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Pre-existing Microfilarial Infections of American Robins (Passeriformes: Turdidae) and Common Grackles (Passeriformes: Icteridae) Have Limited Impact on Enhancing Dissemination of West Nile Virus in Culex pipiens Mosquitoes (Diptera: Culicidae)

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ABSTRACT

Microfilariae (MF) are the immature stages of filarial nematode parasites and inhabit the blood and dermis of all classes of vertebrates, except fish. Concurrent ingestion of MF and arboviruses by mosquitoes can enhance mosquito transmission of virus compared to when virus is ingested alone. Shortly after being ingested, MF penetrate the mosquito's midgut and may introduce virus into the mosquito's hemocoel, creating a disseminated viral infection much sooner than normal. This phenomenon is known as microfilarial enhancement. Both American Robins and Common Grackles harbor MF – i.e., *Eufilaria* sp. and *Chandlerella quiscali* von Linstow, respectively. We compared infection and dissemination rates in *Culex pipiens* L. mosquitoes that fed on birds with and without MF infections that had been infected with West Nile virus (WNV). At moderate viremias, about 10^7 plaque-forming units (pfu)/ml of blood, there were no differences in infection or dissemination rates among mosquitoes that ingested viremic blood from a bird with or without microfilaremia. At high viremias, $>10^{8.5}$ pfu/ml, mosquitoes feeding on a microfilaremic Grackle with concurrent viremia had significantly higher infection and dissemination rates than mosquitoes fed on viremic Grackles without microfilaremia. Microfilarial enhancement depends on the specific virus, MF, and mosquito species examined. How virus is introduced into the hemocoel by MF differs between the avian/WNV systems described here (i.e., leakage) and various arboviruses with MF of the human filarid, *Brugia malayi* (i.e., co-transport). Additional studies are needed to determine if other avian species and their MF are involved in the microfilarial enhancement of WNV in nature.

KEY WORDS: West Nile virus, *Culex pipiens*, American Robin, Common Grackle, filaria, microfilarial enhancement of arboviral transmission

West Nile virus (WNV), a member of the genus *Flavivirus*, family *Flaviviridae,* was introduced into the Americas in 1999 (CDC, 1999). Since then, WNV has spread throughout the Americas and ~3,000 human cases have been reported in the United States every year since 2003 (CDC 2020). While WNV has been isolated from many mosquito species, *Culex pipiens* L. has be incriminated as one of the principal vectors in the eastern United States (Turell et al. 2000, Andreadis 2012). However, many factors affect the ability of a mosquito to serve as a vector (Hardy et al. 1983, Hardy 1988). In order for the mosquito to be a competent vector, the virus must be able to infect the cells of the midgut. The inability of the virus to infect the midgut is known as a "midgut barrier" (Chamberlain and Sudia 1961). If the virus is able to infect the midgut, it still needs to be able to disseminate to the rest of the mosquito's body before it can be transmitted. The inability of a virus to disseminate from the midgut to the hemocoel is known as a "midgut escape barrier" (Kramer et al. 1981, Hardy et al. 1983). For *Cx. pipiens* and WNV, once the midgut barriers are overcome, nearly all individuals are able to transmit WNV by bite (Turell et al. 2000, 2001). Therefore, any mechanism that effectively bypasses midgut barriers will greatly increase the potential transmission of arboviruses by *Cx. pipiens* and other species.

In nature, enzootic transmission of arboviruses, like WNV, probably does not occur in isolation. More likely, transmission cycles of enzootic arboviruses occur within the context of other pathogen transmission cycles circulating within a vertebrate host population. One such example of a biotic interaction is that of arboviruses and pre-existing filarial infections – specifically, blood microfilariae (MF). Mosquitoes and biting midges feeding on blood with concurrent viremias and microfilaremias have consistently been shown to have significantly higher viral dissemination rates (Mellor and Boorman 1980; Turell et al. 1984b; Vaughan and Turell 1996, 2017; Vaughan et al. 1999, 2009; Zytoon et al. 1993) than those that fed on blood

with similar viremias, but without MF. This effect is known as microfilarial enhancement (Vaughan and Turell 1996).

This study examined the potential of microfilarial enhancement of WNV transmission in two bird species known to be susceptible to WNV and to participate in its enzootic transmission within the United States. American Robins (*Turdus migratorius* L.) are believed to be one of the most important amplifying hosts for WNV and are the primary blood source for *Cx. pipiens* in the eastern United States (Molaei et al. 2006, Savage et al. 2007, Simpson, et al. 2009). However, little is known about the prevalence, transmission biology, or the species of filarial nematodes that parasitize Robins. Conversely, the role of Common Grackles (*Quiscalus quiscula* L.) as amplifying hosts for WNV is generally less appreciated but more information exists with regard to its filarial parasites. There are at least two species of filarial nematodes that produce microfilaremia in Grackles and five species that produce microfilaremia in Robins (Table 1). The primary filarial parasite of Grackles is *Chandlerella quiscali* von Linstow. Prevalence of MF infections in Grackle and Robin populations also varies according to location, but overall, prevalence is higher in Grackles than in Robins (Table 1). It should be noted that most blood surveys of birds are conducted during daylight hours. This can result in underestimates of active MF infections. That is because most species of blood-inhabiting avian MF exhibit nocturnal periodicity (Anderson 2000, Hibler 1963, Odetoyinbo 1960, Vaughan et al. 2012). Avian MF may be scarce within the peripheral blood during the day, making infections easy to overlook.

Nocturnal periodicity may also explain why there is a paucity of information regarding the intensity of microfilarial infections in birds. With regard to microfilaremias of *C. quiscali* MF in Grackles, our counts of MF from 34 individual Grackles, taken at night during peak microfilaremia using methods described below, combined with nighttime count data from Odetoyindo (1960) (n = 10 birds), yielded an overall geometric mean of 22.5 MF per 20 µl of

blood during peak microfilaremia. However, the variation among individual Grackles was considerable, ranging from 3 to 896 MF per 20 µl blood. This is undoubtedly reflective of the varying number of reproductive adult worms that may be present in the brains of infected Grackles. For *Eufilaria* infections in Robins, our MF counts based on three individual Robins, yielded a geometric mean of 7 MF per 20 µl blood (range = 1 to 53). This suggests that microfilaremia in Robins may be less intense than in Grackles.

Nocturnal periodicity of avian MF is relevant to WNV transmission because the primary mosquito vectors of WNV – i.e., *Culex* spp. – generally feed on birds at night when MF are most abundant within the peripheral circulation of the bird. Both Robins and Grackles are involved in the enzootic transmission of WNV and are known to harbor MF infections. Thus, if microfilarial enhancement is important in the enzootic transmission of WNV, then Robins and Grackles are appropriate species to investigate.

MATERIALS AND METHODS

Ethical Approval

Research was conducted under IACUC approved protocols from both the University of North Dakota and the US Army Medical Research Institute of Infectious Diseases in compliance with the Animal Welfare Act, PHS Policy, and other Federal statutes and regulations relating to animals and experiments involving animals. Both facilities where this research was conducted were accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International and adhere to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011. Collection, transport, and experimentation with migratory birds were conducted under the authority and approval of a U.S.

Fish & Wildlife scientific collection permit (MB072162) and annual state collecting permits from North Dakota and Minnesota.

Mosquitoes

Two strains of *Cx. pipiens* were used. One (Rutgers) was derived from larvae collected in the 1980's at the Edgeboro Landfill in East Brunswick, NJ, and provided by Dina Fonseca at Rutgers University. It had been maintained at the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) since 2011 (F>200). The second (Area B) was derived from larvae collected at Fort Detrick, MD in June of 2010 (F~30). Both strains were maintained in colonies in a Biosafety Level (BSL)-2 insectary at USAMRIID and maintained at 26ºC with a 16:8 (L:D) photoperiod until used in these studies.

Birds

American Robins (*Turdus migratorius* L., Passeriformes: Turdidae) were collected from Roseau and Pennington Counties, MN, in spring and early summer (April – June) using mist nets. Common Grackles (*Quiscalus quiscala* L., Passeriformes: Icteridae) were collected from Grand Forks Co., ND, in spring (April through May) using wire-mesh ground traps baited with bread cubes. All birds were screened for MF, trypanosomes, haemosporidia (*e.g., Plasmodium*) and antibodies to WNV. Because avian blood MF exhibit nocturnal periodicity (Hibler 1963, Odetoyinbo 1960, Vaughan et al. 2012), birds were held in outdoor cages after capture and bled at ~midnight to detect microfilaremias. For each bird, 30-50 µl of blood was collected into heparinized capillary tube after pricking the brachial wing vein with a sterile 26-gauge syringe needle. Tubes were centrifuged for ~2 minutes in a hematocrit centrifuge. Spun tubes were positioned in the slide holder of a compound microscope and the interface between serum and cell pack was examined at 200x for motile MF and trypanosomes (Collins 1971). To estimate host microfilaremia, the volume of blood in each tube was noted. Then while viewing the

capillary tube at 200x, the tube was slowly rotated within the slide holder with a lightly-moistened finger and the tube moved from the buffy coat out along the entire length of the serum column using the stage control knob. All MF were counted and the total was adjusted for the blood volume within the tube in order to obtain an estimate of the absolute density of MF within the microfilaremic bird from which the sample had been taken. Tubes were then scored with a glass cutter and snapped in two at the blood-serum interface. The cell pack was expelled into a labelled microfuge tube and used for molecular screening of hemosporidian parasites as described by Hellgren et al. (2004). The serum was expelled into a labelled microfuge tube and used for serological testing of WNV antibodies using an epitope-blocking enzyme-linked immunosorbent assay as described by Blivitch et al. (2003). Only birds that tested negative for antibodies against WNV were selected for use in WNV infectivity trials. Select birds were transported in groups of 2-3 birds within modified plastic dog kennels (Petco Animal Supplies, San Diego, CA) via commercial airline from Grand Forks, ND, to Dulles International Airport, VA, where they were driven by car to the USAMRIID facilities and housed in standard bird cages in the BSL-3 suite for an acclimatization period of $\geq$ 24 hour prior to use in experiments. A total of six Robins (=three microfilaremic plus three non-microfilaremic) and nine Grackles (=five microfilaremic plus four non-microfilaremic) were used in the WNV infectivity trials.

Microfilariae in mosquito midguts

The natural vectors for the MF species examined in this study (*C. quiscali* and *Eufilaria* spp.) are *Culicoides* midges, not *Culex* sp. mosquitoes (Anderson 2000, Bain 1980, Hibler 1963, Robinson 1971). Therefore, prior to conducting infectivity studies with WNV, preliminary studies were conducted with microfilaremic birds to determine whether *Cx. pipiens* would ingest MF during blood feeding on microfilaremic birds and if so, how efficient were MF at penetrating the midguts of engorged *Cx. pipiens*. Microfilaremias were determined as described above. Birds

were weighed and anesthetized prior to mosquito exposure. Grackles received intramuscular injections of 2 mg ketamine : 0.4 mg xylazine per 100 g body weight and Robins received 5 mg ketamine : 0.2 mg xylazine per 100 g body weight. Blood-fed mosquitoes were held overnight at ~26°C to allow MF to penetrate the midgut. The next day, mosquitoes were aspirated into 70% ethanol and transferred to chilled saline. The midguts were excised intact and placed into 20 µl of 5% acetic acid (vinegar) to lyse erythrocytes and immobilize the MF. Preparations were placed on microscope slides, covered with coverslips, and the total number of MF in a sample was counted at 200x magnification. To quantify MF penetration, eviscerated carcasses were minced in ~50 µl of buffered saline and preparations were examined for MF at 200x magnification.

Virus

We used the crow 397-99 strain of WNV that was obtained from the brain of a crow, *Corvus brachyrhynchos* Brehm, which died in the Bronx, NY, in September, 1999 (Turell et al. 2000). The virus had been passaged twice in Vero cell culture and stocks frozen at -80°C before use in this study. Mosquito and bird blood suspensions were tested for infectious WNV by plaque assay on African green monkey kidney (Vero) cell monolayers. Procedures were similar to those of Gargan et al. (1983), except that the second overlay, containing neutral red, was added 2, rather than 4, d after the initial assay. Viral titers were expressed as Logarithm_{10} plaque-forming units (pfu) per mosquito or per ml for viremia titers.

Vector Competence Studies

Birds were inoculated subcutaneously with 0.2 ml of WNV ($10^{6.1}$ pfu) in the late afternoon/early evening between 1700 and 1930 h and mosquito feedings were conducted on the birds for three successive nights. Mosquito feedings had to be conducted at night (~1200 to 0130 h) to coincide with nocturnal appearance of MF. Therefore, at intervals of ~30, 54 and 78 h after birds were

injected with WNV, groups of *Cx. pipiens* mosquitoes (6 – 8 days post-eclosion) were fed on anesthetized birds. The Grackles and Robins were anesthetized as described above. Anesthetized birds were placed through the cotton stockinette of individual cylindrical 3.8-liter screen-topped cardboard cages containing ~50 mosquitoes. Mosquitoes were allowed ~30 minutes to engorge on the birds which occurred primarily on the bare skin of the eyelids, periorbital region, and base of the beak. After 30 min, any mosquitoes still attached were gently blown and brushed off and the birds were removed and returned to their respective birdcages to recover. Unfed mosquitoes were removed and placed into a fresh cage and were used to feed on the same bird the following night. Partially-fed mosquitoes were discarded. Following the termination of each feed, six to eight fully engorged mosquitoes were removed from each cage. Three of the mosquitoes were triturated and tested by plaque assay to determine the viremia at the time of feeding (Kading et al. 2014). Three to five of the mosquitoes were dissected to estimate how many MF had been ingested using the methods described above.

To determine infection and dissemination rates, we froze a subsample of the mosquitoes at –20°C for (5 min) at 3, 4, 5, or 7 d after the infectious blood meal. This time period was selected because when *Cx. pipiens* are fed WNV only and held thereafter at 26°C, they rarely develop a disseminated infection before 7 d after the infectious blood meal (Dohm et al. 2002). Earlier-than-normal dissemination of WNV in *Cx. pipiens* fed on dually-infected birds would indicate evidence of microfilarial enhancement. Bodies and legs were triturated separately in 1 ml of diluent. These suspensions were stored at –80°C until tested for virus by plaque assay. Presence of virus in a mosquito's body indicated infection, while virus in the legs indicated the mosquito had a disseminated infection (Turell et al. 1984a).

Host viremia strongly influences resulting viral infection and dissemination rates in mosquitoes (Chamberlain and Sudia 1961, Hardy et al. 1983, Hardy 1988). Therefore, fair

comparisons for outcomes between our two experimental treatments (i.e., singly versus dually infected birds) could only be made between groups of mosquitoes having fed on birds with comparable viremias. The range of host viremias was similar among the six Robins used in these trials but host viremias were more variable with the nine Grackles. Therefore, mosquitoes fed on Grackles were split into two categories for analysis purposes – mosquitoes that had fed on birds with 'moderate' viremias ($10^{6.1}$ to $10^{7.7}$ pfu/ml) and mosquitoes that had fed on birds with 'high' viremia ($\geq 10^{8}$ pfu/ml). Rates of infection and dissemination among groups were distinguished statistically with Fisher exact tests and Chi square tests using a p-value of 0.05.

Serial dilution "spin-and-wash" experiment

To test the potential adherence of WNV to MF, *C. quiscali* MF and virus were harvested from dually infected Grackles at mid-morning following the third day of WNV viremia. Microfilariae of nocturnally periodic filarids typically recede from the peripheral circulation at sunrise and become sequestered in the alveolar capillaries of the lungs until the next night when they leave alveolar capillaries, move into the general circulation, and reappear in peripheral blood (Hawking and Thurston 1951). Thus, enormous numbers of MF can be collected from the lungs of microfilaremic birds during the day. Grackles were humanely euthanized and their lungs were removed. The lungs were lightly teased apart with jeweler's forceps and gently agitated in ~5 ml of Medium 199 with Earle's salts (E-199) to release MF from the capillaries. The chunks of lung were discarded and ~4 ml of the remaining 'slurry' of MF and alveolar tissue was transferred to centrifuge tubes and centrifuged for 5 minutes at 3,000 rpm, resulting in a dark red pellet (blood/tissue) with a white band (MF) just above it. The supernatant and most of the white band containing MF were carefully pipetted and transferred to a clean tube, leaving behind unwanted blood and other host debris. The suspension, now containing WNV and 'semi-purified' MF from dually-infected birds, was brought up to a final volume of 3 ml of E-199. The suspension was

centrifuged as before to pellet the MF. This time, exactly half of the supernatant (1.5ml) was removed and placed into a clean tube. Thus, the two starting tubes contained the same concentration of virus except one contained only virus, whereas the other contained virus and MF. Both tubes were brought up to a final volume of 3 ml E-199 and incubated for 1 hour at room temperature, being gently agitated every 5-10 minutes to ensure mixing of MF and virus. After the incubation, tubes were vortexed and a tiny subsample (10µl) was removed from each tube, acidified in 20 µl vinegar, and the MF were counted, as described above. Tubes were then centrifuged for 5 minutes at 3,000 rpm to pellet the MF and 2.7 ml of the supernatant was removed and stored at -80°C for later viral quantification. The MF pellet and control pellet (i.e., virus only tube) were re-suspended by adding 2.7 ml of fresh medium. Tubes were vortexed for several minutes to ensure mixing and allowed to incubate for 1 to 2 hours, then sampled for MF and centrifuged again at 3,000 rpm to pellet the MF. This procedure of washing the MF with serial 10-fold dilutions was repeated multiple times in order to dilute virus beyond the theoretical limits of detection.

RESULTS

Viremias in Grackles and Robins with and without blood parasitemias

The course of viremia in Robins and Grackles was similar (Fig. 1). Viremias peaked on Days 1 and 2 after inoculation and subsided by Day 3. In terms of blood parasites, the only blood parasites detected in the five microfilaremic Grackles were *C. quiscali* MF; no other adventitious blood parasites were present. The four non-microfilaremic Grackles were free of blood parasites. However, this was not the case with the Robins. Two of the three microfilaremic Robins and two of the non-microfilaremic Robins were also infected with *Plasmodium* (i.e., avian malaria). All three microfilaremic Robins were also infected with trypanosomes whereas none of the non-

microfilaremic Robins were infected with trypanosomes. Two of the Robins were triply infected with MF, *Plasmodium* and trypanosomes. Only one of the six Robins had no blood parasites. Nevertheless, none of these blood parasites appeared to alter WNV levels as the viremias in both Grackles and Robins were similar whether or not they were co-infected with MF, *Plasmodium* or trypanosomes.

Mosquito ingestion of microfilariae

During WNV infectivity studies, 26% of 27 mosquitoes examined ingested *Eufilaria* MF after feeding on the three microfilaremic Robins. The geometric mean number ingested was 1.6 MF; ranging from 1 to 5 MF per mosquito. In contrast, 100% of 55 mosquitoes examined ingested *C. quiscali* MF after feeding on the five microfilaremic Grackles, with a geometric mean of 45.3 MF ingested. Numbers of *C. quiscali* MF ingested were highly variable, even amongst mosquitoes feeding on the same bird at the same time (overall range was 3 to 425 MF per mosquito).

West Nile virus infection in *Cx. pipiens* that had fed on Robins and Grackles

Robins. When *Cx. pipiens* fed on Robins with WNV viremias ~$10^{6.7}$ pfu/ml, about 85% of the mosquitoes became infected. There was no difference (Fisher's exact test, $p = 1.0$) in either infection or dissemination rates between *Cx. pipiens* that fed on Robins with or without *Eufilaria* spp. MF (Table 2). Likewise, there were no differences in WNV infection rates between *Cx. pipiens* fed on Robins with or without blood protozoan infections (Table 3).

Grackles. When fed on Grackles with WNV viremias ~$10^{7.0}$ pfu/ml, about 36% of *Cx. pipiens* mosquitoes became infected (Table 4). While the infection rate was not significantly greater (Fisher's exact test, $p > 0.05$) in the mosquitoes that concurrently ingested *C. quiscali*, this apparent difference was because more of the Area B strain than the Rutgers strain fed on the microfilaremic Grackles and more of the Rutgers strain than the Area B strain fed on the

amicrofilaremic Grackles. When the MF status of the Grackle was ignored, significantly more of the Area B strain (45%, 157/349) were infected than the Rutgers strain (32%, 69/217) (Fisher's exact test, $p = 0.002$). There was no difference in dissemination rates between those *Cx. pipiens* that fed on Grackles with or without *C. quiscali* MF (Fisher's exact test, $p = 1.0$). However, when *Cx. pipiens* fed on Grackles with WNV viremias ~$10^{8.3}$ pfu/ml, both infection and dissemination rates were significantly higher in the mosquitoes that concurrently ingested *C. quiscali* and WNV than in mosquitoes that ingested WNV alone (Fisher's exact test, $p < 0.0001$, Table 5).

Serial dilution "spin-and-wash"

The affinity of WNV to associate with *C. quiscali* MF was examined by harvesting the MF from dually-infected Grackles and washing the MF using repeated cycles of centrifugation and resuspension. Parallel procedures were performed with samples from the exact same birds from which the MF had been removed beforehand. The starting virus concentrations for samples prepared from the two birds were $10^{2.3}$ and $10^{2.6}$ pfu/ml. With each successive centrifugation and 10-fold resuspension, virus concentration in the supernatants of both MF-positive and MF-negative samples diminished in a logarithmic fashion so that by the second or third wash, virus was depleted (Table 6). The MF densities in both MF-positive samples remained essentially the same throughout the spin-and-wash procedure.

DISCUSSION

This study examined whether or not naturally occurring microfilarial infections of American Robins and Common Grackles could enhance the dissemination of WNV into the hemocoel of *Cx. pipiens* mosquitoes. There were several notable findings. First, microfilaremias did not affect the intensity or dynamics of WNV viremia in either bird species (Fig. 1). This differs from

306 reports with retroviral-MF co-infections, where viral loads are higher in microfilaremic versus
307 non-microfilaremic hosts (Dietze et al. 2016, Kroidl et al. 2016).

308 Second, the frequency and numbers of MF ingested were different in mosquitoes when
309 fed on microfilaremic Robins versus microfilaremic Grackles. When fed on microfilaremic
310 Robins, the frequency (26%) and density (1.6 MF/mosquito) of *Eufilaria* MF ingested by
311 mosquitoes were much lower than that observed for mosquitoes fed on microfilaremic Grackles
312 (*i.e,* 100% ingested *C. quiscali* MF at densities ~45 MF/mosquito). To investigate midgut
313 penetration by the MF of these two filarial species, we conducted additional trials using
314 microfilaremic-only birds (*i.e*., no WNV). In those trials, ingested *Eufilaria* MF failed to
315 penetrate *Cx. pipiens* midguts whereas the higher densities of ingested *C. quiscali* MF penetrated
316 the midguts of roughly one in three engorged *Cx. pipiens* (Suppl. Table S1). Since penetration of
317 the midgut is required for microfilarial enhancement to occur, failure to observe midgut
318 penetration by *Eufilaria* MF in Robins suggested that microfilarial enhancement in Robins may
319 not occur or if it does, it is an exceedingly rare event.

320 Third, microfilarial enhancement of WNV dissemination was observed only in a single
321 Grackle that developed a very high viremia. For both Grackles and Robins that produced
322 moderate viremias of ~10^7 pfu/ml, the dissemination rates of WNV in *Cx. pipiens* were similar
323 whether or not a bird was co-infected with MF (Tables 2 and 4). However, when host viremias
324 were high (~$10^{8.5}$ pfu/ml), subsequent infection and dissemination rates were significantly higher
325 in the *Cx. pipiens* that ingested *C. quiscali* MF and WNV as compared to mosquitoes that
326 ingested WNV alone ($p<0.0001$, see Table 5).

327 One obvious mechanism of microfilarial enhancement involves the passive leakage of
328 virus from blood meal into hemocoel through microscopic fissures produced by MF as they
329 penetrate the mosquito midgut. Although this may occur, previous studies indicate that

microfilarial enhancement is not necessarily confined to mere leakage. A more efficient route of virus introduction into the mosquito hemocoel can involve the active association between MF and virus that allows for co-transport of virus by MF into the mosquito hemocoel (Vaughan and Turell 2017). To investigate the potential association and/or adhesion of WNV to avian MF, we conducted spin-and-wash experiments with *C. quiscali* MF. In similar spin-and-wash experiments conducted with two different alphaviruses and the human MF, *Brugia malayi* (Brug), all virus was diluted out of the sample without MF but virus remained present in samples containing *B. malayi* MF for many dilutions past where it should have been diluted out (Vaughan and Turell 2017). However, this was not what we observed with WNV and *C. quiscali* MF. Instead, WNV was easily diluted and washed free of the MF (Table 4), indicating that WNV does not tightly associate or adhere to *C. quiscali* MF during a co-infection. Thus, there is probably minimal co-transport of WNV into the hemocoel of *Cx. pipiens* during midgut penetration by *C. quiscali* MF. Nevertheless, a small amount of blood meal leakage could still occur as the result of MF penetrating the mosquito midgut.

Previous examples of microfilarial enhancement come mostly from work using *Brugia* spp. MF. *Brugia* filarids are mammalian parasites transmitted naturally by mosquitoes and have MF (177 to 230 μm) that are larger than *Eufilaria* MF (95-123 μm) or *C. quiscali* MF (178-193 μm) – species transmitted in nature by *Culicoides* midges, not mosquitoes (Anderson 2000, Bain 1980, Hibler 1963, Robinson 1971). The degree of tissue damage inflicted on *Cx. pipiens* midguts by *C. quiscali* MF after ingestion is unknown, but it is probably less than what occurs with larger MF specifically adapted to develop within mosquitoes (e.g., *Brugia, Wuchereria,* etc.). Even with moderately high viremias, the amount of fluid that "leaks" through the midgut as the result of *C. quiscali* MF penetration may be so small, that it is unlikely to contain even one infectious virion. However, with an extremely high viremia, as observed in the current study,

leakage of virions may become possible and result in enhanced WNV dissemination. Thus, in the case of *C. quiscali* MF, the mechanism of microfilarial enhancement is probably restricted to midgut leakage, and active co-transport of virus by MF (as seen with *B. malayi* MF) is not involved. Microfilarial enhancement of WNV by filarial infections in Grackles may occur, but only when there is very high concentration of virus present in the blood meal (Table 5).

In conclusion, our results suggest that microfilarial infections of Robins probably play no role in enhancing WNV dissemination in *Cx pipiens*. Microfilarial infections in Grackles can enhance WNV dissemination in *Cx. pipiens*, but only in Grackles experiencing very high viremias. Even though these results seem to place a major constraint on microfilarial enhancement of WNV transmission by avian MF, it is important to note that in the case of Grackles, there are populations in the Midwest in which nearly every adult Grackle is microfilaremic with *C. quiscali* (see Table 1). The sheer abundance of this parasite increases the probability that microfilarial enhancement could augment the importance of Grackles as an amplifying host for WNV in some locations. Furthermore, the prevalence of a related microfilarial species, *Chandlerella chitwoodae* Anderson, in American Crows can also be quite high (>60%; Bartlett and Anderson 1980, Robinson 1955, Vaughan et al. 2012). This could be important because the American Crow is a bird species known to develop extremely high viremias at levels that can exceed 10^{10} pfu/ml (Komar et al. 2003, Bunning et al. 2007). Thus, microfilarial enhancement in passerine birds may contribute to a greater or lesser extent to enzootic maintenance of WNV, depending on the bird species and filarial parasite involved.

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Disclaimers:

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Table 1. Prevalence estimates of microfilarial infections within populations of Common Grackles and American Robins throughout the central United States of America, as determined by examination of lung tissue (necropsy) or peripheral blood collected at night.

Bird Species	Locality	n	% MF [1]	Filarial species	Basis for MF identification	Reference
Common Grackle	North Dakota / Minnesota	777	11%	*Chandlerella quiscali*	Morphology, DNA seq.	Vaughan, unpubl. data, 2005 - 2018
			0.1%	*Eufilaria* sp.	Morphology	
	Iowa	112	62%	*Chandlerella quiscali*	Morphology	Odetoyinbo 1960
	Arkansas	71	87%	*Chandlerella quiscali*	Morphology	Johnson 1984
	Illinois	203	98%	*Chandlerella quiscali*	Morphology	Granath 1980
	Indiana	184	100%	*Chandlerella quiscali*	Morphology	Welker 1962
	Ohio	42	24%	*Chandlerella quiscali*	Morphology	Robinson 1961, Buck et al. 1975
American Robin	North Dakota / Minnesota	160	20%	*Eufilaria* sp.	Morphology	Vaughan, unpubl. data, 2005 - 2014
			2%	*Cardiofilaria* sp.	Morphology	
	Illinois	63	8%	*Splendidofilaria* sp.	DNA seq.	Hamer et al. 2013
			3%	*Chandlerella quiscali*	DNA seq.	
			3%	Unidentified sp.	DNA seq.	
	Ohio	11	9%	Unidentified sp.	not reported	Robinson 1961

[1] Percentage of birds containing microfilariae (MF).

Table 2. Rates of West Nile virus (WNV) infection and dissemination in *Culex pipiens* mosquitoes after feeding on infected American Robins with comparable levels of viremia (6.7 ± 0.3 PFU/ml) with or without co-infections of *Eufilaria* sp. microfilariae (MF).

Microfilaria Status of Robin	No. MF ingested[1]	No. of Feeds	No. mosquitoes tested for WNV	Infection Rate[2]	Dissemination Rate[3]
Amicrofilaremic	0	3	62	85% (53)	3% (2)
Microfilaremic	1.6	3	88	84% (74)	2% (2)

[1] At time of the infectious feeding, 26% of 27 mosquitoes examined ingested *Eufilaria* MF after feeding on microfilaremic Robins. The geometric mean number of MF ingested was 1.6 MF per mosquito, with a range of one to five MF per mosquito.

[2] Percent of mosquitoes infected with WNV (number of infected mosquitoes)

[3] Percent of mosquitoes with a disseminated WNV infection (number of mosquitoes with disseminated infections)

Table 3. Rates of West Nile virus (WNV) infection in *Culex pipiens* mosquitoes (Area B strain) after feeding on American Robins with comparable levels of viremias with or without co-infection of blood parasites, including microfilariae (MF), *Plasmodium,* or trypanosomes.

Host Parasite Infection Status	Host Viremia (log PFU/ml)	No. of Feeds	No. Mosquitoes Tested for WNV	Infection Rate[1]	p-value
Plasmodium	7.2 – 7.4	2	34	100% (34)	0.125
No blood parasites	7.3	1	35	91% (32)	
MF + trypanosomes	7.3	1	21	71% (15)	0.057
No blood parasites	7.3	1	35	91% (32)	
MF + *Plasmodium* + trypanosomes	6.7	1	14	86% (12)	0.305
No blood parasites	6.7	1	34	74% (25)	
MF + trypanosomes +/- *Plasmodium*	6.7-7.3	2	69	77% (27)	0.60
No blood parasites	6.7-7.3	2	35	83% (57)	

[1]Percent of mosquitoes infected with WNV (number of infected mosquitoes)

Table 4. Rates of West Nile virus (WNV) infection and dissemination in *Culex pipiens* mosquitoes after feeding on infected Common Grackles with comparable levels of viremia (7.0 ± 0.7 PFU/ml) with or without co-infections of *Chanderella quiscali* microfilariae (MF).

Microfilaria Status of Grackle	No. MF ingested[1]	No. of Feeds	No. mosquitoes tested for WNV	Infection Rate[2]	Dissemination Rate[3]
Amicrofilaremic	0	6	228	34% (78)	5% (11)
Microfilaremic LOW	12-49	5	212	41% (91)	5% (10)
Microfilaremic HIGH	138-143	2	88	47% (41)	1% (1)
Microfilaremic ALL	12-143	7	300	44% (132)	4% (11)

[1] Denotes the range in geometric mean number of MF ingested per mosquito observed in separate feedings. All 27 mosquitoes examined ingested *C. quiscali* MF after feeding on microfilaremic Grackles. Microfilariae densities are grouped by low and high densities. The overall geometric mean number of MF ingested for all feeding combined was 47 MF per mosquito.

[2] Percent of mosquitoes infected with WNV (number of infected mosquitoes)

[3] Percent of mosquitoes with a disseminated WNV infection (number of mosquitoes with disseminated infections)

Table 5. Rates of West Nile virus (WNV) infection and dissemination in *Culex pipiens* mosquitoes after feeding on infected Common Grackles with comparable levels of viremia (8.3 ± 0.4 PFU/ml) with or without co-infections of *Chanderella quiscali* microfilariae (MF).

Microfilaria Status of Grackle	No. MF ingested[1]	No. of Feeds	No. mosquitoes tested for WNV	Infection Rate[2]	Dissemination Rate[3]
Amicrofilaremic	0	2	150	63% (95)	7% (11)
Microfilaremic	83	1	50	100% (50)	46% (23)

[1] All three mosquitoes examined ingested *C. quiscali* MF after feeding on a microfilaremic Grackle. The geometric mean number of MF ingested was 82.7 MF per mosquito, with a range of 77 to 92 MF per mosquito.

[2] Percent of mosquitoes infected with WNV (number of infected mosquitoes)

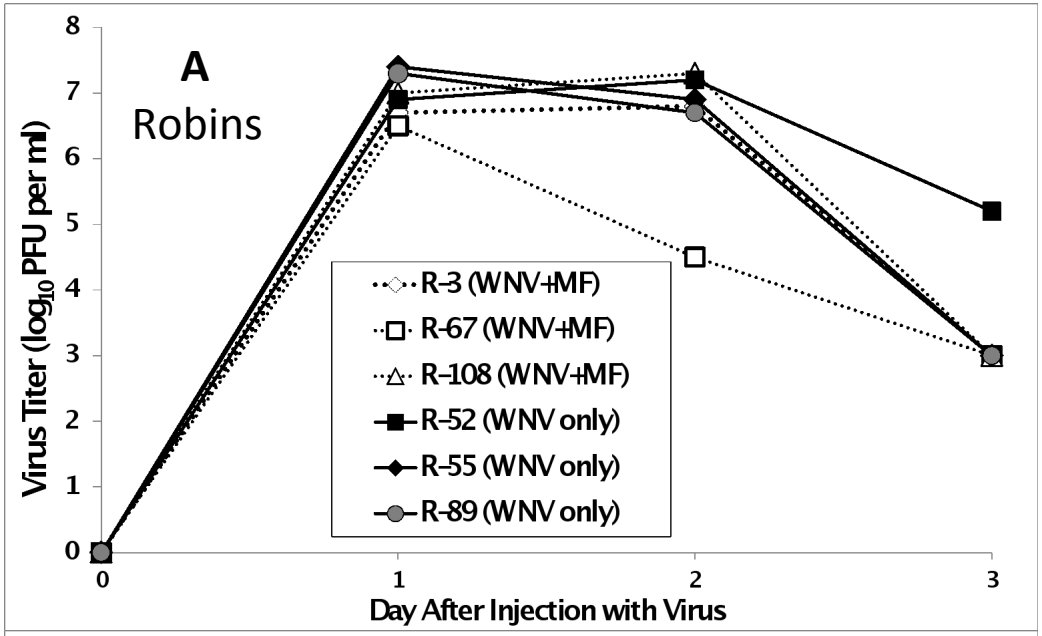
[3] Percent of mosquitoes with a disseminated WNV infection (number of mosquitoes with disseminated infections)

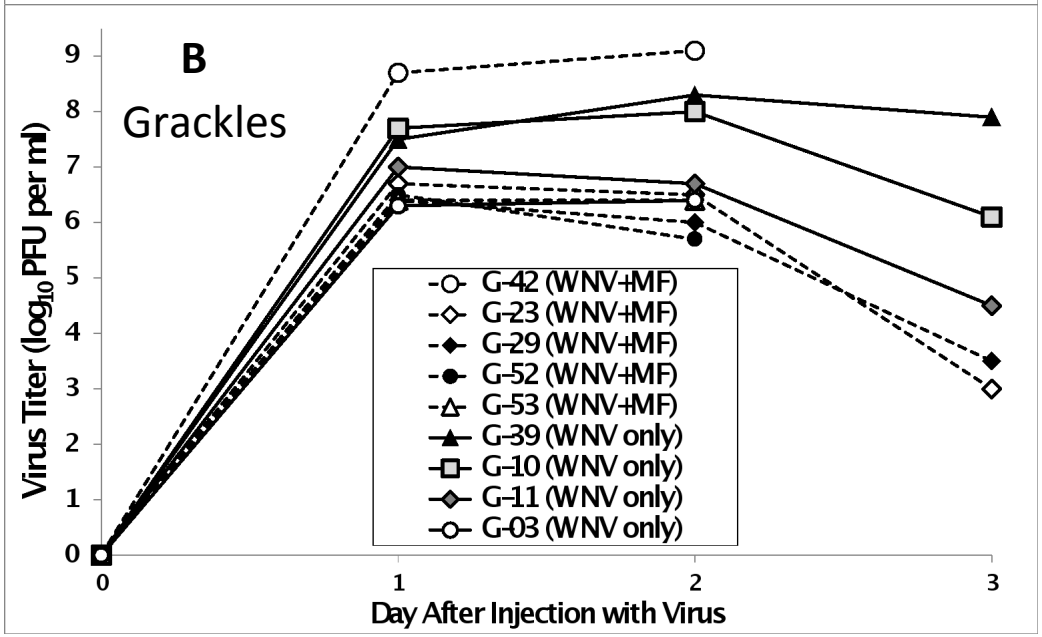
Table 6. Lack of virus adherence and affinity to *Chandlerella quiscali* microfilariae (MF) within two microfilaremic grackles 3 days after they were experimentally infected with West Nile virus (WNV). Microfilariae were harvested from the lungs of co-infected grackles and washed by repeated cycles of centrifugation, removal of supernatant, and resuspension.

Bird No.		Virus titer (log_{10} PFU per ml) of supernatant at each dilution					
		Stock	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
29	WNV - MF removed	2.6	1.7	0	0	0	NT
	WNV with MF	2.6	1.9	1.0	0	0	NT
	MF density per 10µl	-	81±1	-	59±1	-	92±16
23	WNV - MF removed	2.4	1.4	0	0	0	NT
	WNV with MF	2.3	1.5	0	0	0	NT
	MF density per 10µl	-	137±5	-	95±7	-	197±61

Figure Legend

Fig. 1. A. Course of viremia in six adult American Robins inoculated subcutaneously (0.2 ml) with $10^{6.1}$ PFU of West Nile virus (Crow 397-99 strain). B. Course of viremia in nine adult Common Grackles inoculated subcutaneously (0.2 ml) with $10^{6.1}$ PFU of West Nile virus (Crow 397-99 strain). Birds dually-infected with West Nile virus and microfilariae are denoted with dashed lines. Birds infected with only West Nile virus are denoted with solid lines.


A
Robins
Virus Titer (log10 PFU per ml)
Day After Injection with Virus
R-3 (WNV+MF)
R-67 (WNV+MF)
R-108 (WNV+MF)
R-52 (WNV only)
R-55 (WNV only)
R-89 (WNV only)


B
Grackles
Virus Titer (log10 PFU per ml)
Day After Injection with Virus
G-42 (WNV+MF)
G-23 (WNV+MF)
G-29 (WNV+MF)
G-52 (WNV+MF)
G-53 (WNV+MF)
G-39 (WNV only)
G-10 (WNV only)
G-11 (WNV only)
G-03 (WNV only)

Supplemental Table 1. Examination of the ingestion of *Eufilaria* sp. and *Chandlerella quiscali* microfilariae (MF) by *Culex pipiens* mosquitoes fed on microfilaremic Robins and Grackles respectively, and ability of ingested MF to penetrate the midguts of engorged mosquitoes within 12 hours after mosquitoes had fed.

Bird (MF species)	Bird ID	Host microfilaremia (no# MF per 3 μl)	No. mosquitoes examined	% mosquitoes ingesting MF	No. MF ingested per mosquito (range) [1]	No. MF penetrating midgut (range)	Prevalence of MF penetration
Robin (*Eufilaria* sp.)	C67	1.8	30	80%	2.0 (1-8)	0	0%
Robin (*Eufilaria* sp.)	C109	7.8	32	100%	4.2 (1-16)	0	0%
Robin (*Eufilaria* sp.)	C3	13.2	40	100%	7.8 (1-27)	0	0%
Grackle (*C. quiscali*)	304	11.7	26	96%	6.9 (1-37)	1.0 (1)	4%
Grackle [2] (*C. quiscali*)	303	103.8	49	100%	28.8 (1-347)	1.9 (1-12)	37%

[1] Excludes zero counts, *i.e.*, density equals the geometric mean number (range) of MF in the blood meals of those mosquitoes that ingested MF.

[2] Data from Vaughan et al. 2012.