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Laurence Mousson, Estelle Martin, Karima Zouache, Yoann Madec, Patrick Mavingui, Anna-Bella Failloux

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1            ***Wolbachia* modulates Chikungunya replication in *Aedes albopictus***

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4            L. MOUSSON,<sup>\*</sup> E. MARTIN,<sup>\*</sup> K. ZOUACHE,<sup>#</sup> Y. MADEC,<sup>†</sup> P. MAVINGUI<sup>#</sup> and A.-B.

5

   FAILLOUX<sup>\*</sup>

6

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8

9            <sup>\*</sup> Institut Pasteur, Génétique moléculaire des Bunyavirus, 25-28 rue du Dr Roux, F-75724

10          Paris cedex 15, France

11          <sup>†</sup> Institut Pasteur, Unité de Recherche et d'Expertise Epidémiologie des Maladies Emergentes,

12          25-28 rue du Dr Roux, F-75724 Paris cedex 15, France

13          <sup>#</sup> Université Lyon, F-69022, Lyon, France, Université de Lyon 1, Villeurbanne, CNRS,

14          UMR5557, Ecologie Microbienne, Lyon, France

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18          **Running title:** *Wolbachia* and CHIKV interference

19

20          ( <sup>\*</sup> ) Corresponding author:

21          Anna-Bella FAILLOUX

22          Institut Pasteur, Génétique moléculaire des Bunyavirus, 25-28 rue du Dr Roux, F-75724 Paris

23          cedex 15, France.

24          FAX: 00 33 1 40 61 31 51. E-mail: afaillou@pasteur.fr

25 **Abstract**

26 The *Aedes albopictus* mosquito has been involved as the principal vector of recent major  
27 outbreaks due to the chikungunya virus (CHIKV). The species is naturally infected by two  
28 strains of *Wolbachia* (*wAlbA* and *wAlbB*). *Wolbachia* infections are thought to have spread  
29 by manipulating the reproduction of their hosts; cytoplasmic incompatibility is the mechanism  
30 used by *Wolbachia* to invade natural populations of many insects including *Ae. albopictus*.  
31 Here, we report a study on the effects of removing *Wolbachia* from *Ae. albopictus* on CHIKV  
32 replication and examine the consequences of CHIKV infection on some life-history traits  
33 (survival and reproduction) of *Wolbachia*-free *Ae. albopictus*. We found that *Wolbachia*-free  
34 mosquitoes maintained a highly heterogeneous CHIKV replication compared to *Wolbachia*-  
35 infected individuals. In *Wolbachia*-infected *Ae. albopictus*, the regular increase of CHIKV  
36 followed by a steady viral load from day 4 post-infection onwards was concomitant with a  
37 decline in *Wolbachia* density. This profile was also detected when examining the two key  
38 organs for viral transmission, the midgut and the salivary glands. Moreover, *Wolbachia*-free  
39 *Ae. albopictus* was not altered in life history traits such as survival, oviposition and hatching  
40 characteristics whether infected or not with CHIKV. We found that *Wolbachia* is not essential  
41 for viral replication, its presence could lead to optimize replication from day 4 post-infection  
42 onwards, coinciding with a decrease in *Wolbachia* density. *Wolbachia* may regulate viral  
43 replication in *Ae. albopictus*, with consequences on survival and reproduction.

## 44 Introduction

45

46 The Asian tiger mosquito, *Aedes albopictus* (Skuse), originates from the forests of South-East  
47 Asia (Smith 1956). This species does not display any ecological specialization, and has  
48 succeeded in colonizing temperate zones such as the United States (Sprenger &  
49 Wuithiranyagool 1986) and Europe (Scholte & Schaffner 2007), and is currently invading  
50 African countries (Fontenille & Toto 2001; Toto *et al.* 2003; Coffinet *et al.* 2007). *Ae.*  
51 *albopictus* was incriminated as the main vector of chikungunya virus (CHIKV) in the Indian  
52 Ocean region in 2005-2006 (Delatte *et al.* 2008). In the Reunion Island, a variant of CHIKV  
53 harboring an A226V substitution in the E1 glycoprotein (E1-226V) (Schuffenecker *et al.*  
54 2006) was demonstrated to be efficiently transmitted by *Ae. albopictus* (Vazeille *et al.* 2007).

55 First discovered in the *Culex pipiens* mosquito in 1924 (Hertig 1936), the *Wolbachia*  
56 endosymbiont is widely found in natural populations of *Ae. albopictus* (Ahantarig *et al.*  
57 2008). Most populations are multi-infected with two different *Wolbachia* strains designated  
58 *wAlbA* and *wAlbB* (Dobson *et al.* 2001; Kittayapong *et al.* 2002; Zhou *et al.* 1998). Most  
59 *Wolbachia* infections in insects are thought to have spread by host reproductive alterations  
60 leading to successful increase of bacterial transmission through the female germline (Werren  
61 1997). These manipulations can be described as one of two classes. Sex-ratio-distorting  
62 strains increase the production of daughters at the expense of sons by male killing (Husrt *et*  
63 *al.* 1999), feminizing genetic males (Rousset *et al.* 1992) or inducing parthenogenesis  
64 (Stouthamer *et al.* 1993). Other strains induce cytoplasmic incompatibility (CI) (Hoffmann &  
65 Turelli 1997) that is known to occur in *Ae. albopictus* (Kambhampati *et al.* 1993). When a  
66 *Wolbachia*-infected male mates with an uninfected female, eggs or embryos die and this leads  
67 to a decrease in the fitness of uninfected females. CI is due to a failure in histone deposition in

68 the male pronucleus after fertilization (Landmann *et al.* 2009) and to a delay in the nuclear  
69 envelope breakdown of the paternal chromatin nucleus, leading to improper condensation of  
70 chromatin in the embryo and embryonic death (Tram & Sullivan 2002). In females,  
71 *Wolbachia* infections rescue the modified pronucleus so that normal karyogamy and  
72 development can continue. Typically, modification and rescue components are specific for  
73 different *Wolbachia* strains: one *Wolbachia* strain is often unable to rescue the modification  
74 induced by a different strain. CI penetrance is particularly high in mosquitoes and is typically  
75 associated with a 90-100% failure of eggs to hatch (Dutton & Sinkins 2005), making  
76 *Wolbachia* a promising candidate for vector control programs (Sinkins & Gould 2006;  
77 McMeniman *et al.* 2009).

78 Vertical transmission of symbionts is more efficient in healthy hosts as the fitness of  
79 both interacting partners is directly linked to each other. Consequently, vertically transmitted  
80 symbionts are in conflict with horizontally transmitted pathogens (Haine 2008). Thus,  
81 *Wolbachia* may protect the host by restricting the uptake or development/replication of a  
82 secondary horizontally transmitted pathogen. Whilst the two parasites may be viewed as  
83 competing for the host in some sense, both the horizontally transmitted pathogen (e.g., a  
84 virus) and the vertically transmitted parasite (*Wolbachia*) may benefit from the interaction.  
85 *Drosophila melanogaster* is commonly concomitantly infected with *Wolbachia* and a viral  
86 pathogen, Drosophila C virus. The bacterial infection renders the flies more resistant to the  
87 virus, reducing the viral load (Hedges *et al.* 2008; Teixeira *et al.* 2008). The induced resistance  
88 to natural viral pathogens may explain *Wolbachia* prevalence in natural populations  
89 (Kittayapong *et al.* 2002).

90 *Wolbachia* is usually facultative (secondary symbionts) in arthropods as aposymbiotic  
91 individuals are not affected physiologically. Here, we describe experiments in which we

92 sought to examine (i) the effects of removing *Wolbachia* from *Ae. albopictus* on CHIKV  
93 replication, and (ii) the consequences of CHIKV infection on some life-history traits (survival  
94 and reproduction) of *Wolbachia*-free *Ae. albopictus*.

95

96

## 97 **Materials and Methods**

98

### 99 *Mosquitoes*

100 The ALPROV strain of *Ae. albopictus* from La Reunion Island is naturally infected with  
101 *wAlbA* and *wAlbB* (Tortosa *et al.* 2008). The 3<sup>rd</sup> generation mosquitoes was maintained using  
102 standard conditions at 28°C ± 1°C, 80 % of relative humidity, and under a 16/8 light/dark  
103 cycle. Eggs were hatched in water and larvae were reared in pans containing one yeast tablet  
104 per liter of dechlorinated tap water. For adult mosquito maintenance, a constant supply of  
105 10% sucrose was provided. Females were fed on mice three times a week (OF1 mice obtained  
106 from Charles River laboratories, France) and eggs were collected weekly. All experiments  
107 involving live vertebrates were performed in compliance with French and European  
108 regulations and according to the Institute Pasteur guidelines for laboratory animal husbandry  
109 and care.

110

### 111 *Antibiotic treatment to clear Wolbachia*

112 The ALPROV *Ae. albopictus* strain was treated with tetracycline and rifampicin to obtain  
113 *Wolbachia*-free ALPROV. Larvae were raised for four successive generations with 10 mg/L  
114 (F3), 20 mg/mL (F4), and two times 40 mg/mL of tetracycline (F5 and F6). Briefly, eggs were  
115 hatched and first instar larvae were placed in a tetracycline solution until the pupal stage. The

116 adults hence obtained were reared at 28°C and blood-fed to obtain fresh eggs of the next  
117 generation. After each treatment, 10 adults were tested by quantitative PCR for *Wolbachia*  
118 infection. After the last larval treatment, one treatment of adults (F6) consisted of introducing  
119 a solution of rifampicin (2.5 g/L) dissolved in 10% sucrose into the cage. After one generation  
120 of amplification (F7), the 8<sup>th</sup> generation of ALPROV resulting from four generations of  
121 antibiotic treatment and two generations of amplification was used for experiments.

122

### 123 *Virus production*

124 The CHIKV (E1-226V) was kindly provided by the French National Reference Center for  
125 Arboviruses at the Institut Pasteur. This strain presented an A->V change at position 226 in  
126 the E1 glycoprotein (E1-226V) (Schuffenecker *et al.* 2006). Stock virus was produced  
127 following three passages on *Ae. albopictus* C6/36 cells (Figure S1), being harvested and  
128 stored at -80°C. The titer of the frozen stock virus was estimated as 10<sup>9</sup> plaque-forming units  
129 (PFU)/mL.

130

### 131 *Experimental oral infections*

132 Blood-meals were prepared as follows: 1 mL of viral suspension in L-15 medium  
133 supplemented with 2 % fetal bovine serum (FBS), was added to 2 mL of washed rabbit  
134 erythrocytes supplemented with ATP (5 x 10<sup>-3</sup> M) as a phagostimulant. The infectious blood,  
135 at a titer of 10<sup>7.5</sup> PFU/mL, was transferred to a glass feeder maintained at 37°C and placed on  
136 top of the mesh of a plastic box containing 60 of 1-week-old female mosquitoes that had been  
137 starved for 24 hours prior to the infection experiment. After 15 min of feeding, engorged  
138 females were sorted on ice and transferred to cardboard containers. Females were fed with

139 10% sucrose at 28°C. The entire feeding period lasted one hour during which time no  
140 significant change in the viral titer in glass feeders occurred.

141

#### 142 *Nucleic acid extraction and quantitative PCR*

143 Individual mosquitoes and dissected organs (midguts and salivary glands) were used to  
144 extract total nucleic acids. Extraction was done with NucleoSpin® RNA/DNA buffer set  
145 (Macherey-Nagel) coupled to the NucleoSpin® RNA II kit that enables the isolation of both  
146 RNA and DNA. RNA was used to determine viral load by quantitative RT-PCR, and DNA to  
147 measure *Wolbachia* (*wAlbA* and *wAlbB*) density and actin gene content by quantitative PCR.  
148 To measure viral load at different days post-infection (pi), five females were killed every day  
149 until day 14 pi. After total RNA extraction, a one-step RT-PCR reaction was performed with  
150 a Power SYBR® Green RNA-to-CT™ one step kit (Applied Biosystem) in a volume of 25  
151 µL containing 2 µL RNA template, 12.5 µL 2X Power SYBR® Green I RT-PCR Mix, 0.25  
152 µL sense primer (0.1 µM), 0.25µL anti-sense primer (0.1 µM), 0.2 µL RT enzyme mix and 9.8  
153 µL of ddH<sub>2</sub>O. Primers selected in the E2 structural protein coding region were: sense  
154 Chik/E2/9018/+ (CACCGCCGCAACTACCG) and anti-sense Chik/E2/9235/-  
155 (GATTGGTGACCGCGGCA). The PCR program was: 48°C for 30 min, 95°C for 10 min; 40  
156 cycles of 95°C for 15 s, 60°C for 1min, and 72°C for 30 s; 95°C for 20 s with a final ramping  
157 of 19 min 59 sec. The size of the PCR product was 217 bp. A standard curve was generated  
158 using duplicates of 10-fold serial dilutions of RNA synthetic transcripts. Quantification of  
159 viral RNA was achieved by comparing the threshold cycle (Ct) values of samples to those of  
160 standards according to the  $\Delta C_t$  analysis. One Log of infectious viral particles corresponds to  
161 1-2 Log RNA virus (Figure S2). To quantify *Wolbachia* (*wAlbA* and *wAlbB*) and actin gene,  
162 total DNA was extracted and used for quantitative PCR. For standardization between



163 *Wolbachia* specific genes and mosquito genes, a plasmid (qQuantAlb) kindly provided by  
164 Tortosa *et al.* (2008) that contains the three loci *wAlbA*, *wAlbB*, and the *Ae. albopictus* actin  
165 gene was used. The plasmid was serially diluted to build a standard curve. Primers were:  
166 actAlb-dir (GCA AAC GTG GTA TCC TGA C) and actAlb-rev (GTC AGG AGA ACT  
167 GGG TGC T), QAdir1 (GGG TTG ATG TTG AAG GAG) and QArev2 (CAC CAG CTT  
168 TTA CTT GAC C), 183F (AAG GAA CCG AAG TTC ATG) and QBrev2 (AGT TGT GAG  
169 TAA AGT CCC), for *wAlbA*, *wAlbB* and actin, respectively. From 60  $\mu$ l of DNA solution  
170 extracted from one mosquito, 2  $\mu$ l of DNA was mixed with 0.3  $\mu$ M of each primer and 12.5  $\mu$ l  
171 of FastStart Universal SYBR Green Master (Rox). PCR was run for 40 cycles (95°C for 10  
172 min, 95°C for 15 sec, 60°C for 1 min). A new standard curve was built for each run, so  
173 signals could be normalized with the nuclear actin reference. The mean number of genomes of  
174 *wAlbA* and *wAlbB* was given per actin copies.

175

#### 176 *Female life history traits*

177 Two traits were examined: survival and reproduction (oviposition/egg hatching). Dead  
178 mosquitoes were scored every day to estimate the female life duration following exposure to a  
179 blood-meal. Their infection status was checked by quantitative RT-PCR to estimate the viral  
180 RNA load. Oviposition was examined by assessing three parameters: (i) the time from the  
181 blood-meal to the female's first egg laying, (ii) the number of eggs laid per female, (iii) the  
182 time between the first egg laying and female death. Hatching was studied by estimating: (i)  
183 the hatching capacity (the proportion of mosquitoes with at least one egg hatched relative to  
184 mosquitoes which have laid) and (ii) the hatching rate (the number of eggs hatched compared  
185 to the number of eggs laid per mosquito).

186

187 *Measurement of vertical transmission efficiency*

188 Adults resulting from the progeny of *Wolbachia*-free ALPROV exposed to a blood-meal  
189 containing E1-226V CHIKV (see above) were screened to detect viral RNA by quantitative  
190 RT-PCR in pools of adults.

191

192 *Statistical analysis.*

193 Kaplan-Meier survival curves were used to describe survival in CHIKV-infected and  
194 uninfected mosquitoes, and these curves were compared using the logrank test. The CHIKV  
195 load estimated in females at their death was compared using an analysis of variance according  
196 to the life duration divided into three categories ( $\leq 5$  days, 6-10 days, and 11-15 days).

197 For *Wolbachia*-free mosquitoes, exposed or not to an infectious blood-meal with E1-226V  
198 CHIKV, the time to the first egg laying was also described using Kaplan-Meier estimates, and  
199 survival curves were compared using the logrank test. Then the effect of CHIKV infection on  
200 the total number of eggs laid was investigated using a negative binomial regression model.  
201 This model is relevant when analyzing incidence, as it enables the control for life duration,  
202 and effectively provides incidence rate ratios (IRR) and their 95% confidence intervals. The  
203 significance level of the covariate was tested using Wald's test. We also estimated the time  
204 between the first oviposition and mosquito death, using Kaplan-Meier estimates, and  
205 compared these curves using the logrank test.

206 For each mosquito strain, the hatching capacity was studied through the assessment of the  
207 proportion of mosquitoes with at least one hatched egg. These proportions were compared  
208 using a Fisher's exact test. Hatching rates, i.e. the proportion of hatched eggs among all eggs  
209 laid by a given mosquito, were compared using an analysis of variance according to the status  
210 of infection (infectious blood-meal or non-infectious blood-meal).

211 All statistical analyses were performed using the STATA software (StataCorp LP, Texas,  
212 USA).

213

214

## 215 **Results**

216

### 217 *Removing Wolbachia from infected mosquitoes by antibiotic treatments*

218 Attempts to clear *Wolbachia* from a doubly-infected *Ae. albopictus* were only successful  
219 when both larvae and adults were treated. Rearing larvae under different concentrations of  
220 tetracycline (10, 20 and 40 mg/mL) from the F3 to the F6 generation did not totally clear the  
221 *Wolbachia* infection. An additional treatment of adult mosquitoes with rifampicin was  
222 necessary to completely remove the bacteria. PCR assays of the F8 generation demonstrated  
223 that all adults tested were *Wolbachia*-free (Figure 1) as compared to positive signals detected  
224 on untreated individuals.

225

### 226 *CHIKV replication in Wolbachia-infected and Wolbachia-free mosquitoes*

227 For *Wolbachia*-infected *Ae. albopictus*, the number of viral RNA copies increased after  
228 exposure to the blood-meal containing CHIKV (Figure 2A); from  $10^{5.7} \pm 10^{0.2}$  viral RNA  
229 copies/mosquito (trial 1) or  $10^{6.2} \pm 10^{0.2}$  viral RNA copies/mosquito (trial 2) at day 0 pi to  
230  $10^{8.5} \pm 10^{0.3}$  viral RNA copies/mosquito (trial 1) or  $10^{8.9} \pm 10^{0.4}$  viral RNA copies/mosquito  
231 (trial 2) at day 4 pi. This number stayed steady until day 14 pi:  $10^{8.3} \pm 10^{0.1}$  viral RNA  
232 copies/mosquito (trial 1) and  $10^{8.5} \pm 10^{0.2}$  viral RNA copies/mosquito (trial 2). The two trials  
233 that concerned *Wolbachia*-infected *Ae. albopictus* gave similar profiles of viral replication  
234 (Figure 2A). By contrast, *Wolbachia*-free *Ae. albopictus* exhibited a high heterogeneity in the

235 number of viral RNA harbored by mosquitoes (Figure 2B); despite a roughly similar kinetic  
236 of replication with an increase from ingestion until day 4 pi [ $10^{7.3} \pm 10^{1.3}$  viral RNA  
237 copies/mosquito (trial 1) and  $10^{8.7} \pm 10^{0.5}$  viral RNA copies/mosquito (trial 2)] followed by a  
238 plateau until day 14 pi. Some mosquitoes were able to efficiently sustain viral replication  
239 beyond this time, whereas others presented slightly less viral RNA than that ingested (Figure  
240 2B).

241

#### 242 *Variation of Wolbachia density following CHIKV infection*

243 The relative numbers of *Wolbachia* are presented as a ratio of gene copy numbers of  
244 *Wolbachia* to host *actin*. At day 0 pi, when *Wolbachia*-infected *Ae. albopictus* ingested an  
245 infectious blood-meal containing E1-226V CHIKV, the Log number of *wAlbA* and *wAlbB*  
246 per mosquito was close to 1:  $10^{1.0} \pm 10^{0.01}$  for *wAlbA* strain and  $10^{1.0} \pm 10^{0.02}$  for *wAlbB* strain.  
247 At day 2 pi, these numbers started to decrease to gradually reach  $10^{0.6} \pm 10^{0.07}$  *wAlbA* and  
248  $10^{0.5} \pm 10^{0.08}$  *wAlbB* at day 14 pi (Figure 3A). Conversely, for *Wolbachia*-infected mosquitoes  
249 having ingested a non-infectious blood-meal, *Wolbachia* densities did not vary substantially  
250 (Figure 3B) from day 0 pi ( $10^{1.0} \pm 10^{0.02}$  *wAlbA* and  $10^{0.9} \pm 10^{0.03}$  *wAlbB*) to day 14 pi ( $10^{1.0} \pm$   
251  $10^{0.04}$  *wAlbA* and  $10^{0.9} \pm 10^{0.08}$  *wAlbB*).

252

#### 253 *Viral and bacterial densities in midguts and salivary glands*

254 To examine the microbial density in the midgut and the salivary glands, each organ was  
255 dissected and tested separately. The load of viral RNA in *Wolbachia*-infected *Ae. albopictus*  
256 was found to reach a maximum at day 3 pi in the midgut ( $10^{8.8} \pm 10^{0.5}$  viral RNA) and at day 4  
257 pi in the salivary glands ( $10^{2.5} \pm 10^{0.5}$  viral RNA) (Figure 4A). After this, values varied from  
258  $10^{8.9} \pm 10^{0.1}$  viral RNA (day 4 pi) to  $10^{9.3} \pm 10^{0.1}$  viral RNA (day 12 pi) in midguts, and from

259  $10^{2.4} \pm 10^{0.8}$  viral RNA (day 5 pi) to  $10^{2.7} \pm 10^{0.6}$  viral RNA (day 12 pi) in the salivary glands  
260 (Figure 4A). As *Wolbachia*-free *Ae. albopictus* individuals exhibited high variability in viral  
261 load (see above), no measurement was performed on these organs.

262 When examining the two organs, we only considered *Wolbachia*-positive organs as the  
263 bacteria were not detectable in some of them. Thus, the mean number of *wAlbA* strain tends  
264 to decrease from  $10^{1.0} \pm 10^{0.1}$  at day 1 pi to 0 at day 7 pi in midguts, and from  $10^{0.9} \pm 10^{0.5}$  at  
265 day 2 pi to 0 at day 7 pi in the salivary glands (Figure 4B). In contrast, after only a slight  
266 reduction, the relative number of *wAlbB* strain remained stable at around  $10^{0.5}$  *Wolbachia* per  
267 *actin* copies (Figure 4C).

268

269 *CHIKV infection and life-history traits of Ae. albopictus cleared of Wolbachia*

270 *Female survival after CHIKV infection*

271 In *Wolbachia*-free *Ae. albopictus*, the mean ( $\pm$  standard deviation) lifespan of CHIKV-  
272 infected mosquitoes was  $11.6 \pm 7.0$  days (trial 1) and  $8.4 \pm 5.3$  days (trial 2) (see Table). This  
273 lifespan was slightly increased in CHIKV-uninfected mosquitoes:  $14.6 \pm 11.9$  days and  $9.6 \pm$   
274  $6.3$  days for trial 1 and trial 2, respectively (see Table). Nevertheless, survival was not  
275 significantly different between CHIKV-infected and uninfected mosquitoes (logrank test:  $p =$   
276  $0.08$  and  $p = 0.022$  in trials 1 and 2, respectively) (Figure 5).

277

278 *CHIKV load in females according to lifespan*

279 *Wolbachia*-free *Ae. albopictus* females were categorized according to their life duration of:  
280  $\leq 5$ , 6 to 10, and 11 to 15 days. Females living more than 16 days were not considered in this  
281 study, as they were so few. The CHIKV viral load distribution in these three categories of  
282 mosquitoes is presented in Figure 6. Using an ANOVA, lifespan had a significant effect on

283 the CHIKV viral load ( $p = 0.004$  and  $p < 10^{-4}$  in trials 1 and 2, respectively). In both trials, the  
284 CHIKV viral load was higher in females living 6 to 10 days than in females living less or  
285 equal to 5 days. On the other hand, the CHIKV viral load was similar in females living 6 to 10  
286 days to those living 11 to 15 days.

287

### 288 *Oviposition*

#### 289 *Time to first egg laying*

290 The proportions of *Wolbachia-free* mosquitoes that laid eggs when exposed to CHIKV  
291 (68.9% and 73.3% in trials 1 and 2, respectively) were close to those that were not exposed to  
292 CHIKV (47.4% and 82% in trials 1 and 2, respectively). Although *Wolbachia-free*  
293 mosquitoes exposed to CHIKV laid eggs slightly earlier (see Table), the Kaplan-Meier  
294 estimates did not show any significant difference in the time from the blood-meal to egg  
295 laying ( $p = 0.05$  in trial 1 and  $p = 1$  in trial 2).

#### 296 *Number of eggs laid per mosquito*

297 Using a negative binomial regression model, no significant difference was found in the  
298 number of eggs laid, between *Wolbachia-free* mosquitoes that had taken an infectious blood-  
299 meal and those that received a non-infectious blood-meal (incidence rate ratio [IRR] (95% CI)  
300 of 1.16 (0.75 – 1.79)) (see Table).

#### 301 *Time between first oviposition and mosquito death*

302 Time between the first oviposition and mosquito death was estimated using Kaplan-Meier  
303 survival curves. No significant difference between mosquitoes exposed and those not exposed  
304 to CHIKV was observed in either trial ( $p = 0.21$  and  $p = 0.90$  in trials 1 and 2, respectively)  
305 (see Table).

#### 306 *Hatching characteristics*

307 *Proportion of mosquitoes with at least one egg hatched*

308 When comparing the proportion of mosquitoes with at least one egg hatched, using the  
309 Fisher's exact test, no significant difference was found between mosquitoes exposed to a non-  
310 infectious blood-meal and those exposed to an infectious blood-meal ( $p = 0.66$  and  $p = 0.84$  in  
311 trials 1 and 2, respectively) (see Table).

312 *Hatching rate per female*

313 Using an ANOVA, the hatching rate was not found to be significantly different in CHIKV-  
314 infected than in uninfected mosquitoes ( $p = 0.43$  and  $p = 0.23$  in trials 1 and 2, respectively).  
315 It should be noted that overall the number of eggs laid and the hatching rate of eggs were  
316 surprisingly low. The BSL-3 conditions in which the experiments were carried out might  
317 explain this, as these two traits have higher values in regular insectaries (data not shown).

318

319 *Vertical transmission efficiency*

320 In both trials, no viral RNA was detected in offspring whose *Wolbachia*-free parents had been  
321 exposed to CHIKV. A total of 1054 offspring (528 males and 526 females) resulting from 66  
322 females in trial 1 and 1070 individuals (538 males and 532 females) from 87 females in trial 2  
323 were negative for CHIKV as monitored by PCR.

324

325

326 **Discussion**

327

328 We have shown that the clearance of *Wolbachia* infection from one line of *Ae. albopictus*  
329 originated from La Reunion Island induced distinct CHIKV replication profiles: some  
330 individuals of *Wolbachia*-free mosquitoes harbored less viral RNA and others hosted 10,000

331 times more viral RNA than the amount ingested. In contrast in *Wolbachia*-infected *Ae.*  
332 *albopictus*, a homogeneous profile of viral replication concomitant with a decrease in  
333 *Wolbachia* density was observed. This profile was also detected when examining the two key  
334 organs for viral transmission, midgut and salivary glands. Nevertheless, removing *Wolbachia*  
335 did not induce any significant changes of mosquito response to infection by CHIKV. Indeed,  
336 life history traits, survival, oviposition and hatching characteristics did not differ between  
337 *Wolbachia*-free mosquitoes that had been exposed and those not exposed to viral infection.

338

### 339 **CHIKV infection leads to a decrease of *Wolbachia* density in *Ae. albopictus***

340 When infected with CHIKV, *Ae. albopictus* harboring *Wolbachia* became the site of intensive  
341 viral replication yielding an ~1000-fold increase in viral RNA copies at day 4 pi (Figure 2A).  
342 Concomitantly, the *Wolbachia* load decreased from day 2 to day 5 pi at a time when viral  
343 replication was increasing (Figure 3A). The *Wolbachia* load was 3 times less from day 5 pi.  
344 (~0.5 log). The *Wolbachia* decrease might result from competition for resources with  
345 replicating CHIKV in mosquito cells. Since both *Wolbachia* and CHIKV occupy the same  
346 niche, i.e. the cell cytoplasm, the presence of *Wolbachia* could reduce the pool of amino acids  
347 available to ensure the achievement of the viral cycle. Thus, the intensive phase of CHIKV  
348 replication in mosquitoes coincides with a decrease of *Wolbachia* densities. The decline was  
349 not observed when *Wolbachia*-infected mosquitoes ingested a non-infectious blood-meal  
350 (Figure 3B). Moreover, it has been shown that in insecticide-resistant *Culex pipiens*,  
351 *Wolbachia* densities tended to increase, suggesting that resistant mosquitoes suffering from a  
352 physiological resistance cost might control *Wolbachia* loads less efficiently (Berticat *et al.*  
353 2002). This pattern was not observed from our data, which suggests a different relationship in  
354 *Ae. albopictus* dealing with multiple infection, virus plus bacteria, in accordance with the



355 work of Tortosa *et al.* (2008). Indeed, naturally occurring *Wolbachia* strains are proved to  
356 present antiviral protection in insects (Teixera *et al.* 2008; Hedges *et al.* 2008). Virus particles  
357 accumulate more slowly and virus induced mortality is delayed. *Wolbachia* density plays an  
358 important role for antiviral protection. Thus, high densities may be important for antiviral  
359 protection resulting from a competition between virus and bacteria for limited host resources  
360 (Osborne *et al.* 2009). Nevertheless, *Wolbachia*-mediated antiviral protection is not  
361 ubiquitous (Osborne *et al.* 2009). It is therefore likely that the interactions between *Wolbachia*  
362 and viruses impact on the distribution of both microbes in insect populations. It has been  
363 proposed that a life-shortening strain, *Wolbachia pipientis* (wMelPop) transfected in *Ae.*  
364 *aegypti* might be used to alter mosquito population age structure, thereby reducing arbovirus  
365 transmission without eradicating the mosquito population (McMeniman *et al.* 2009).  
366 Moreover, wMelPop probably causes tissue damages which leads to reduced blood-feeding  
367 success (Turley *et al.* 2009).

368 One question that needed to be addressed was whether this effect was detectable in all organs,  
369 given that *Wolbachia* is widespread throughout tissues. Salivary glands and the midgut have  
370 both been reported to be target tissues for *Wolbachia* infection in *Ae. albopictus* (Zouache *et*  
371 *al.* 2009a). In addition, CHIKV must infect and subsequently pass through the epithelium of  
372 the mosquito midgut and then reach the salivary glands for further replication before  
373 transmission can occur. We showed that the two organs, midgut and salivary glands, sustained  
374 CHIKV replication in agreement with the pattern obtained when examining entire  
375 mosquitoes: an increase of viral load from day 0 to day 4 pi and a plateau from day 5 pi  
376 onwards (Figure 4). In addition, the load of *Wolbachia* detected in both organs decreased over  
377 time as we have shown in the entire body. Thus, CHIKV replication might interfere with  
378 *Wolbachia* densities hosted in the midgut and salivary glands of *Ae. albopictus*. Conversely, it

379 has been shown that the strain wMelPop-CLA transfected in *Ae. aegypti* reduces the ability of  
380 dengue and chikungunya viruses to establish high infections in the mosquito suggesting an  
381 interference effect of *Wolbachia* with the pathogen through the expression of some immune  
382 effector genes (Moreira *et al.* 2009). To control viral infection, insects can activate immune  
383 signaling pathways such as Toll (Sanders *et al.* 2005; Xi *et al.* 2008), JAK/STAT (Souza-  
384 Neto *et al.* 2009) or Imd/JNK (Sanders *et al.* 2005) that may in turn also affect the bacterial  
385 symbionts (for a review on mosquito antiviral responses to arboviruses, Fragkoudis *et al.*  
386 2009). Thus, the competence of *Ae. albopictus* naturally infected by *Wolbachia* to CHIKV is  
387 suggested to be related to lower densities of *Wolbachia* limiting its ability to effectively  
388 interfere with virus replication. It is not clear whether *Wolbachia* is able to remain a benign  
389 symbiont simply by maintaining a very low replication rate independently of the host cell  
390 cycle, or whether the bacteria actively coordinates its replication with that of the host through  
391 unknown mechanisms. Most studies tend to suggest that *Wolbachia* may simply be slow  
392 replicators. However, work of Ruang-Areerate *et al.* (2004) supports the existence of  
393 synchrony between *Wolbachia* replication and that of its host cells in *Ae. albopictus*.

394

### 395 **CHIKV infection does not affect life history traits of *Wolbachia*-free *Ae. albopictus***

396 We found that infection with CHIKV did not significantly affect mosquito survival, female  
397 oviposition and egg hatching of *Wolbachia*-free mosquitoes, as no significant differences was  
398 found between CHIKV-infected females and CHIKV-uninfected females (see Table and  
399 Figure 5). Conversely, we found increased life spans with *Wolbachia*-infected *Ae. albopictus*  
400 regardless of the infection with CHIKV (unpublished data). These results are in agreement  
401 with a theory predicting that as a vertically transmitted bacterium, *Wolbachia* should be

402 selected to increase its transmission by providing fitness benefits to its host (Lipsitch *et al.*  
403 1995).

404 Furthermore, removing *Wolbachia* from *Ae. albopictus* necessitated four generations of  
405 antibiotic treatments: larval treatment with tetracycline for three generations and both  
406 larval/adult treatment with tetracycline and rifampicin for one generation. These two  
407 antibiotics differ in their modes of action; tetracycline affects protein synthesis while  
408 rifampicin inhibits prokaryotic DNA-dependent RNA polymerase (Raoult & Drancourt 1991).  
409 Tetracycline alone failed to completely clear *Wolbachia*. This ineffectiveness of tetracycline  
410 treatment may come from either the potential resistance of *Wolbachia* (Kambhampati *et al.*  
411 1993) or the inability of the antibiotic to reach all *Wolbachia* cell niches. Treatments  
412 associating tetracycline and rifampicin generated *Wolbachia*-free individuals, avoiding a  
413 requirement of establishing isofemale lines to produce aposymbiotic lines. Their maintenance  
414 would have been difficult with a higher mortality due to an increased homozygosity of  
415 deleterious loci generated by inbreeding effects. However, *Wolbachia* may not be the only  
416 bacteria removed by antibiotic treatments. Other intracellular bacteria could be affected by  
417 antibiotic treatment (Zouache *et al.* 2009b), contributing to the observed effects on CHIKV  
418 replication in mosquitoes. Indeed, the bacteria *Acinetobacter* has been detected in the midgut  
419 and salivary glands of *Ae. albopictus* females (Zouache *et al.* 2009a). Our repeated treatments  
420 with antibiotics did not succeed to completely remove bacteria of the genus *Acinetobacter*  
421 from *Ae. albopictus* (data not shown). Interestingly, a recent study has shown that  
422 *Acinetobacter antiviralis* sp. nov. from Tobacco plant roots was able to produce an antiviral  
423 compound with inhibitory effects on tobacco mosaic virus multiplication (Lee *et al.* 2009).  
424 Thus, the role of other bacteria hosted by *Ae. albopictus* on the transmission of CHIKV  
425 should be investigated.

426

427 **Removing *Wolbachia* does not reduce enough *Ae. albopictus* lifespan to affect the**  
428 **transmission of CHIKV**

429 A female mosquito must survive longer than the extrinsic incubation period to successfully  
430 contribute to pathogen transmission (Hardy *et al.* 1983). This time period lasts from pathogen  
431 ingestion to potential infectivity; this period has been estimated to be two days for *Ae.*  
432 *albopictus* infected with CHIKV (Dubrulle *et al.* 2009). Mosquito survival is therefore  
433 considered a critical component of a vector's population capacity for pathogen transmission.  
434 Thus, removing *Wolbachia* does not reduce enough the mosquito lifespan to alter the  
435 transmission efficiency of CHIKV. However, when transinfected into a naturally *Wolbachia*-  
436 free *Aedes aegypti*, the main vector of several arboviruses, *Wolbachia* wMelPop strain is able  
437 to shorten the adult mosquito lifespan, with the potential to reduce disease transmission  
438 (Brownstein *et al.* 2003). CHIKV replication in *Ae. aegypti* exhibits a similar profile to that in  
439 *Wolbachia*-infected *Ae. albopictus* (data not shown). Horizontal gene transfer from  
440 *Wolbachia* to *Ae. aegypti* has been reported which might explain this similarity (Klasson *et al.*  
441 2009). The transfer of some genes from *Wolbachia* to *Ae. aegypti* is probably accomplished  
442 through a mechanism of transfer *via* nuclear-phage recombination.

443 Although the distribution patterns of *Wolbachia* vary among hosts, *Wolbachia* is consistently  
444 found in the gonads (Dobson *et al.* 1999). It is from this organ that *Wolbachia* ensures its  
445 transmission to subsequent generations *via* eggs. As for other vertically transmitted  
446 intracellular symbionts, *Wolbachia* must maintain a replication rate that does not exceed that  
447 of its host cells if it is to remain benign. Bacterial densities in the ovaries have a direct bearing  
448 on transmission efficiencies. Therefore, too high densities of *Wolbachia* in the ovaries could  
449 cause a reduction in reproductive fitness. It has been shown that in *Drosophila*, low densities

450 of *Wolbachia* in ovaries are sufficient to secure perfect transmission of bacteria to progenies.  
451 Thus, an attenuation of fitness costs could be explained by concomitant reductions in  
452 *Wolbachia* replication rates in ovaries (McGraw *et al.* 2002). In addition, the absence of  
453 *Wolbachia* does not favor maternal transmission of CHIKV as no virus has been detected in  
454 2124 mosquito offspring (1,054 adults in the trial 1 and 1,070 in the trial 2). Moreover, when  
455 analyzing *Wolbachia*-infected *Ae. albopictus* from the Reunion Island, Vazeille *et al.* (2009)  
456 did not succeed in detecting CHIKV under laboratory conditions. Vertical transmission of  
457 CHIKV might be a rare and uncommon phenomenon (Mourya 1987) as we did not find any  
458 infected individuals in the progenies of infected females. Even rare, vertical transmission is  
459 described as one mechanism for alphavirus maintenance in nature (Dhileepan *et al.* 1996;  
460 Fulhorst *et al.* 1994).

461  
462 Our results pertaining to CHIKV replication in *Wolbachia*-infected *Ae. albopictus* adds to the  
463 perception that the response of a host to a particular pathogen also depends on the presence of  
464 other microorganisms as described in the case of *Ae. aegypti* infected with dengue virus (Xi *et*  
465 *al.* 2008). The infection by *Wolbachia* may alter the intracellular environment to allow its  
466 survival within the host. For example, *Wolbachia* could interfere with iron in a way that limits  
467 oxidative stress and cell death, thus promoting its persistence within host cells (Kremer *et al.*  
468 2009). Here, we have shown that in spite of the fact that *Wolbachia* is not essential for viral  
469 replication, its presence could lead to optimize replication from day 4 pi onwards, coinciding  
470 with a decrease in *Wolbachia* density. Thus the presence of the symbiont could maintain the  
471 complexity of the viral population. Whether this observation could be extrapolated to other  
472 viruses remains to be determined.

473

474

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620

621

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633 **Figure Legends**

634

635 **Figure 1.** *Wolbachia* densities,  $wAlbA$  (■) and  $wAlbB$  (◆), across treatments with antibiotics.  
636 Larval treatments were done with tetracycline from generations F3 to F5 and both larval/adult  
637 treatments with tetracycline/rifampicin from generations F6 to F8. To quantify *Wolbachia*,  
638 total DNA was extracted and used for quantitative PCR. The mean number of genomes of  
639  $wAlbA$  and  $wAlbB$  was given per *actin* copies.

640

641 **Figure 2.** Variations of viral loads in *Wolbachia*-infected (A) and *Wolbachia*-free (B) *Ae.*  
642 *albopictus* after exposure to a blood-meal with CHIKV E1-226V. Two trials were carried out  
643 and measures were done with 5 females sacrificed at different days pi. Individual mosquitoes  
644 were used to extract both DNA and RNA. RNA was used to determine viral load by  
645 quantitative RT-PCR, and DNA to measure *Wolbachia* density and *actin* gene content by  
646 quantitative PCR. The mean number of genomes of  $wAlbA$  and  $wAlbB$  was given per *actin*  
647 copies.

648

649 **Figure 3.** Variations of  $wAlbA$  and  $wAlbB$  in *Wolbachia*-infected *Ae. albopictus* after  
650 exposure to an infectious blood-meal with CHIKV E1-226V (A) or to a non-infectious blood-  
651 meal (B). Two trials were carried out and measures were done with 5 females sacrificed at  
652 different days pi. DNA was extracted from each individual mosquito to measure *Wolbachia*  
653 density and *actin* gene by quantitative PCR. The mean number of genomes of  $wAlbA$  and  
654  $wAlbB$  was given per *actin* copies. These low levels of  $wAlbA$  and  $wAlbB$  which are not  
655 found in field-collected mosquitoes could be the consequence of laboratory rearing  
656 conditions.

657

658 **Figure 4.** Densities of viral RNA (A), *wAlbA* (B) and *wAlbB* (C) in midguts and salivary  
659 glands of *Wolbachia*-infected *Ae. albopictus* after exposure to an infectious blood-meal with  
660 CHIKV E1-226V. Five females were dissected at different days post-infection. From each  
661 female, organs were dissected and treated individually to extract both DNA and RNA. RNA  
662 was used to determine viral load by quantitative RT-PCR, and DNA to measure *Wolbachia*  
663 density and *actin* gene content by quantitative PCR. The mean number of genomes of *wAlbA*  
664 and *wAlbB* was given per *actin* copies.

665

666 **Figure 5.** Survival of *Wolbachia*-free *Ae. albopictus* after exposure to an infectious or non-  
667 infectious blood-meal with CHIKV at a titer of  $10^{7.5}$  PFU/mL. Dead mosquitoes were scored  
668 every day to estimate the female life duration following exposure to a blood-meal.

669

670 **Figure 6.** CHIKV loads in *Wolbachia*-free *Ae. albopictus* according to life duration.  
671 Mosquitoes were exposed to an infectious blood-meal at a titer of  $10^{7.5}$  PFU/mL. Dead  
672 mosquitoes were collected and their infection status was checked by quantitative RT-PCR to  
673 estimate the viral RNA load.

674

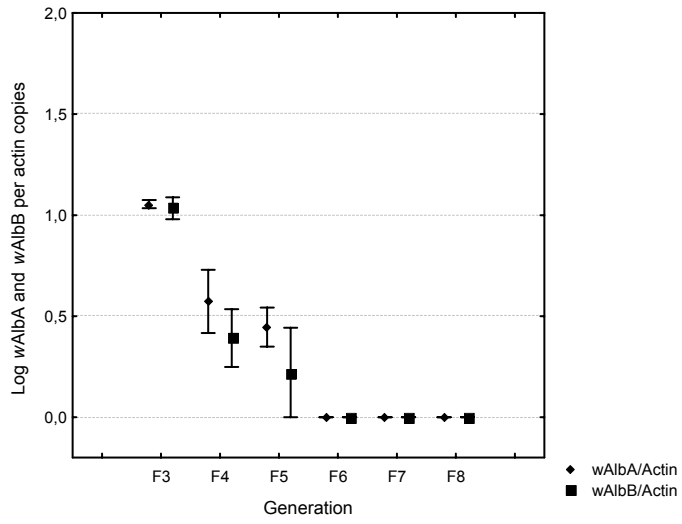
675

676

**Table.** Life duration, oviposition characteristics (delay to laying, number of laid eggs and time between first oviposition and female death) and hatching characteristics (proportion of females with at least one egg hatched and hatching rate per female) of *Wolbachia*-free *Ae. albopictus* from the Reunion Island after exposure to a blood-meal (non-infectious or infectious with CHIKV E1-226V).

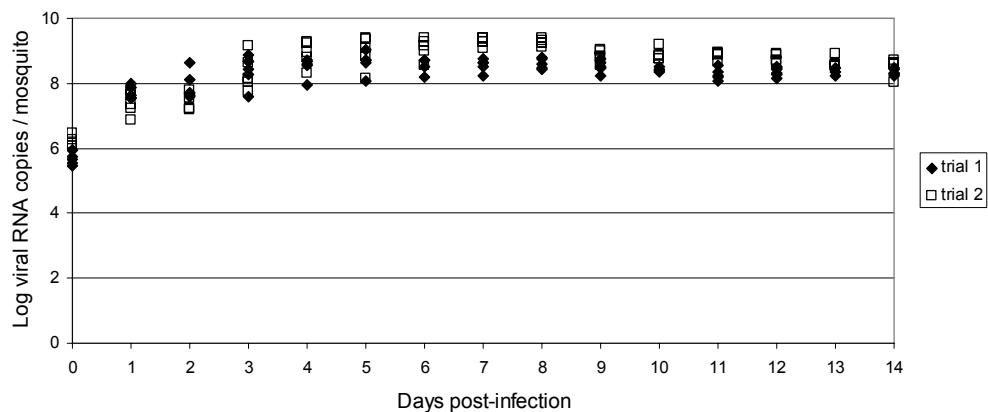
Blood-meal	Life duration ± SD (N)		Oviposition (in days)						Hatching (%)			
			Delay to laying ± SD (N)		Number of laid eggs ± SD (N)		Time between first oviposition and female death ± SD (N)		Proportion of females with at least one egg hatched (N)		Hatching rate per female ± SD (N)	
	1 <sup>*</sup>	2 <sup>#</sup>	1	2	1	2	1	2	1	2	1	2
Non-infectious	14.6 ± 11.9 (19)	9.6 ± 6.3 (50)	9.4 ± 10.3 (9)	6.2 ± 3.8 (41)	26.3 ± 31.6 (19)	35.4 ± 24.6 (50)	9.9 ± 13.6 (9)	3.7 ± 4.8 (41)	77.8 (9)	75.6 (41)	27.9 ± 24.2 (9)	31.6 ± 27.3 (41)
Infectious with E1-226A	11.6 ± 7.0 (132)	8.4 ± 5.3 (161)	6.3 ± 4.0 (91)	5.2 ± 2.0 (118)	33.2 ± 31.0 (132)	32.5 ± 29.2 (161)	6.1 ± 6.0 (91)	3.7 ± 4.3 (118)	76.9 (91)	77.1 (118)	35.7 ± 28.6 (91)	25.8 ± 26.2 (118)

N, number of female analysed; SD, standard deviation; \*, trial 1; #, trial 2.

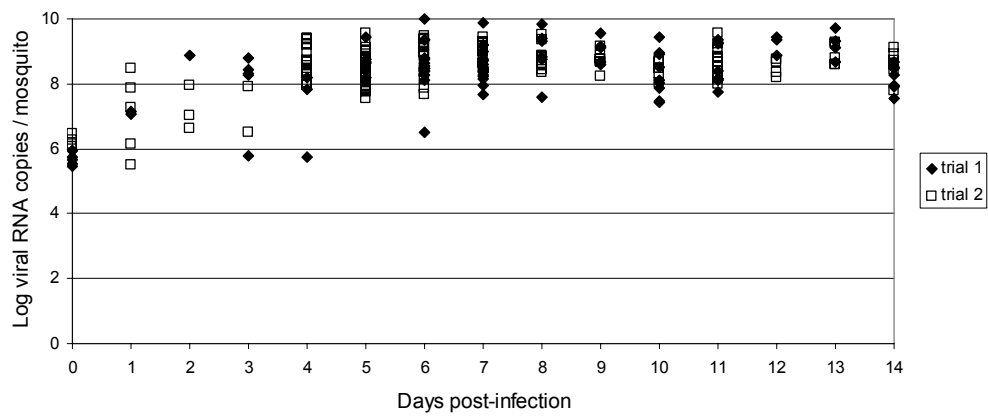




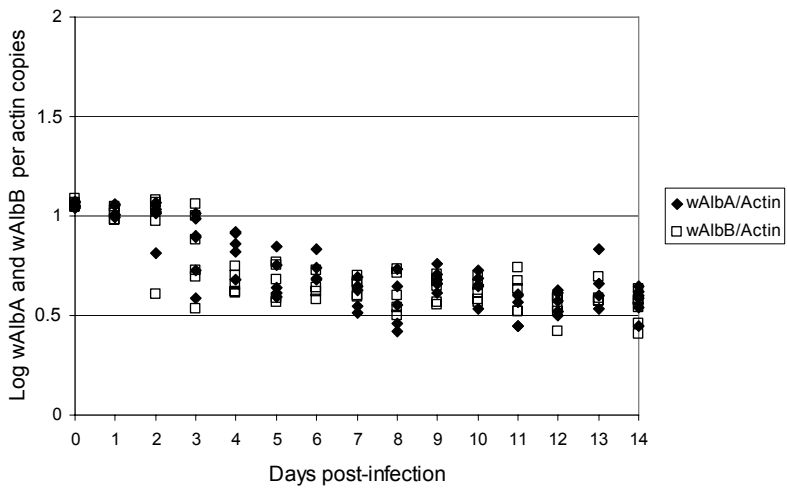
(A)



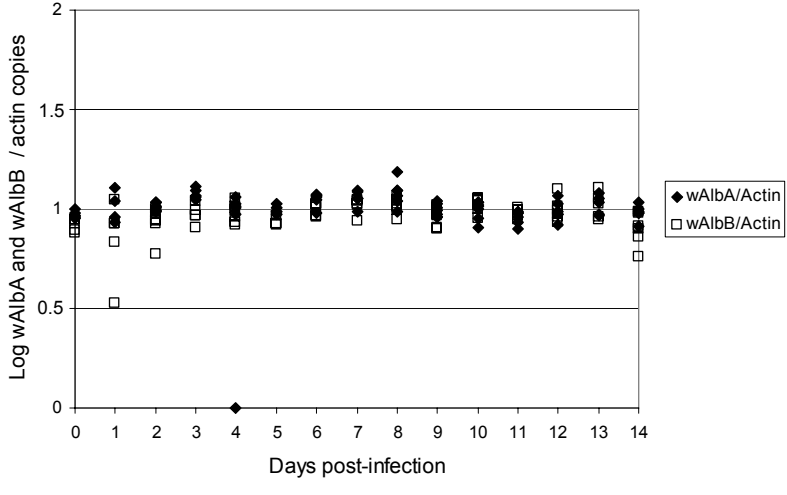
(B)

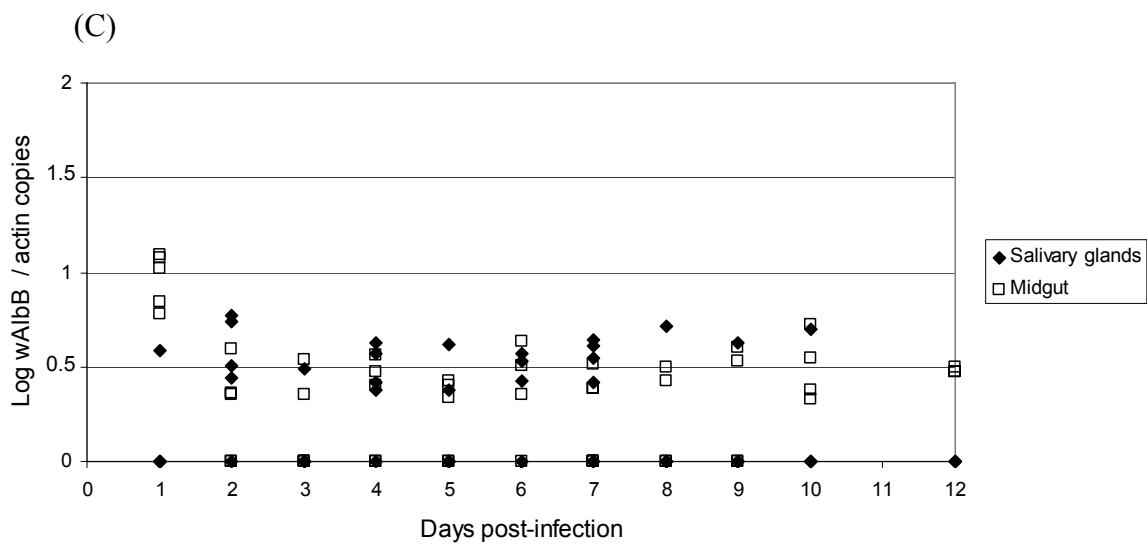
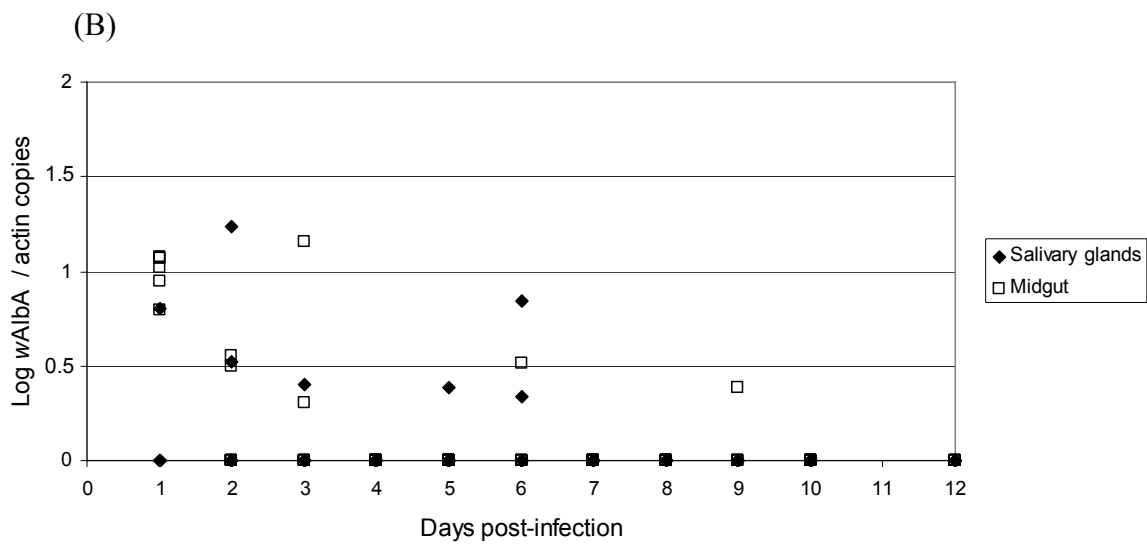
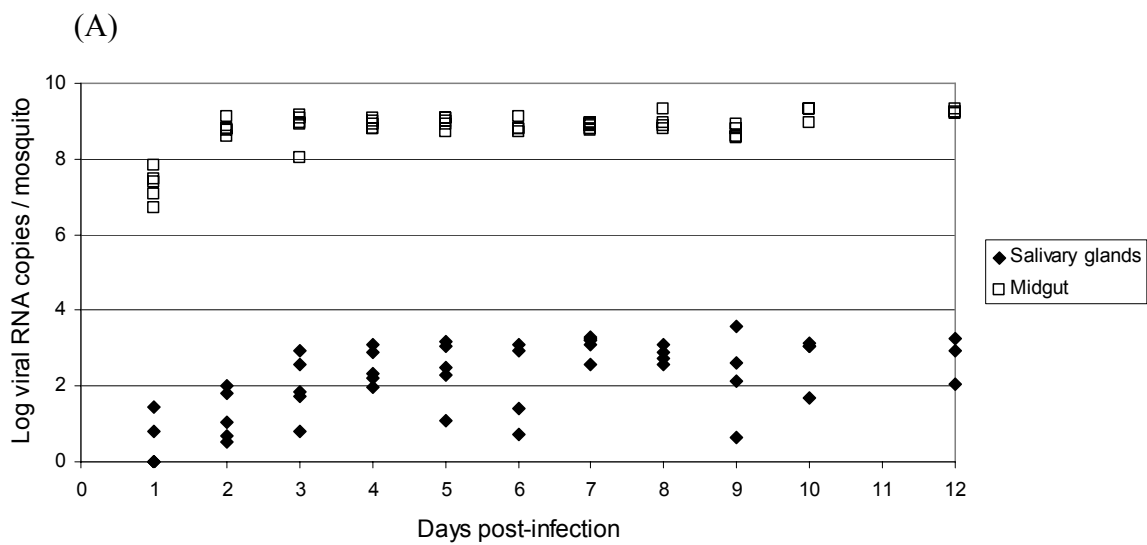


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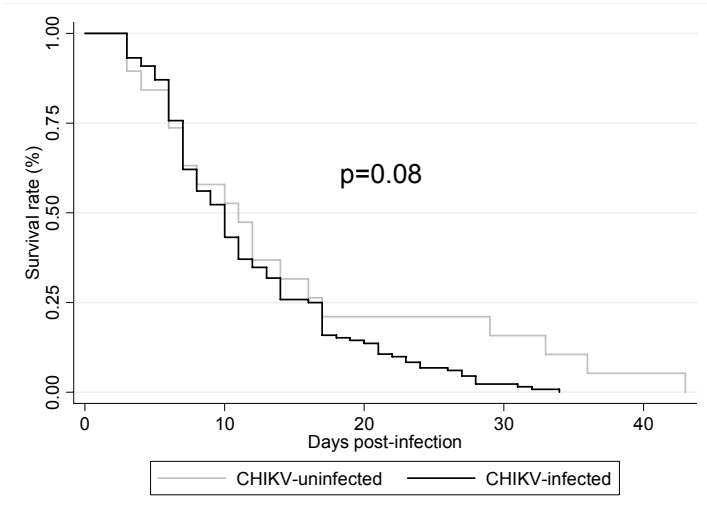


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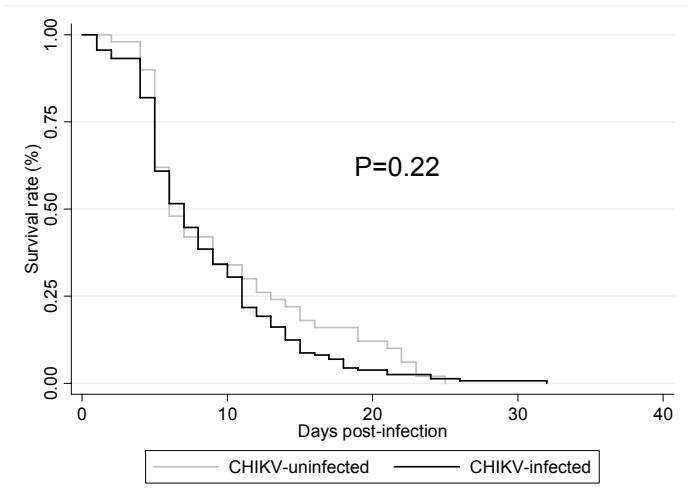




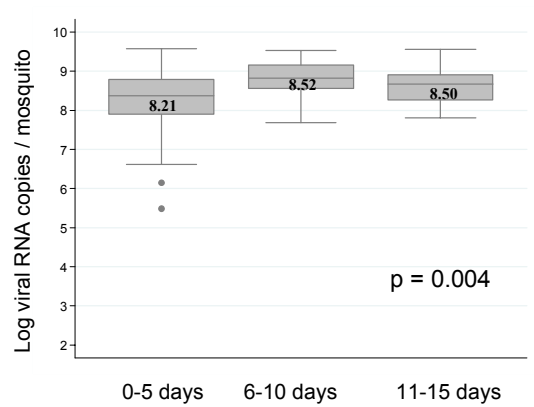
Trial 1



Trial 2



## Trial 1



## Trial 2

