes feeding on grackles would be involved in MF enhancement (VC_{YES}) whereas the remaining 85% would not (VC_{NO}). Thus:

$$\text{'Actual } VC_{YES} \text{' = } (0.15) (VC_{YES}) + (0.85) (VC_{NO}) .$$

These calculations were performed for each different region in the United States where estimates of MF prevalence exist (Fig. 1). The data for MF penetration were obtained during this study (see below).

Results

Passerine MF

In total, 144 songbirds were examined in 2005, comprised mostly of common grackle ($n = 58$), American robin ($n = 26$), and red-winged blackbird (*Agelaius phoeniceus* L.) ($n = 26$). Five of the 10 bird species examined had MF infections including; common grackle (11/58), American robin (4/26), brown-headed cowbird (*Molothrus ater* Boddaert) (1/6), blue jay (*Cyanocitta cristata* L.) (1/3), and eastern kingbird (1/3). Three types of MF were observed. Common grackle harbored sheathed MF with blunt tails and moved in an undulating, snake-like fashion (Fig. 2a). Adult worms were recovered from the ventricles of the brains of microfilaremic grackles and identified morphologically as *Chandlerella quiscali* (von Linstow), the filarioid most commonly found in common grackle (Anderson 2000). The four microfilaremic robins and the cowbird, blue jay, and kingbird harbored smaller ($\approx 100 \mu\text{m}$), unsheathed MF with pointed tails that moved in an erratic, often jerky fashion (Fig. 2b). Six additional robins collected in 2011 and 2012 harbored a third MF type that were long ($>250 \mu\text{m}$), unsheathed, with tapering pointed tails and moved in coiling/uncoiling fashion. The small MF were morphologically identical to taxonomic descriptions given for MF of the genus *Eufilaria* Seurat (Anderson 2000, Bartlett and Anderson 1980, Bartlett 2008) and the large MF were morphologically identical to taxonomic descriptions given for MF in the genus *Cardiofilaria* Strom (Bartlett 2008). Efforts to identify these filarioids to species by recovering adult worms from microfilaremic birds were unsuccessful. Two grackles and one of the robins each harbored more than one type of MF, indicative of multiple infections.

Dual Exposure of Birds to MF and WNV

All bird species examined had antibody to WNV. Seroprevalences ranged from 17% in brown-headed cowbird ($n = 6$) to 100% in eastern kingbird ($n = 3$). Evidence of dual exposure (i.e., birds having both MF and antibodies to WNV) was detected in common grackle, American robin, and eastern kingbird (Table 3). The prevalence of dual exposure in these bird species did not differ from what would be predicted based on the combined prevalences of MF and sero-positivity (Fisher exact tests; $P > 0.05$). Grackles and robins are common songbirds in Grand Forks, ND, during the summer (J.A. Vaughan, personal observation) and both produce high viremias when infected with WNV (Komar et al. 2003), making them important local amplifying hosts for WNV. A significantly higher percentage of grackles had antibodies to WNV (67%) than did robins (38%) (Fisher's exact test; $P < 0.03$), suggesting that local grackles had been more frequently exposed to the bites of infected vectors than were robins.

Nocturnal Periodicity of Grackle MF (*Chandlerella quisquali*)

Peak microfilaremiams among grackles varied by as much as 45-fold, ranging from four MF (Bird 307) to 185 MF (Bird 302) per 20 μ l blood (Fig. 3). Even so, the patterns of periodicity were similar. Microfilaremiams were nil at 8:00 p.m., but rose steadily throughout the night, reaching a peak at 2:00 to 4:00 a.m. Microfilaremiams rapidly subsided to almost nothing by sunrise (6:00 a.m.) (Fig. 3). These patterns were similar to those reported previously for *Ch. quisquali* MF (Odetoyinbo 1960). Thus, nocturnal periodicity of *Ch. quisquali* MF limits the phenomenon of MF enhancement in common grackle to those vector species that feed at night.

Likelihood of Mosquitoes Feeding on Birds at Night

The main mosquito species collected in Mosquito Magnets were *Aedes vexans* (Meigen), *Ae. dorsalis* (Meigen), and *Cx. tarsalis* Coquillett. Mean abundances of host-seeking *Cx. tarsalis* were significantly higher at night than during the day (paired t -test on \log_{10} transformed data; $t = 5.3$; $df = 8$; $P = 0.0007$), indicating that *Cx. tarsalis* is primarily a "night-biting" species (Table 4). There were no significant differences between the mean numbers of host-seeking *Ae. vexans* or *A. dorsalis* caught during the day versus the night (paired t -tests; $P > 0.10$), indicating that these floodwater species hunt both day and night. Sixty-eight of 79 (86%) bloodmeals extracted from field-collected mosquitoes provided sufficient quality of host DNA to make accurate determinations (Table 5). Although most *Ae. vexans* (68%) collected had fed on mammals, a substantial proportion (26%) had fed on common grackle and one had fed on American Robin. Although sample sizes were small, it was clear that other *Aedes* species and *Cx. pipiens* also fed on common grackle. The combined results of MF periodicity studies (Fig. 3), day versus night sampling (Table 4), and bloodmeal analysis (Table 5) indicate that local mosquitoes, even mammophilic floodwater species such as *Ae. vexans*, and *Ae. dorsalis*, fed on grackles (and possibly robins) and hunted at night when MF are circulating in peripheral blood of the birds.

MF Penetration of Mosquito Midguts

All five of the wild-caught mosquito species tested were susceptible to midgut penetration by co-indigenous *Ch. quisquali* MF (Table 6). However, the prevalence of penetration differed among mosquito species, densities of MF ingested, and in some cases, between the two feeding techniques. *Ae. dorsalis* and *Ae. vexans* experienced higher rates of midgut penetration when *Ch. quisquali* MF were ingested from a membrane feeder than from a live bird. Perhaps the lack of blood coagulation in the membrane feeders facilitated MF motility and penetration through the midgut whereas normal coagulation within bloodmeals taken from a live host hindered MF motility and penetration. Whatever the reason, the different outcomes suggest that live bird feedings should be the method of choice to assess MF penetration in nature. When fed on a live bird, the rates of MF penetration in *Ae. vexans*, *Ae. dorsalis*, *Ae. flavescens* (Muller), and *Cx. tarsalis* were all <20%.

Because of the importance of *Cx. pipiens* as a major vector of WNV in the eastern United States, additional feeding trials were conducted with a recently colonized strain of *Cx. pipiens*, comparing the abilities of three different genera of passerine MF to penetrate *Cx. pipiens* midguts (Table 7). When mosquitoes fed on microfilaremic birds at night, the proportion of midguts penetrated by *Chandlerella* (37%) and *Eufilaria* (60%) MF did not differ significantly from one another ($\chi^2 = 2.55$; $P = 0.11$). In contrast, when *Cx. pipiens* fed on a microfilaremic robin harboring *Cardiofilaria* MF, none of the ingested MF penetrated the midguts. This may have been a function of the extremely low intensity of *Cardiofilaria* microfilaremia (2 MF per 20 μ l) within the robin. Microfilaremias within the *Chandlerella*-infected grackle (693 MF per 20 μ l) and the *Eufilaria*-infected junco (938 MF per 20 μ l) were much higher. Indeed when data for all three MF species were pooled, there was a significant relationship between densities of MF ingested and numbers penetrating the midgut ($F = 13.00$; $df = 1, 82$; $P = 0.0005$). Thus, the intensity of a host's microfilaremia may be important in determining its relative potential to contribute to MF enhancement. Nevertheless, these results demonstrated that at least two species of passerine MF possess the ability to penetrate the midgut of *Cx. pipiens* when ingested from a live host. The grackle MF, *Ch. quisquali*, can penetrate the midgut of *Cx. tarsalis*, albeit to a lesser extent.

Effect of *Ch. quisquali* MF on *Cx. pipiens* Mortality

To determine if *Ch. quisquali* MF penetration of the midgut causes excessive mortality in *Cx. pipiens*, survivorship of the same cohort of mosquitoes used in the midgut penetration studies described above was monitored for 5 d after blood feeding. Of 354 engorged *Cx. pipiens* mosquitoes, only 15 (4%) died, all within 32 h after feeding. The remaining mosquitoes survived and oviposited normally. Fourteen of the 15 dead mosquitoes were “red mosquitoes,” indicating that midgut integrity had been disrupted and that blood had leaked into the hemocoel. The dead mosquitoes were carefully dissected and found to contain significantly more MF in their hemocoels (geometric mean = 15; range = 3–71) than their seemingly healthy counterparts dissected the day before (geometric mean = 2; range = 1–12) (Wilcoxon Rank Sum Test; $P < 0.001$). These results indicate that ingested *Ch. quisquali* MF had negligible effect on the overall mortality of *Cx. pipiens* but in those few cases where mortality did occur, the amount of host blood leaking into the hemocoel was so great that it was plainly visible and probably toxic to the mosquito.

Vectorial Capacity

The five parameters necessary to calculate vectorial capacity were available from literature sources for *Cx. pipiens* and *Cx. tarsalis* (Tables 1 and 2). For each mosquito species, separate calculations were performed for each of six regions in the mid-west United States where estimates on *Ch. quiscalis* MF prevalence in common grackles were available (Fig. 1). For both *Cx. pipiens* and *Cx. tarsalis*, the inclusion of potential MF effects yielded significantly higher values of vectorial capacity than when MF effects were excluded from the calculations (one-sample *t*-tests; *P* values <0.008) (Table 8). When MF effects were included, the average vectorial capacity for *Cx. pipiens* ($VC_{YES} = 0.43 \pm 0.17$) increased over three times that of the baseline vectorial capacity value ($VC_{NO} = 0.13$). For *Cx. tarsalis*, the difference between the average VC_{YES} value and the VC_{NO} value was not as divergent (i.e., 1.4-fold difference). One reason for this relates to the lower efficiency of *Ch. quiscalis* MF in penetrating the midguts of *Cx. tarsalis* (i.e., 12%; Table 6) compared with that in *Cx. pipiens* (i.e., 37%; Table 7). In addition, the baseline vector competence of *Cx. tarsalis* for the NY-99 strain of WNV is already so high (VC_{NO} ; $b = 0.82$) that MF have little net effect on increasing vector competence (VC_{YES} ; $b = 1.00$). Thus, the effects of MF enhancement in *Cx. tarsalis* (at least as pertains to *Ch. quiscalis* MF and grackles) would seem to be confined to decreasing the extrinsic incubation period in a small proportion of mosquitoes. However, the theoretical effects of MF enhancement in *Cx. pipiens* are much greater and may help explain why WNV was able to spread so rapidly across the eastern half of North America where *Cx. pipiens* is the predominate vector.

Discussion

Laboratory studies have demonstrated that MF enhancement of arboviral transmission can increase the susceptibility of marginally susceptible mosquito species to infection with arboviruses, and at the same time, can accelerate the infectious process within refractory and fully susceptible mosquitoes alike. Central to the concept's validity is the existence in nature of vertebrates that are infected concurrently with MF and arbovirus during the time when vectors are feeding. Throughout the world, examples of multiple parasitism are common (Petney and Andrews 1998) and there is enormous geographic overlap between many arboviral and filarioid infections. Filarioid infections are chronic, often life-long infections. Once individuals within a vertebrate population become microfilaremic, they have the potential to serve as dually infected hosts should they later acquire arboviral infections.

This is exactly the scenario that played out for many songbird populations when WNV expanded outward from its point of introduction in New York City during the early to mid-2000s. Parasitological surveys of American crow, blue jay, black-billed magpie, and common grackle conducted before the arrival of WNV indicate that there were extremely high prevalences of pre-existing MF infection among these WNV-susceptible species over a large expanse of North America (Fig. 1). In areas where nonimmune bird populations had MF prevalences approaching 100% (e.g., Indiana, Illinois), it is probable that dually infected birds constituted a major part of the infectious reservoir for WNV for those bird species.

However, the existence of dual infections by itself does not satisfy all the requirements needed for MF enhancement to occur. An important aim of this study was to determine the

degree of interaction occurring between mosquitoes and the MF of songbirds in nature. We found that the common grackle and American robin were naturally exposed to both MF and WNV infections (Table 3) and that local mosquitoes fed on these bird species (Table 5) and fed during the night (Table 4) when nocturnally periodic MF were present in the peripheral blood (Fig. 3). Importantly, we observed that mosquito ingestion of two taxonomically distant genera of passerine MF (i.e., *Chandlerella* [subfamily: Splendofilariinae] and *Eufilaria* [subfamily: Lemdaninae]) resulted in penetration of mosquito midguts (Tables 6 and 7); even though mosquitoes are not the vectors for *Chandlerella* or *Eufilaria* and do not support their development. These findings underscore the notion that MF species not normally associated with mosquitoes can, nevertheless, penetrate the midguts of mosquitoes. This has been observed in other MF/mosquito species combinations. For example, Santos et al. (2006) showed that *Litomosoides chagasfilhoi* de Moraes Neto, Lanfredi and de Souza, a mite-borne filarioid of neotropical rodents, penetrated and disrupted the midgut of *Cx. quinquefasciatus* Say mosquitoes fed on microfilaremic rodents. Similarly, Hibler (1963) exposed two black-billed magpies (*Pica pica* L.), each with a quadruple infection of *Splendofilaria picacardina* Hibler, *S. caperata* Hibler, *Ch. striatospicula* Hibler, and *E. longicaudata* Hibler MF, to a total of 2,000 co-indigenous *Cx. tarsalis* mosquitoes during peak MF periodicity (=midnight). He reported that; “no larval development occurred even though each mosquito obtained an average of 100 MF, many of which penetrated the midgut. Living MF were found in the abdomen and thorax up to 12 h after ingestion, after which all of the MF were dead.” These filarioid species only complete their development in *Culicoides* (Hibler 1963). Likewise, Vaughan et al. (2007) reported that *Mansonella ozzardi* (Manson), a *Culicoides*-borne filarioid of humans, readily penetrated the midgut of a co-indigenous strain of *Ae. aegypti* (L.) mosquitoes fed directly on microfilaremic people. Thus, a mosquito species does not have to be a competent vector for the filarioid parasite to have a potential role in MF enhancement (Vaughan et al. 1999). The MF only have to penetrate the gut. Subsequent development is irrelevant.

Another requirement is that MF penetration of the midgut must introduce enough virus into the hemocoel to establish a disseminated infection. The appearance of dead and dying red *Cx. pipiens* mosquitoes produced by a high intensity of *Ch. quiscalis* MF penetration (mean = 15 MF) clearly indicates that copious amounts of bloodmeal leakage can occur as the result of *Ch. quiscalis* MF passage through the midgut. However, in these trials such a high intensity of MF penetration was rare and the more 'atypical' intensity of MF penetration averaged only two MF per midgut (Table 7). Although this might seem low, it is comparable to penetration intensities reported in two previous trials (means ranged from 1 to 3 MF) wherein three *Aedes* spp. mosquitoes were fed on gerbils concurrently infected with *Brugia* spp. MF and different arboviruses (Vaughan and Turell 1996, Vaughan et al. 1999). In those trials, midgut penetration by even one or two *Brugia* MF was enough to produce significantly higher rates of disseminated infections in two of the three *Aedes* mosquitoes than when mosquitoes fed on singly infected gerbils with equivalent viremias but no microfilaremias. This suggests that if *Cx. pipiens* fed on a dually infected grackle (i.e., microfilaremic and viremic), one or two penetrating *Ch. quiscalis* MF could potentially introduce enough co-ingested WNV into the hemocoel to result in disseminated infection and cause MF enhancement. Assuming that MF-induced introduction of virus into the

hemocoel would reduce the EIP of WNV in *Cx. pipiens* by over half, as it does in the dengue virus/*B. malayi* MF/*Ae. aegypti* system (Turell et al. 1987, Vaughan et al. 2009), then the effect on viral amplification could be considerable. In our calculations, the theoretical vectorial capacity for WNV in *Cx. pipiens* feeding on grackle populations was over three times higher than would be expected if calculations were based on vector competence and EIP values derived from clean, nonmicrofilaremic grackles.

In this report, we identified a naturally occurring system where there appears to be sufficient interaction among the vertebrate (common grackle), parasite (*Ch. quiscalis* MF), vector (*Culex pipiens*), and virus (WNV) to warrant further studies on MF enhancement. There may be many other naturally occurring systems in which MF enhancement could influence arboviral transmission. Indeed, the very first experimental model of MF enhancement (Mellor and Boreham 1980) alluded to a naturally occurring system that is both economically important and easily studied, that is, bluetongue virus in cattle. Over the past decade, Europe has experienced multiple invasions of different serotypes of bluetongue virus, all vectored by *Culicoides* midges. Throughout Europe, *Culicoides* (and notably, black flies) also transmit bovine onchocerciasis. The prevalence of *Onchocerca* spp. MF in the dermis of European cattle are substantial, that is, Austria (131/345; Safar-Hermann and Supperer 1983) Germany (175/438; Dohnal et al. 1990), Wales (130/463; Trees et al. 1987), and Finland (77/209; Solismaa et al. 2008). If bovine onchocerciasis were shown experimentally to accelerate the EIP of bluetongue virus in the midge vector, then antihelminthic treatment of cattle herds may prove to be an effective supplement to traditional bluetongue management strategies (i.e., vector control).

Studies of MF enhancement in natural systems may lead to the recognition of additional, important amplifying host species. For example, the American robin is considered a major amplifying host of WNV based on the disproportionate amount of *Cx. pipiens* feeding on this avian species (Kilpatrick et al. 2006, Hamer et al. 2009, Savage et al. 2007, Molaei et al. 2006). However, other bird species not as frequently fed upon but having high prevalence of MF infection (i.e., common grackle), may also be important contributors to viral amplification if vector competence and/or the EIP for WNV is enhanced in mosquitoes that feed on dually infected hosts. Likewise, MF enhancement may produce unanticipated vector species. For example, researchers in Louisiana amplified WNV RNA from three species of ornithophilic *Culicoides* midges at concentrations comparable to that found in *Culex* mosquito vectors (Sabio et al. 2006). The ability of *Culicoides* to transmit WNV remains unknown. However, even if it were found that *Culicoides* had impenetrable midgut barriers to WNV, it is very likely that their midguts would be penetrated by passerine MF because *Culicoides* are primary vectors for passerine MF (Anderson 2000). The first priority in defining the role of MF enhancement in this particular system would be to determine whether or not ornithophilic *Culicoides* possess salivary gland barriers to WNV, that is, conduct transmission studies with intrathoracically inoculated midges.

Without a fuller understanding of the potential role of MF enhancement in naturally occurring systems, cryptic, potentially important amplifying hosts, and vectors may be overlooked and our understanding of enzootic transmission of arboviruses may be incomplete.

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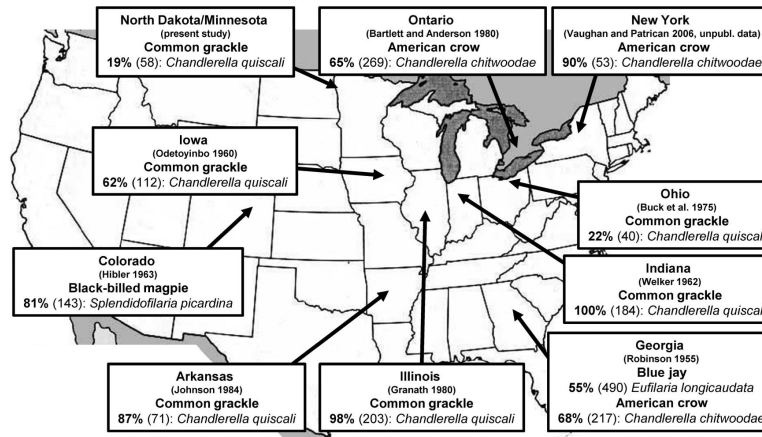


Fig. 1. Prevalence (number birds examined) of microfilarial infections in North American populations of American crow, blue jay, black-billed magpie, and common grackle. Parasite species are in italics and references are in parentheses.

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Fig. 2.
(A) Sheathed *Chanderella quisquali* microfilaria (150 µm long) from common grackle; (B)
Unsheathed *Eufilaria* sp. microfilaria (100 µm long) from American robin.

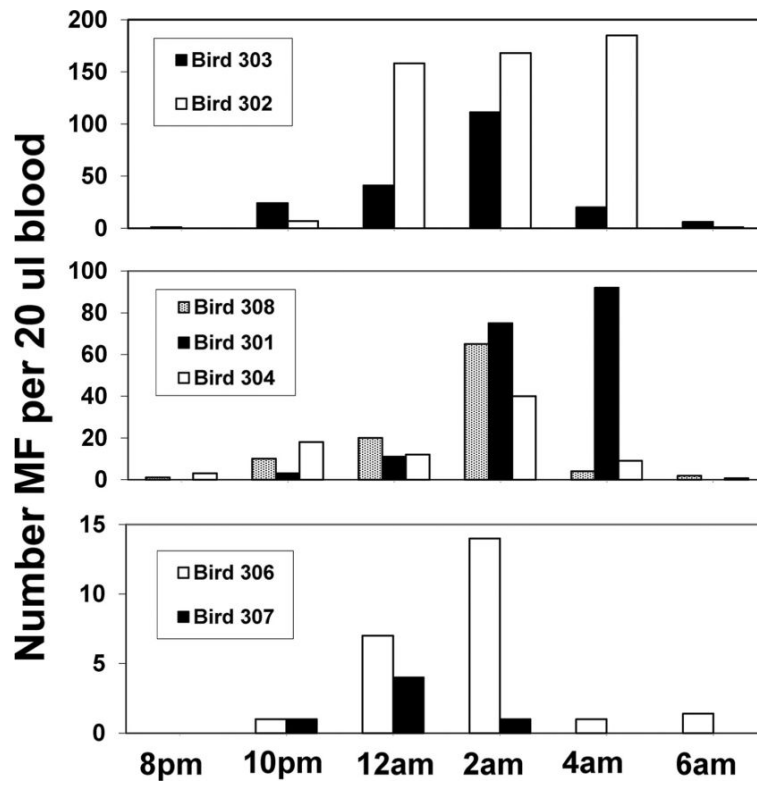


Fig. 3.
Nocturnal periodicity of *Chandlerella quisicali* MF in common grackles.

Parameter estimates used in calculations of vectorial capacity for WNV in *Culex pipiens* mosquitoes feeding on common grackles whereby MF enhancement is invoked (VC_{YES}) or not invoked (VC_{NO})

Table 1

Scenario	Biting rate (ma) ^a	Avian host preference (a) ^b	Vector competence (b) ^c	Daily Survival (P) ^d	EIP (T) ^e
VC _{YES}	1.53	0.186	0.87	0.90	5 d
VC _{NO}	1.53	0.186	0.17	0.90	12 d

^a Biting rate (no. bites/bird/night)—based on bird-baited mosquito traps (Darbro and Harrington 2006).

^b Avian host preference—based on bird blood index of 0.93 (Molaei et al. 2006) divided by gonotrophic value of 5 d (Reisen et al. 1992).

^c Vector competence—based on experimental transmission rates of an unpassed isolate of WNV (Crow 397–99 strain) obtained from the brain of a crow that died in the epicenter (i.e., New York, NY) during the initial year of the WNV outbreak (i.e., 1999) and using *Cx. pipiens* reared from larvae collected 20 km away from the epicenter. Values for vector competence represent viral transmission rates of mosquitoes that were either orally exposed to WNV (VC_{NO}; i.e., 31/178) or were intrathoracically inoculated and/or had disseminated infections (VC_{YES}; i.e., 21/24) (data are combined from Turell et al. 2000, Turell et al. 2001).

^d Daily survival—based on mark-release-recapture with *Cx. pipiens* in Washington, DC (Jones et al. 2012). Mortality of *Cx. pipiens* because of ingestion of *Ch. quitiscali* MF was assumed to be negligible (~4%; see text).

^e Extrinsic incubation period (EIP)—the 'normal' EIP (i.e., VC_{NO}) of 12 d is based on orally exposed *Cx. pipiens* held at 26°C where dissemination rate exceeded 15% (Dohm et al. 2002, Anderson et al. 2008). The MF-enhanced EIP (i.e., VC_{YES}) of 5 d was calculated as 42.4% of normal EIP, based on the avg reductions of EIP observed with dengue 1 virus development within *Ae. aegypti* fed blood that was either dually infected (virus + *Burkholderia malayi* MF) or singly infected (virus only) (Vaughan et al. 2009).

Parameter estimates used in calculations of vectorial capacity for WNV in *Culex tarsalis* mosquitoes feeding on common grackles whereby MF enhancement is invoked (VC_{YES}) or not invoked (VC_{NO})

Table 2

Scenario	Biting rate (ma) ^a	Avian host preference (a) ^b	Vector competence (b) ^c	Daily survival (P) ^d	EIP (T) ^e
VC _{YES}	14.1	0.176	1.00	0.80	5 d
VC _{NO}	14.1	0.176	0.82	0.80	12 d

^a Biting rate (no. bites/bird/night)—based on the avg no. of engorged *Culex tarsalis* captured over 25 trap-nights by net traps baited with four species of wild birds (Anderson and Brust 1995, 1997; Lura et al. 2012).

^b Avian host preference—based a bird blood index of 0.88 during the summer months in California (Thiemann et al. 2011) divided by a gonotrophic value of 5 d (McHugh 1990).

^c Vector competence—based on experimental transmission rates of WNV (Crow 397–99 strain) for orally exposed *Cx. tarsalis* (VC_{NO}; i.e., 58/71) and for disseminated infections of *Cx. tarsalis* (VC_{YES}; i.e., 6/6) (Turell et al. 2002).

^d Daily survival—based on parity rates of wild-caught *Cx. tarsalis* in Imperial and Coachella Valleys, CA, during the summer months (Reisen et al. 1995).

^e Extrinsic incubation period (EIP)—the `normal' EIP (i.e., VC_{NO}) of 12 d is based on orally exposed *Cx. tarsalis* held at 26°C where dissemination rate exceeded 50% (Anderson et al. 2012). The MF-enhanced EIP (i.e., VC_{YES}) of 5 d was calculated as 42.4% of normal EIP, based on the avg reductions of EIP observed with dengue 1 virus development within *Ae. aegypti* fed blood that was either dually infected (virus + *Brugia malayi* MF) or singly infected (virus only) (Vaughan et al. 2009).

Table 3

Prevalence of common grackles, American robins, and eastern kingbirds having microfilarial infections, antibodies to West Nile virus (WNV), and concurrently microfilaremic and sero-positive to WNV (=dually exposed)

Species	Microfilaremic (%)	WNV seropositive (%)	Dually exposed (%)
Common grackle	11/58 (19%)	36/54 (67%)	9/54 (17%)
American robin	4/26 (15%)	10/26 (33%)	2/26 (8%)
Eastern kingbird	1/3 (33%)	3/3 (100%)	1/3 (33%)

Red River Valley; northeast North Dakota and northwest Minnesota, summer 2005.

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Table 4

Geometric mean (95% CL) of host-seeking mosquitoes collected in a Mosquito Magnet trap over nine 24 h sampling intervals

Mosquito species	Sunrise to sunset (day)	Sunset to sunrise (night)
<i>Aedes vexans</i>	9 (4, 22)	12 (4, 33)
<i>Aedes dorsalis</i>	7 (4, 14)	13 (5, 32)
<i>Culex tarsalis</i>	4 (1, 10)	67 (29, 156)

Collections were taken at sunrise and at sunset. Grand Forks, ND, July, 2005.

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Table 5
 Number of bloodmeals identified to host species for engorged mosquitoes collected in Grand Forks County, ND, summer 2005 and Steele County, ND, summer 2010 (*Culex pipiens*)

Host species	<i>Aedes vexans</i>	<i>Aedes dorsalis</i>	<i>Aedes triseriatus</i>	<i>Culiseta inornata</i>	<i>Culex tarsalis</i>	<i>Culex pipiens</i>	Total
Common grackle	10	2	1			3	16
American robin	1						1
Chipping sparrow					1		1
Pigeon				1	5		6
Chicken	1			1	2		4
Deer	21			5	1		27
Cow	3	1		1			5
Human	2	1	1	3	1		8
Total	38	4	2	11	10	3	68

Table 6

Penetration and movement across the mosquito midgut by *Chandlerella quisicali* microfilariae (MF) when ingested by co-indigenous, wild-caught mosquitoes

Mosquito species	Type of feeding	MF ingested per mosquito (range)	MF penetrating midgut (range)	Prevalence of MF penetration (n)
<i>Aedes vexans</i>	Membrane-high density	3211 (420–48,880)	6 (1–176)	42% (40)
	Membrane-low density	24 (1–350)	2 (1–4)	9% (34)
	Live bird	16 (6–68)	0	0% (14)
<i>Aedes dorsalis</i>	Membrane-high density	1073 (396–5205)	65 (3–670)	67% (12)
	Membrane-low density	52 (1–292)	5 (1–113)	43% (14)
	Live bird	26 (3–88)	1 (1–2)	16% (25)
<i>Aedes foveolatus</i>	Live bird	70 (22–136)	2	17% (6)
<i>Aedes triseriatus</i>	Membrane-high density	1406 (607–4380)	4 (1–7)	50% (4)
	Membrane-low density	142 (48–375)	1	17% (6)
<i>Culex tarsalis</i>	Membrane-high density	2060 (500–12,125)	3 (1–9)	15% (52)
	Membrane-low density	145 (1–432)	1 (1–3)	12% (33)
	Live bird	21 (1–156)	1	12% (40)

Mosquitoes were either fed MF harvested from bird lungs, mixed with bird blood, and placed into prewarmed membrane feeders (=membrane) or were allowed to feed directly on anesthetized microfilaremic birds at night (=live bird).

Penetration and movement across the mosquito midgut by three different genera of passerine microfilariae (MF) when ingested by laboratory-reared *Culex pipiens* mosquitoes

Table 7

MF species	Bird host	Type of feeding	MF ingested per mosquito (range)	MF penetrating midgut (range)	Prevalence of MF penetration (n)
<i>Chandlereilla quiscai</i>	Grackle	Membrane high density	1028 (587–1487)	4 (1–13)	100% (9)
		Membrane-low density	37 (17–62)	1 (1–2)	25% (16)
<i>Cardiofilaria</i> spp.	Robin	Live bird	29 (1–347)	2 (1–12)	37% (49)
		Membrane-low density	5 (2–9)	1	11% (9)
<i>Eufilaria</i> spp.	Junco	Live bird	3 (1–5)	0	0% (19)
		Live bird	71 (48–125)	1 (1–2)	60% (15)

Mosquitoes were either fed MF harvested from bird lungs, mixed with bird blood, and placed into prewarmed membrane feeders (=membrane) or were allowed to feed directly on anesthetized microflaemic birds at night (=live bird).

Theoretical vectorial capacity values computed for *Culex pipiens* and *Cx. tarsalis* mosquitoes feeding on West Nile virus-infected common grackles whereby the potential effect of concurrent *Chandlerella quisquali* microfilarinias (MF) on increasing the vector competence and simultaneously reducing the extrinsic incubation of the virus has either been invoked (VC_{YES}) or has been ignored (VC_{NO}) in the calculations

Table 8

State	Reported MF prevalence (n)	<i>Culex pipiens</i>		<i>Culex tarsalis</i>	
		VC _{YES}	VC _{NO}	VC _{YES}	VC _{NO}
Ohio	22% (40)	0.23	0.13	0.71	0.63
Indiana	100% (184)	0.59	0.13	0.99	0.63
Illinois	98% (203)	0.58	0.13	0.98	0.63
Iowa	62% (112)	0.42	0.13	0.85	0.63
Arkansas	87% (71)	0.54	0.13	0.95	0.63
North Dakota	19% (58)	0.22	0.13	0.70	0.63
Average (±SD)	80% (668)	0.43 ± 0.17	0.13	0.86 ± 0.13	0.63

Calculations take into account the regional variation in MF prevalence (see Fig. 1) and different rates of MF penetration between *Cx. pipiens* and *Cx. tarsalis* (see Tables 6 and 7). See Appendix for parameter estimations.