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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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2 **Pre-existing microfilarial infections of American Robins (Passeriformes: Turdidae) and**

3 **Common Grackles (Passeriformes: Icteridae) have limited impact on enhancing**

4 **dissemination of West Nile virus in *Culex pipiens* mosquitoes (Diptera: Culicidae)**

5

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18 SHORT TITLE: VAUGHAN ET AL.: **Avian microfilariae and WNV transmission**

19

20

21 **ABSTRACT**

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

41
42 **KEY WORDS:** West Nile virus, *Culex pipiens*, American Robin, Common Grackle, filaria,
43 microfilarial enhancement of arboviral transmission

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

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

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

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

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

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

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

103

104 **MATERIALS AND METHODS**

105 **Ethical Approval**

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

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

116 **Mosquitoes**

117 Two strains of *Cx. pipiens* were used. One (Rutgers) was derived from larvae collected in the
118 1980's at the Edgeboro Landfill in East Brunswick, NJ, and provided by Dina Fonseca at
119 Rutgers University. It had been maintained at the United States Army Medical Research Institute
120 of Infectious Diseases (USAMRIID) since 2011 (F>200). The second (Area B) was derived from
121 larvae collected at Fort Detrick, MD in June of 2010 (F~30). Both strains were maintained in
122 colonies in a Biosafety Level (BSL)-2 insectary at USAMRIID and maintained at 26°C with a
123 16:8 (L:D) photoperiod until used in these studies.

124 **Birds**

125 American Robins (*Turdus migratorius* L., Passeriformes: Turdidae) were collected from Roseau
126 and Pennington Counties, MN, in spring and early summer (April – June) using mist nets.

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

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

155 **Microfilariae in mosquito midguts**

156 The natural vectors for the MF species examined in this study (*C. quisquali* and *Eufilaria* spp.) are
157 *Culicoides* midges, not *Culex* sp. mosquitoes (Anderson 2000, Bain 1980, Hibler 1963, Robinson
158 1971). Therefore, prior to conducting infectivity studies with WNV, preliminary studies were
159 conducted with microfilaremic birds to determine whether *Cx. pipiens* would ingest MF during
160 blood feeding on microfilaremic birds and if so, how efficient were MF at penetrating the
161 midguts of engorged *Cx. pipiens*. Microfilaremias were determined as described above. Birds

162 were weighed and anesthetized prior to mosquito exposure. Grackles received intramuscular
163 injections of 2 mg ketamine : 0.4 mg xylazine per 100 g body weight and Robins received 5 mg
164 ketamine : 0.2 mg xylazine per 100 g body weight. Blood-fed mosquitoes were held overnight at
165 ~26°C to allow MF to penetrate the midgut. The next day, mosquitoes were aspirated into 70%
166 ethanol and transferred to chilled saline. The midguts were excised intact and placed into 20 µl
167 of 5% acetic acid (vinegar) to lyse erythrocytes and immobilize the MF. Preparations were
168 placed on microscope slides, covered with coverslips, and the total number of MF in a sample
169 was counted at 200x magnification. To quantify MF penetration, eviscerated carcasses were
170 minced in ~50 µl of buffered saline and preparations were examined for MF at 200x
171 magnification.

172 **Virus**

173 We used the crow 397-99 strain of WNV that was obtained from the brain of a crow, *Corvus*
174 *brachyrhynchos* Brehm, which died in the Bronx, NY, in September, 1999 (Turell et al. 2000).
175 The virus had been passaged twice in Vero cell culture and stocks frozen at -80°C before use in
176 this study. Mosquito and bird blood suspensions were tested for infectious WNV by plaque assay
177 on African green monkey kidney (Vero) cell monolayers. Procedures were similar to those of
178 Gargan et al. (1983), except that the second overlay, containing neutral red, was added 2, rather
179 than 4, d after the initial assay. Viral titers were expressed as Logarithm₁₀ plaque-forming units
180 (pfu) per mosquito or per ml for viremia titers.

181 **Vector Competence Studies**

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

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

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

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

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

218 **Serial dilution “spin-and-wash” experiment**

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

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

248

249 **RESULTS**

250 **Viremias in Grackles and Robins with and without blood parasitemias**

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

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

263 **Mosquito ingestion of microfilariae**

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

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

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

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

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

290 **Serial dilution "spin-and-wash"**

291 The affinity of WNV to associate with *C. quiscalis* MF was examined by harvesting the MF from
292 dually-infected Grackles and washing the MF using repeated cycles of centrifugation and
293 resuspension. Parallel procedures were performed with samples from the exact same birds from
294 which the MF had been removed beforehand. The starting virus concentrations for samples
295 prepared from the two birds were $10^{2.3}$ and $10^{2.6}$ pfu/ml. With each successive centrifugation and
296 10-fold resuspension, virus concentration in the supernatants of both MF-positive and MF-
297 negative samples diminished in a logarithmic fashion so that by the second or third wash, virus
298 was depleted (Table 6). The MF densities in both MF-positive samples remained essentially the
299 same throughout the spin-and-wash procedure.

300

301 **DISCUSSION**

302 This study examined whether or not naturally occurring microfilarial infections of American
303 Robins and Common Grackles could enhance the dissemination of WNV into the hemocoel of
304 *Cx. pipiens* mosquitoes. There were several notable findings. First, microfilaremiias did not affect
305 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. quisquali* MF at densities \sim 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. quisquali* 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 $\sim 10^7$ 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 ($\sim 10^{8.5}$ pfu/ml), subsequent infection and dissemination rates were significantly higher
325 in the *Cx. pipiens* that ingested *C. quisquali* 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

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

344 Previous examples of microfilarial enhancement come mostly from work using *Brugia*
345 spp. MF. *Brugia* filarids are mammalian parasites transmitted naturally by mosquitoes and have
346 MF (177 to 230 μm) that are larger than *Eufilaria* MF (95-123 μm) or *C. quisquali* MF (178-193
347 μm) – species transmitted in nature by *Culicoides* midges, not mosquitoes (Anderson 2000, Bain
348 1980, Hibler 1963, Robinson 1971). The degree of tissue damage inflicted on *Cx. pipiens*
349 midguts by *C. quisquali* MF after ingestion is unknown, but it is probably less than what occurs
350 with larger MF specifically adapted to develop within mosquitoes (e.g., *Brugia*, *Wuchereria*,
351 etc.). Even with moderately high viremias, the amount of fluid that “leaks” through the midgut as
352 the result of *C. quisquali* MF penetration may be so small, that it is unlikely to contain even one
353 infectious virion. However, with an extremely high viremia, as observed in the current study,

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

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

374

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393 **References Cited**

394

395 **Anderson, R. C. 2000.** Nematode Parasites of Vertebrates. Their Development and
396 Transmission. 2nd ed. CABI Publishing NY.

397 **Andreadis, T. G. 2012.** The contribution of *Culex pipiens* complex mosquitoes to transmission
398 and persistence of West Nile virus in North America. J. Am. Mosq. Cont. Assoc. 28: 137-
399 151.

400 **Bain O. 1980.** Two filariae of the genus *Eufilaria* in *Turdus merula*; development in *Culicoides*
401 *nubeculosus*. [French]. Ann. Parasitol. Hum. Comp. 55: 583-590.

402 **Bartlett, C. M. and R. C. Anderson. 1980.** Filarioid nematodes (Filarioidea: Onchocercidae) of
403 *Corvus brachyrhynchos* Brehm in southern Ontario, Canada and a consideration of the
404 epizootiology of avian filariasis. Syst. Parasitol. 2: 77-102.

405 **Blitvich, B. J., R. A. Bowen, N. L. Marlenee, R. A. Hall, M. L. Bunning, and B. J. Beaty.**
406 **2003.** Epitope-blocking enzyme-linked immunosorbent assays for detection of West Nile
407 virus antibodies in domestic mammals. J. Clin. Microbiol. 41: 2676-2679.

408 **Bunning, M. L., P. E. Fox, R. A. Bowen, N. Komar, G .J. Chang, T. J. Speaker, M. R.**
409 **Stephens, N. Nemeth, N. A. Panella, S. A. Langevin, P. Gordy, M. Teehee, P. R.**
410 **Bright, and M. J. Turell. 2007.** DNA vaccination of the American crow (*Corvus*
411 *brachyrhynchos*) provides partial protection against lethal challenge with West Nile
412 virus. Avian Dis. 51: 573-577.

413 **Chamberlain, R. W., and W. D. Sudia. 1961.** Mechanism of transmission of viruses by
414 mosquitoes. Ann. Rev. Entomol. 6: 371-390.

415 **Collins, J. D. 1971.** The detection of microfilariae using the capillary hematocrit tube methods.
416 Trop. Animal Health Prod. 3: 23-25.

- 417 **Centers for Disease Control and Prevention (CDC), 1999.** Outbreak of West Nile-like viral
418 encephalitis—New York, 1999. *MMWR* 48: 845–849.
- 419 **Centers for Disease Control and Prevention (CDC), 2020.**
420 <https://www.cdc.gov/westnile/statsmaps/cumMapsData.html>
- 421 **Dietze K. K., U. Dittmer, D. K. Koudaimi, S. Schimmer, M. Reitz, M. Breloer, and W.**
422 **Hartmann. 2016.** Filariae-retrovirus co-infection in mice is associated with suppressed
423 virus-specific IgG immune response and higher viral loads. *PLoS Negl. Trop. Dis.* 2016
424 Dec 6;10(12): e0005170.
- 425 **Dohm D. J., M. L. O'Guinn, and M. J. Turell. 2002.** Effect of environmental temperature on
426 the ability of *Culex pipiens* (Diptera: Culicidae) to transmit West Nile virus. *J. Med.*
427 *Entomol.* 39: 221-225.
- 428 **Gargan, T. P., II, C. L. Bailey, G. A. Higbee, A. Gad, and S. El Said. 1983.** The effect of
429 laboratory colonization on the vector pathogen interactions of Egyptian *Culex pipiens*
430 and Rift Valley fever virus. *Am. J. Trop. Med. Hyg.* 32: 1154-1163.
- 431 **Granath, W. O. 1980.** Fate of the wild avian filarioid nematode *Chandlerella quisicali*
432 (Onchocercidae: Filarioidea) in the domestic chicken. *Poultry Sci.* 59: 996-1000.
- 433 **Hamer, G. L., T. K. Anderson, G. E. Berry, A. P. Makohon-Moore, J. C. Crafton, J. D.**
434 **Brawn, A. C. Dolinski, B. L. Krebs, M. O. Ruiz, P. M. Muzzall, T. L. Goldberg, and**
435 **E. D. Walker. 2013.** Prevalence of filarioid nematodes and trypanosomes in American
436 robins and house sparrows, Chicago USA. *Internat. J. Parasitol: Parasites Wildlife.* 2: 42-
437 49.
- 438 **Hardy, J. L. 1988.** Susceptibility and resistance of vector mosquitoes, pp. 87-126. *In* T. P.
439 Monath [ed.], *The arboviruses: epidemiology and ecology*, vol. 1. CRC, Boca Raton, FL.

- 440 **Hardy, J. L., E. J. Houk, L. D. Kramer, and W. C. Reeves. 1983.** Intrinsic factors affecting
441 vector competence of mosquitoes for arboviruses. *Annu. Rev. Entomol.* 28: 229–262.
- 442 **Hawking, F., and J. P. Thurston. 1951.** The periodicity of microfilariae. I. The distribution of
443 microfilariae in the body. *Trans. Royal Soc. Trop. Med. Hyg.* 45: 307-328.
- 444 **Hellgren, O., J. Waldenstrom, and S. Bensch. 2004.** A new PCR assay for simultaneous
445 studies of *Leucocytozoon*, *Plasmodium*, and *Haemoproteus* from avian blood. *J. Parasitol.*
446 90: 797-802.
- 447 **Hibler, C. P. 1963.** Onchocercidae (Nematoda: Filarioidea) of the American magpie *Pica pica*
448 *hudsonia* (Sabine) in northern Colorado. Ph.D. dissertation, Colorado State University.
- 449 **Johnson, A. A. 1984.** Helminths of common grackles (*Quiscalus quisicali-versicolor*, Vieillot) in
450 central Arkansas. *Arkansas Acad. Sci. Proc.* 38: 53-55.
- 451 **Kading, R. C., B. J. Biggerstaff, G. Young, and N. Komar. 2014.** Mosquitoes used to draw
452 blood for arbovirus viremia determinations in small vertebrates. *PLoS ONE* 9: e99342.
- 453 **Komar, N., S. Langevin, S. Hinten, N. Nemeth, E. Edwards, D. Hettler, B. Davis, R. Bowen,**
454 **and M. Bunning. 2003.** Experimental infection of North American birds with the New
455 York 1999 strain of West Nile virus. *Emerg. Infect. Dis.* 9: 311-322.
- 456 **Kramer, L. D., J. L. Hardy, S. B. Presser, and E. J. Houk. 1981.** Dissemination barriers for
457 western equine encephalomyelitis virus in *Culex tarsalis* infected after ingestion of low
458 viral doses. *Am. J. Trop. Med. Hyg.* 30: 190-197.
- 459 **Kroidl I., E. Saathoff, L. Maganga, W. H. Makunde, A. Hoerauf, C. Geldmacher, P.**
460 **Clowes, L. Maboko, and M. Hoelscher. 2016.** Effect of *Wuchereria bancrofti* infection
461 on HIV incidence in southwest Tanzania: a prospective cohort study. *Lancet* 388: 1912-
462 1920.

- 463 **Molaei, G., T. G. Andreadis, P. M. Armstrong, J. F. Anderson, C. R. Vossbrinck. 2006.**
464 Host feeding patterns of *Culex* mosquitoes and West Nile virus transmission,
465 Northeastern United States. *Emerg. Infect. Dis.* 12: 468-474. doi:
466 10.3201/eid1203.051004.
- 467 **Mellor, P. S., and J. Boorman. 1980.** Multiplication of bluetongue virus in *Culicoides*
468 *nubeculosus* (Meigen) simultaneously infected with the virus and the microfilariae of
469 *Onchocerca cervicalis* (Railliet & Henry), *Ann. Trop. Med. Parasitol.* 74: 463-469, DOI:
470 10.1080/00034983.1980.11687368
- 471 **Odetoyinbo, J. A. 1960.** Biology of *Splendidofilaria quiscali* (von Linstow, 1904) n. comb.
472 (Nematoda: Onchocercidae). Ph.D. dissertation. Iowa State University, Ames.
- 473 **Robinson, E. J. 1955.** Observations on the epizootiology of filarioid infections in two species of
474 the avian family Corvidae. *J. Parasitol.* 41: 209-214.
- 475 **Robinson, E. J. 1971.** *Culicoides crepuscularis* (Malloch) (Diptera: Ceratopogonidae) as a host
476 for *Chandlerella quiscali* (Von Linstow, 1904) *comb. n.* (Filarioidea: Onchocercidae). *J.*
477 *Parasitol.* 57: 772-776.
- 478 **Savage, H. M. et al. 2007.** Host choice and West Nile virus infection rates in blood-fed
479 mosquitoes, including members of the *Culex pipiens* complex, from Memphis and Shelby
480 County, Tennessee, 2002–2003. *Vector-Borne Zoonotic Dis.* 2007; 7: 365–386. doi:
481 10.1089/vbz.2006.0602
- 482 **Simpson, J. E., C. M. Folsom-O'Keefe, J. E. Childs, L. E. Simons, T. G. Andreadis, and M.**
483 **A Diuk-Wasser. 2009.** Avian host selection by *Culex pipiens* in experimental trials.
484 *PLoS One.* 4(11):e7861. doi: 10.1371/journal.pone.0007861.

- 485 **Turell, M. J., T. P. Gargan II, and C. L. Bailey. 1984a.** Replication and dissemination of Rift
486 Valley fever virus in *Culex pipiens*. *Am. J. Trop. Med. Hyg.* 33: 176-181.
- 487 **Turell, M. J., P. A. Rossignol, A. Spielman, C. A. Rossi, and C. L. Bailey. 1984b.** Enhanced
488 arboviral transmission by mosquitoes that concurrently ingested microfilariae. *Science*
489 (Wash. D.C.) 225: 1039-1041.
- 490 **Turell, M. J., T. N. Mather, A. Spielman, and C. L. Bailey. 1987.** Increased dissemination of
491 dengue 2 virus in *Aedes aegypti* associated with concurrent ingestion of microfilariae of
492 *Brugia malayi*. *Am. J. Trop. Med. Hyg.* 37: 197-201.
- 493 **Turell, M.J., M.L. O'Guinn, and J. Oliver. 2000.** Potential for New York mosquitoes to
494 transmit West Nile Virus. *Am. J. Trop. Med. Hyg.* 62: 413-414.
- 495 **Turell, M. J., M. L. O'Guinn, D. J. Dohm, and J. W. Jones. 2001.** Vector competence of
496 North American mosquitoes (Diptera: Culicidae) for West Nile virus. *J. Med. Entomol.*
497 38: 130-134.
- 498 **Vaughan, J. A., and M. J. Turell. 1996.** Dual host infections: enhanced infectivity of eastern
499 equine encephalitis virus to *Aedes* mosquitoes mediated by *Brugia* microfilariae. *Am. J.*
500 *Trop. Med. Hyg.* 54: 105-109.
- 501 **Vaughan, J. A. and M. J. Turell. 2017.** *Brugia malayi* microfilariae transport alphaviruses
502 across the mosquito midgut. *PLoS One.* 12:e0172309. doi:
503 10.1371/journal.pone.0172309. eCollection 2017.
- 504 **Vaughan, J. A., M. Trpis, and M. J. Turell. 1999.** *Brugia malayi* microfilariae (Nematoda:
505 Filaridae) enhance the infectivity of Venezuelan equine encephalitis virus to *Aedes*
506 mosquitoes (Diptera: Culicidae). *J. Med. Entomol.* 36: 758-763.

507 **Vaughan, J. A., D. A. Focks, and M. J. Turell. 2009.** Simulation models examining the effect
508 of Brugian filariasis on dengue epidemics. *Am. J. Trop. Med. Hyg.* 80: 44-50.

509 **Vaughan, J. A., J. O. Mehus, C. M. Brewer, D. K. Kvasager, S. Bauer, J. L. Vaughan, H. K.
510 Hassan, T. R. Unnasch, and J. A. Bell. 2012.** Theoretical potential of passerine
511 filariasis to enhance the enzootic transmission of West Nile virus. *J. Med. Entomol.* 49:
512 1430-1441.

513 **Welker, G. W. 1962.** Helminth parasites of the common grackle, *Quiscalis quiscula versicolor*
514 Veillot in Indiana. Ph.D. dissertation, The Ohio State University.

515 **Zytoon, E. M., H. I. el-Belbasi, and T. Matsumura. 1993.** Mechanism of increased
516 dissemination of chikungunya virus in *Aedes albopictus* mosquitoes concurrently
517 ingesting microfilariae of *Dirofilaria immitis*. *Am. J. Trop. Med. Hyg.* 49: 201-207. doi:
518 10.4269/ajtmh.1993.49.201.

519

520

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%	<i>Chandlerella quisicali</i>	Morphology, DNA seq.	Vaughan, unpubl. data, 2005 - 2018
			0.1%	<i>Eufilaria</i> sp.	Morphology	
	Iowa	112	62%	<i>Chandlerella quisicali</i>	Morphology	Odetoyinbo 1960
	Arkansas	71	87%	<i>Chandlerella quisicali</i>	Morphology	Johnson 1984
	Illinois	203	98%	<i>Chandlerella quisicali</i>	Morphology	Granath 1980
	Indiana	184	100%	<i>Chandlerella quisicali</i>	Morphology	Welker 1962
	Ohio	42	24%	<i>Chandlerella quisicali</i>	Morphology	Robinson 1961, Buck et al. 1975
American Robin	North Dakota / Minnesota	160	20%	<i>Eufilaria</i> sp.	Morphology	Vaughan, unpubl. data, 2005 - 2014
			2%	<i>Cardiofilaria</i> sp.	Morphology	
	Illinois	63	8%	<i>Splendidofilaria</i> sp.	DNA seq.	Hamer et al. 2013
			3%	<i>Chandlerella quisicali</i>	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
<i>Plasmodium</i>	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 + <i>Plasmodium</i> + trypanosomes	6.7	1	14	86% (12)	0.305
No blood parasites	6.7	1	34	74% (25)	
MF + trypanosomes +/- <i>Plasmodium</i>	6.7-7.3	2	69	77% (27)	0.60
No blood parasites	6.7-7.3	2	35	83% (57)	

¹Percent 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)

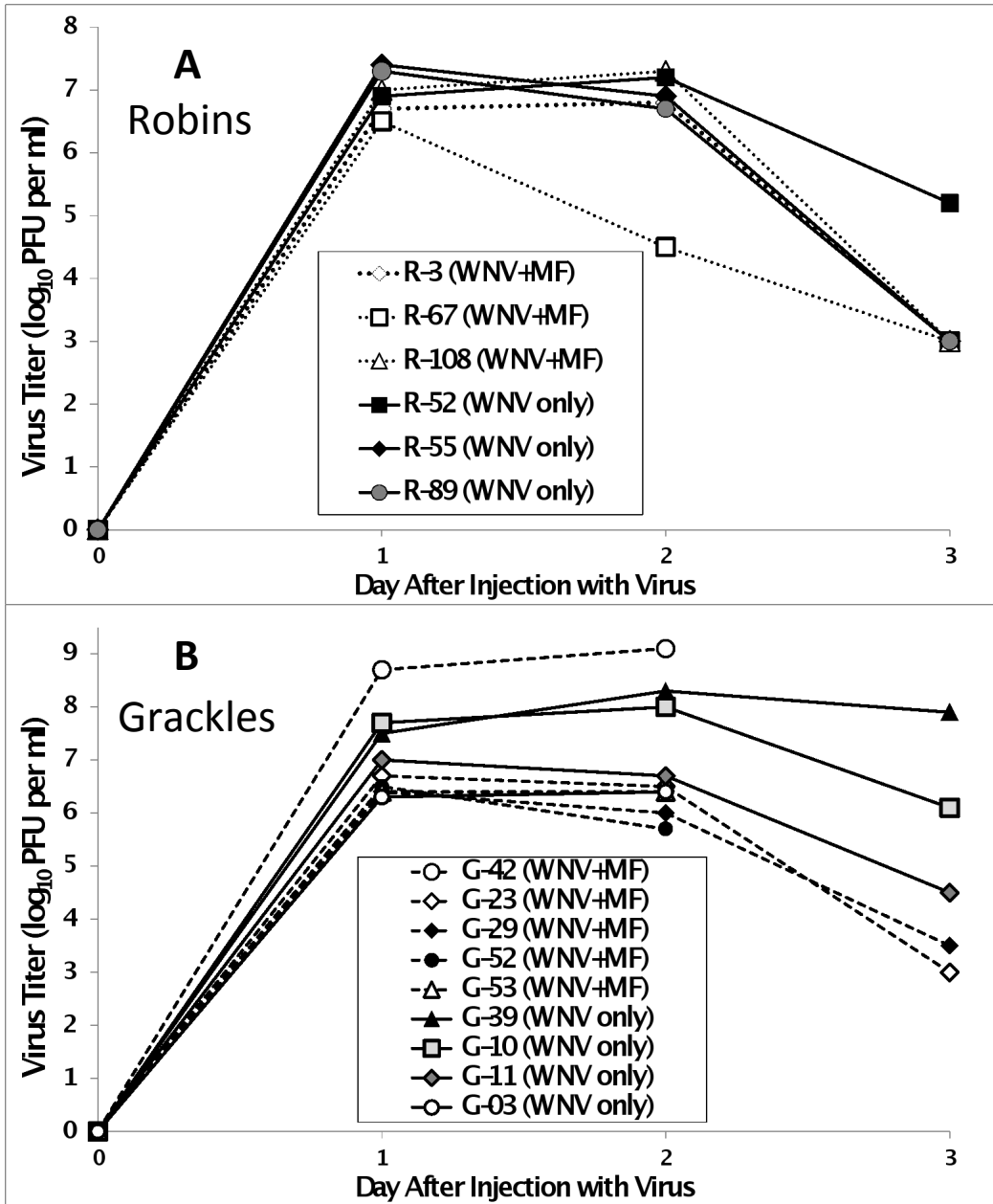
³ Percent of mosquitoes with a disseminated WNV infection (number of mosquitoes with disseminated infections)

Table 6. Lack of virus adherence and affinity to *Chandlerella quisquali* 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 10 μ l	-	81 \pm 1	-	59 \pm 1	-	92 \pm 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 10 μ l	-	137 \pm 5	-	95 \pm 7	-	197 \pm 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.



Supplemental Table 1. Examination of the ingestion of *Eufilaria* sp. and *Chandlerella quisicali* 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 μ l)	No. mosquitoes examined	% mosquitoes ingesting MF	No. MF ingested per mosquito (range) ¹	No. MF penetrating midgut (range)	Prevalence of MF penetration
Robin (<i>Eufilaria</i> sp.)	C67	1.8	30	80%	2.0 (1-8)	0	0%
Robin (<i>Eufilaria</i> sp.)	C109	7.8	32	100%	4.2 (1-16)	0	0%
Robin (<i>Eufilaria</i> sp.)	C3	13.2	40	100%	7.8 (1-27)	0	0%
Grackle (<i>C. quisicali</i>)	304	11.7	26	96%	6.9 (1-37)	1.0 (1)	4%
Grackle ² (<i>C. quisicali</i>)	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.