

1 ***Wolbachia* effects on Rift Valley fever virus infection in *Culex tarsalis* mosquitoes**

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22

## 23 **Abstract**

24 Innovative tools are needed to alleviate the burden of mosquito-borne diseases, and strategies  
25 that target the pathogen instead of the mosquito are being considered. A possible tactic is the use  
26 of *Wolbachia*, a maternally inherited, endosymbiotic bacterium that can suppress diverse  
27 pathogens when introduced to naive mosquito species. We investigated effects of somatic  
28 *Wolbachia* (strain *wAlbB*) infection on Rift Valley fever virus (RVFV) in *Culex tarsalis*  
29 mosquitoes. When compared to *Wolbachia*-uninfected mosquitoes, there was no significant  
30 effect of *Wolbachia* infection on RVFV infection, dissemination, or transmission frequencies,  
31 nor on viral body or saliva titers. Within *Wolbachia*-infected mosquitoes, there was a modest  
32 negative correlation between RVFV body titers and *Wolbachia* density, suggesting that  
33 *Wolbachia* may suppress RVFV in a density-dependent manner in this mosquito species. These  
34 results are contrary to previous work in the same mosquito species, showing *Wolbachia*-induced  
35 enhancement of West Nile virus infection rates. Taken together, these results highlight the  
36 importance of exploring the breadth of phenotypes induced by *Wolbachia*.

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## 38 **Author Summary**

39 An integrated vector management program utilizes several practices, including pesticide  
40 application and source reduction, to reduce mosquito populations. However, mosquitoes are  
41 developing resistance to some of these methods and new control approaches are needed. A novel  
42 technique involves the bacterium *Wolbachia* that lives naturally in many insects. *Wolbachia* can  
43 be transferred to uninfected mosquitoes and can block pathogen transmission to humans.  
44 Additionally, *Wolbachia* is maternally inherited, allowing it to spread quickly through uninfected  
45 field populations of mosquitoes. We studied the impacts of *Wolbachia* on Rift Valley fever virus  
46 (RVFV) in the naturally uninfected mosquito, *Culex tarsalis*. *Wolbachia* had no effects on the

47 ability of *Culex tarsalis* to become infected with or transmit RVFV. High densities of *Wolbachia*  
48 were associated with no virus infection or low levels of virus, suggesting that *Wolbachia* might  
49 suppress RVFV at high densities. These results contrast with our previous study that showed  
50 *Wolbachia* enhances West Nile virus infection in *Culex tarsalis*. Together, these studies highlight  
51 the importance of studying *Wolbachia* effects on a variety of pathogens so that control methods  
52 are not impeded.

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## 61 **Introduction**

62 Globally, mosquito-borne diseases are a major health burden. To decrease mosquito  
63 populations, control programs often use integrated vector management practices including  
64 adulticide and larvicide application, source reduction, and biological control [1]. However, these  
65 mosquito control methods are losing efficacy due to increasing insecticide resistance and  
66 changes in mosquito behavior [2–4]. With these concerns, novel and sustainable control methods  
67 are under investigation, including strategies that target the pathogen instead of the mosquito  
68 [5,6]. *Wolbachia* is a maternally-inherited endosymbiotic bacterium that infects a large number  
69 of insects and other invertebrates [7]. Infection by *Wolbachia* is not innocuous; its presence  
70 within a host can cause broad effects on host physiology. For example, natural *Wolbachia*  
71 infections in fruit flies protect against pathogen-induced mortality [8,9]. When experimentally  
72 transferred to uninfected mosquitoes, *Wolbachia* can suppress infection or transmission of  
73 viruses, *Plasmodium* parasites, and filarial nematodes [10–13]. *Wolbachia* also manipulates host  
74 reproduction in ways that allow it to spread through and persist in insect populations [14].

75 Investigations using *Wolbachia*-infected mosquitoes as a control method for dengue virus  
76 are underway [15], and field trials in Australia have indicated that *Wolbachia* can spread to near-  
77 fixation in naturally uninfected populations of *Aedes aegypti* mosquitoes [16,17]. These  
78 *Wolbachia*-infected *Ae. aegypti* populations can persist years after release, and mosquitoes retain  
79 the dengue virus-blocking phenotype [18]. Similar field experiments are being conducted in  
80 several other countries, but not all have reported successful replacement of the uninfected  
81 population with *Wolbachia*-infected mosquitoes [19].

82 The effects of *Wolbachia*-induced pathogen interference may differ depending on  
83 mosquito species, *Wolbachia* strain, pathogen type, and environment conditions [20–22]. For

84 example, in *Anopheles gambiae*, transient somatic infection of the *Wolbachia* strain wAlbB  
85 inhibits *Plasmodium falciparum* but enhances *Plasmodium berghei* parasites [22,23].  
86 Enhancement phenotypes have been observed in *Anopheles*, *Culex*, and *Aedes* mosquitoes, and  
87 across several malaria species and virus families [20,22,24–27]. Thus, it is important to examine  
88 the range of *Wolbachia*-induced phenotypes so that efficacy of disease control efforts using  
89 *Wolbachia*-induced pathogen interference are not impeded.

90 Previous work has demonstrated that transient *Wolbachia* infections in *Culex tarsalis*  
91 enhance West Nile virus (WNV) infection rates. To better understand the range of *Wolbachia*-  
92 induced phenotypes, we investigated the effects of *Wolbachia* on Rift Valley fever virus (RVFV)  
93 infection in *Cx. tarsalis*. RVFV is a member of the genus *Phlebovirus* in the family Bunyaviridae  
94 and is predominately a disease of domestic ruminants that causes severe economic losses in the  
95 livestock industry and human morbidity in Africa and the Middle East [28–30]. Additionally,  
96 models and laboratory studies have suggested the United States may have environmental  
97 conditions and mosquito vectors that would permit RVFV introduction and invasion [31–34].  
98 *Culex tarsalis* are abundant in the western U.S. and are highly competent laboratory vectors for  
99 RVFV [33–35]. We assessed the ability of *Wolbachia* to affect RVFV infection, dissemination,  
100 and transmission within *Cx. tarsalis* at two time points and evaluated relationships between viral  
101 titer and *Wolbachia* density in mosquitoes.

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## 103 **Materials and Methods**

104 Ethics statement

105 Mosquitoes were maintained on commercially available human blood using a membrane  
106 feeder (Biological Specialty Corporation, Colmar, PA). RVFV experiments were performed  
107 under biosafety-level 3 (BSL-3) and arthropod-containment level 3 (ACL3) conditions.

108 Research at the U.S. Army Medical Research Institute of Infectious Diseases  
109 (USAMRIID) was conducted under an Institutional Animal Care and Use Committee (IACUC)  
110 approved protocol in compliance with the Animal Welfare Act, PHS Policy, and other federal  
111 statutes and regulations relating to animals and experiments involving animals. This facility  
112 where this research was conducted is accredited by the Association for Assessment and  
113 Accreditation of Laboratory Animal Care, International and adheres to the principles stated in the  
114 *Guide for the Care and Use of Laboratory Animals*, National Research Council, 2011. The  
115 USAMRIID IACUC specifically approved this study.

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117 Mosquitoes and *Wolbachia*

118 The *Culex tarsalis* colony used for all experiments was derived from field mosquitoes  
119 collected in Yolo County, CA in 2009. Mosquitoes were reared and maintained at  $27^{\circ}\text{C} \pm 1^{\circ}\text{C}$ ,  
120 12:12 hr light:dark diurnal cycle at 80% relative humidity in 30×30×30 cm cages. The *wAlbB*  
121 *Wolbachia* strain was purified from *An. gambiae* Sua5B cells, according to published protocols  
122 [36]. *Wolbachia* viability and density was assessed using the LIVE/DEAD BacLight Bacterial  
123 Viability Kit (Invitrogen, Carlsbad, CA) and a hemocytometer. The experiment was replicated  
124 three times and *wAlbB* concentrations were as follows: replicate one,  $2.5 \times 10^9$  bacteria/ml;  
125 replicate two,  $2.5 \times 10^9$  bacteria/ml; replicate three,  $5.0 \times 10^9$  bacteria/ml.

126 Two- to 4-day-old adult female *Cx. tarsalis* were anesthetized with  $\text{CO}_2$  and  
127 intrathoracically injected with approximately 0.1  $\mu\text{l}$  of either suspended *wAlbB* or Schneider's

128 insect media (Sigma Aldrich, Saint Louis, MO) as a control. Mosquitoes were provided with  
129 10% sucrose *ad libitum* and maintained at 27°C in a growth chamber.

130

131 Vector competence for RVFV

132 RVFV strain ZH501 was isolated from the blood of a fatal human case in Egypt in 1977  
133 [37]. Adult female Syrian hamsters were inoculated intraperitoneally with 0.2 ml of a suspension  
134 containing RVFV in diluent (10% heat-inactivated fetal bovine serum in Medium 199 with  
135 Earle's salts [Invitrogen], sodium bicarbonate, and antibiotics) containing approximately  $10^5$   
136 plaque-forming units (PFU) per ml of RVFV. Approximately 28–30 hr post-inoculation, infected  
137 hamsters were anesthetized with a suspension of ketamine, acepromazine, and xylazine. A single  
138 viremic hamster was placed across two 3.8-liter cardboard cages containing either *Wolbachia*-  
139 infected *Cx. tarsalis* or control-injected *Cx. tarsalis*, treatments to which the experimenter was  
140 blinded. Mosquitoes were allowed to feed for one hour. After this period, hamsters were  
141 removed, a blood sample taken to determine viremia, and hamsters were euthanized.

142 After feeding, mosquitoes were anesthetized with CO<sub>2</sub> and examined for feeding status;  
143 partially or non-blood fed females were discarded. For all replicates, one blood fed mosquito  
144 from each treatment was sampled to test for input viral titers. Mosquitoes were sampled at 7 and  
145 14 days post-blood feeding, where they were anesthetized with CO<sub>2</sub>, and had their legs removed.  
146 Bodies and legs were placed separately into 2-ml microcentrifuge tubes (Eppendorf, Hauppauge,  
147 NY) containing 1 ml of mosquito diluent (20% heat-inactivated fetal bovine serum [FBS] in  
148 Dulbecco's phosphate-buffered saline, 50 µg/ml penicillin streptomycin, and 2.5 µg/ml  
149 fungizone). Prior to placement into microcentrifuge tubes, saliva was collected from mosquito  
150 bodies on day 14 by positioning the proboscis of each mosquito into a capillary tube containing

151 approximately 10  $\mu$ l of a 1:1 solution of 50% sucrose and FBS. After 30 minutes, the contents  
152 were expelled in individual microcentrifuge tubes containing 0.3 ml of mosquito diluent, and  
153 bodies were placed in individual microcentrifuge tubes containing 1 ml of mosquito diluent. A 5  
154 mm stainless steel bead (Qiagen, Valencia, CA) was placed into microcentrifuge tubes  
155 containing mosquito bodies and legs, homogenized in a mixer mill (Retsch, Haan, Germany) for  
156 30 seconds at 24 cycles per second, and centrifuged for 1 minute at 8000 rpm. All mosquito  
157 bodies, legs, and saliva were stored at -80°C until assayed.

158         Samples were tested for RVFV infectious particles by plaque assay on Vero cells  
159 according to previous published protocols [38]. Serial dilutions were prepared for all mosquito  
160 body, leg, and saliva samples. One hundred microliters of each dilution was inoculated onto  
161 Vero cell culture monolayers. Inoculated plates were incubated at 37°C for 1 hr and an agar  
162 overlay was added (1X EBME, 0.75% agarose, 7% FBS, 1% penicillin streptomycin, and 1%  
163 nystatin). Plates were incubated at 37°C for 4 days and then a second overlay (1X EBME, 0.75%  
164 agarose, and 4% neutral red) was added. Plaques were counted 24 hr after application of the  
165 second overlay and titers calculated.

166

#### 167 Quantitative real-time PCR of *Wolbachia* density

168         To evaluate relationships between *Wolbachia* density and RVFV titer, we measured  
169 *w*AlbB levels in individual mosquitoes. DNA was extracted from 200  $\mu$ l of mosquito body  
170 homogenate using the DNeasy blood and tissue kit (Qiagen) and used as template for qPCR on a  
171 Rotor-Gene Q (Qiagen) with the PerfeCta SYBR FastMix kit (Quanta Biosciences, Beverly,  
172 MA) or on ABI 7500 with Power SYBR green master mix (Applied Biosystems, Foster City,  
173 CA). The qPCR assays were performed in 10 $\mu$ l reactions and amplification was carried out using



174 a standardized program at 95°C for 5 min, and 40 cycles of 95°C for 10 sec, 60°C for 15 sec, and  
175 72°C for 10 sec. *Wolbachia* DNA was amplified with primers Alb-GF and Alb-GR [39] and was  
176 normalized to the *Cx. tarsalis* actin gene by using qGene software [24,40]. qPCRs were  
177 performed in duplicate.

178

## 179 Statistical analyses

180 Infection, dissemination, and transmission rates were compared between *Wolbachia*-  
181 infected and control *Cx. tarsalis*, and between replicates with Fisher's exact tests. Due to  
182 violations of assumptions needed for parametric tests, Mann-Whitney U was used to compare the  
183 following data sets: RVFV body titers between *Wolbachia*-infected and control mosquitoes,  
184 RVFV body titers between RVFV-positive saliva and RVFV-negative saliva, RVFV body titers  
185 over time, and *Wolbachia* density over time. Unpaired t-tests were used to analyze data that  
186 passed normality tests, including the comparison of RVFV saliva titers between *Wolbachia*-  
187 infected and control mosquitoes. To determine relationships between *Wolbachia* density and  
188 RVFV body titer, the Spearman rank correlation test was used, as assumptions for Pearson  
189 correlation were violated. All statistical analyses were performed in GraphPad Prism version 7  
190 for Windows (GraphPad Software, San Diego, CA).

191

## 192 Results

### 193 Vector competence for RVFV

194 For all replicates, one blood fed mosquito from each treatment was tested for input  
195 RVFV titers on the day of blood feeding. Time 0 results for *Wolbachia*-infected *Cx. tarsalis*  
196 were as follows: replicate 1,  $2.50 \times 10^2$ ; replicate 2,  $7.00 \times 10^6$ ; replicate 3,  $1.00 \times 10^{2.0}$ . Time 0

197 results for control *Cx. tarsalis* were as follows: replicate 1,  $5.00 \times 10^2$ ; replicate 2,  $1.05 \times 10^7$ ;  
198 replicate 3,  $1.00 \times 10^2$ . Viremias in the three hamsters were  $10^4$ ,  $10^9$ , and  $10^3$  PFU/ml,  
199 respectively.

200 To determine RVFV vector competence of *Wolbachia*-infected and *Wolbachia*-  
201 uninfected *Cx. tarsalis*, we examined frequencies of RVFV-positive bodies, legs, and saliva (Fig.  
202 1). Infection rate is the proportion of mosquito bodies that contained infectious RVFV.  
203 Dissemination and transmission rates are the proportion of infected mosquitoes with RVFV  
204 positive legs and saliva, respectively. Additionally, transmission rates are also displayed as the  
205 proportion of all tested mosquitoes with RVFV positive saliva. Three replicates were performed,  
206 and individual data from those experiments are available in Table S1. Hamster viremia in  
207 replicate three was low and resulted in low mosquito infection rates. Replicate two infection  
208 frequencies were significantly higher than replicate one for both treatments and at both day 7 and  
209 day 14 ( $P < 0.0001$ ). However, across replicates and time points, *Wolbachia*-infected *Cx. tarsalis*  
210 infection, dissemination, and transmission rates did not differ significantly from *Wolbachia*-  
211 uninfected *Cx. tarsalis* (Fig. 1, Table S1). Thus the data was pooled for further analysis.

212 RVFV body and saliva titers were determined for *Wolbachia*-infected and control *Cx.*  
213 *tarsalis*. There were no significant differences in RVFV body titer or saliva titer between  
214 *Wolbachia*-infected and control *Cx. tarsalis* at either day 7 or day 14 (Fig. 2). Additionally, both  
215 *Wolbachia*-infected and uninfected *Cx. tarsalis* that transmitted RVFV had significantly higher  
216 RVFV body titers than non-transmitting mosquitoes (Fig S1).

217

218 Quantitative real-time PCR of *Wolbachia* (*wAlbB*) density

219 *Wolbachia* density in each mosquito was determined by qPCR. We analyzed  
220 relationships between *Wolbachia* density and RVFV body titer and combined data from all  
221 replicates. Overall, there was a moderate, negative correlation between *Wolbachia* density and  
222 RVFV body titer at both day 7 and 14 (Fig. 3). *Wolbachia* density was also compared across  
223 time; *Wolbachia* concentration at day 14 was significantly higher than at day 7, consistent with  
224 *Wolbachia* replication in mosquitoes (Fig S3).

225

## 226 **Discussion**

227 *Wolbachia* infection can have varied effects on viruses and parasites transmitted by  
228 mosquitoes. These effects can include moderate to complete pathogen inhibition, as well as  
229 pathogen enhancement [17,22,24,41,42]. In a previous study, we found that *Wolbachia* strain  
230 *wAlbB* enhanced WNV infection frequency in *Cx. tarsalis* [24], although in that study, viral  
231 infection titers were not measured. To understand how widespread the *Wolbachia*-induced  
232 enhancement phenotype is in *Cx. tarsalis*, we studied *wAlbB* effects on RVFV, an important  
233 arthropod-borne virus with potential to invade the United States [43,44]. In contrast to our  
234 previous results, we found that *wAlbB* did not affect RVFV body or saliva titers, nor RVFV  
235 infection, dissemination, or transmission frequencies in *Cx. tarsalis*.

236 *Wolbachia*-mediated effects on pathogens may depend on *Wolbachia* density. Several  
237 studies have reported that high densities of *Wolbachia* are more likely than low densities to block  
238 viruses in *Drosophila* spp. and mosquitoes [45–48]. Similarly, we found a moderate, negative  
239 correlation between RVFV body titer and *Wolbachia* density. High *Wolbachia* levels were  
240 associated with RVFV negative mosquitoes and very low RVFV body titers. The low numbers of  
241 mosquitoes at the high *Wolbachia* densities may explain why we did not see a *Wolbachia* effect

242 on population level vector competence measures. However, our correlation data suggests that in  
243 this system, *Wolbachia* may suppress RVFV in a density-dependent manner.

244 In this *Cx. tarsalis*-*wAlbB* system, we have reported different effects of *Wolbachia* on  
245 vector competence for WNV and RVFV [24]. Other studies have found similar differences in  
246 *Wolbachia* phenotypes and suggested they may depend on various factors including  
247 environmental conditions, and pathogen type [20,49]. RVFV and WNV belong to different virus  
248 families and could interact with the mosquito host environment and *Wolbachia* in different ways.  
249 For example, a recent study suggested that the mosquito JAK/STAT pathway may not have the  
250 same antiviral effects on closely related viruses [50]. Although the mosquitoes in our two studies  
251 have the same genetic background, they were reared in separate facilities and may have different  
252 microbiomes that may explain differences in vector competence [51]. Another variable that  
253 could explain these differences may involve differing blood composition. In the WNV study, we  
254 fed mosquitoes on defibrinated bovine blood in a membrane feeder whereas in this study, we fed  
255 mosquitoes on live hamsters. Previous studies have suggested that artificial feeding and  
256 anticlotting agents may affect various processes within the mosquito [52,53].

257 Our study was performed with an adult microinjection model that generates mosquitoes  
258 transiently infected with *Wolbachia*. It remains to be seen whether this model reflects  
259 relationships between *Wolbachia* and viruses in *Cx. tarsalis* in a stable infection system.  
260 However, a recent study showed that both stable and transient *wAlbB* infections in *Ae. aegypti*  
261 produced similar results [45]. This suggests that our transient infection model may correlate with  
262 a stable infection in *Cx. tarsalis*.

263 These studies illustrate the importance of understanding what phenotypes *Wolbachia*  
264 influences, and future studies should seek to understand the mechanisms underlying them.

265

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270

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426 **Figure legends**

427 **Fig. 1. Effects of *Wolbachia* infection on RVFV vector competence frequencies in *Cx.***

428 *tarsalis*.

429 RVFV infection 7 and 14 days post-feeding (A), dissemination 7 and 14 days post-feeding (B),  
430 and transmission rates 14 days post-feeding (C) were compared between *Wolbachia*-infected and  
431 control *Cx. tarsalis*. Bars represent data pooled from three replicates. Error bars denote binomial  
432 confidence intervals. See Table S1 for replicate-specific analyses.

433

434 **Fig. 2. Comparison of RVFV body and saliva titers between *Wolbachia*-infected and control**

435 *Cx. tarsalis*.

436 At both 7 and 14 days post-blood meal, there are no significant differences in RVFV body titers  
437 of *Wolbachia*-infected *Cx. tarsalis* compared to control *Cx. tarsalis*. All replicates are combined  
438 in this figure; separate replicates are provided in supplementary materials (Fig S2). Bars  
439 represent medians and bolded numbers above the data points denote sample sizes.

440

441 **Fig. 3. Correlation between RVFV body titer and *Wolbachia* levels in *Cx. tarsalis***

442 *Wolbachia* levels were normalized to the host gene actin. Normalized *Wolbachia* levels and  
443 RVFV body titer for each mosquito were plotted and analyzed with the Spearman rank  
444 correlation test to determine relationships. There was a moderate, negative correlation between  
445 RVFV body titer and *Wolbachia* levels at both day 7 (A) and day 14 (B) post-blood feeding (Fig.  
446 3). Data for all replicates were combined; see Table S2 for replicate-specific raw data.

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451 **S1 Table. Vector competence of *Cx. tarsalis* following a RVFV blood meal.**

452 RVFV infection, dissemination, and transmission frequencies were compared between

453 *Wolbachia*-infected and control mosquitoes. Replicates are displayed individually.

454

455 **S1 Fig. Comparison of RVFV body titers in *Cx. tarsalis* with virus present or absent in the**  
456 **saliva.**

457 RVFV body titers were compared between mosquitoes that tested positive or negative for RVFV

458 in their saliva. For both *Wolbachia*-infected and control *Cx. tarsalis*, mosquitoes positive for

459 RVFV in the saliva had significantly higher RVFV body titers compared to mosquitoes negative

460 for virus in the saliva There was no significant difference in RVFV body titer of transmitters

461 between *Wolbachia*-infected and control mosquitoes ( $p=0.7692$ ). Data was analyzed with Mann-

462 Whitney U and bars represent medians.

463

464 **S2 Fig. Comparison of RVFV body titers between treatments by replicate.**

465 RVFV body titers were compared between *Wolbachia*-infected and control mosquitoes for

466 replicates 1 (A), 2 (B), and 3 (C). In all replicates, there were no significant differences in RVFV

467 body titer between *Wolbachia*-infected and control mosquitoes. Data did not pass assumptions

468 for normality and were analyzed with Mann-Whitney U, and sample sizes are denoted above

469 data points.

470

471 **S3 Fig. *Wolbachia* density over time.**

472 *Wolbachia* levels for each mosquito, determined by qPCR, were combined across all three  
473 replicates. *Wolbachia* levels are significantly higher at day 14 compared to day 7. Due to  
474 violations of normality, Mann-Whitney U was used for comparisons, bars are medians, and  
475 numbers above data points are sample sizes.







