Wolbachia modulates Chikungunya replication in Aedes albopictus.

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

Wolbachia modulates Chikungunya replication in *Aedes albopictus*

L. MOUSSON,[*] E. MARTIN,[*] K. ZOUACHE,[#] Y. MADEC,[†] P. MAVINGUI[#] and A.-B. FAILLOUX[*]

[*] Institut Pasteur, Génétique moléculaire des Bunyavirus, 25-28 rue du Dr Roux, F-75724 Paris cedex 15, France

[†] Institut Pasteur, Unité de Recherche et d'Expertise Epidémiologie des Maladies Emergentes, 25-28 rue du Dr Roux, F-75724 Paris cedex 15, France

[#] Université Lyon, F-69022, Lyon, France, Université de Lyon 1, Villeurbanne, CNRS, UMR5557, Ecologie Microbienne, Lyon, France

Keywords: Aedes albopictus, chikungunya, *Wolbachia*, real-time PCR, life history traits

Running title: *Wolbachia* and CHIKV interference

(*) Corresponding author:
Anna-Bella FAILLOUX
Institut Pasteur, Génétique moléculaire des Bunyavirus, 25-28 rue du Dr Roux, F-75724 Paris cedex 15, France.
FAX: 00 33 1 40 61 31 51. E-mail: afaillou@pasteur.fr

Abstract

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

Introduction

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

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

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

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

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

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

Materials and Methods

Mosquitoes

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

Antibiotic treatment to clear Wolbachia

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

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

Virus production

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

Experimental oral infections

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

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

Nucleic acid extraction and quantitative PCR

Individual mosquitoes and dissected organs (midguts and salivary glands) were used to extract total nucleic acids. Extraction was done with NucleoSpin® RNA/DNA buffer set (Macherey-Nagel) coupled to the NucleoSpin® RNA II kit that enables the isolation of both RNA and DNA. RNA was used to determine viral load by quantitative RT-PCR, and DNA to measure *Wolbachia* (*w*AlbA and *w*AlbB) density and actin gene content by quantitative PCR. To measure viral load at different days post-infection (pi), five females were killed every day until day 14 pi. After total RNA extraction, a one-step RT-PCR reaction was performed with a Power SYBR® Green RNA-to-CT^{TM} one step kit (Applied Biosystem) in a volume of 25 µL containing 2 µL RNA template, 12.5 µL 2X Power SYBR® Green I RT-PCR Mix, 0.25 µL sense primer (0.1 µM), 0.25µL anti-sense primer (0.1 µM), 0.2 µL RT enzyme mix and 9.8 µL of ddH_2O. Primers selected in the E2 structural protein coding region were: sense Chik/E2/9018/+ (CACCGCCGCAACTACCG) and anti-sense Chik/E2/9235/- (GATTGGTGACCGCGGCA). The PCR program was: 48°C for 30 min, 95°C for 10 min; 40 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 of 19 min 59 sec. The size of the PCR product was 217 bp. A standard curve was generated using duplicates of 10-fold serial dilutions of RNA synthetic transcripts. Quantification of viral RNA was achieved by comparing the threshold cycle (Ct) values of samples to those of standards according to the ΔC_t analysis. One Log of infectious viral particles corresponds to 1-2 Log RNA virus (Figure S2). To quantify *Wolbachia* (*w*AlbA and *w*AlbB) and actin gene, 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 *w*AlbA, *w*AlbB, 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 *w*AlbA, *w*AlbB and actin, respectively. From 60 µl of DNA solution
170 extracted from one mosquito, 2 µl of DNA was mixed with 0.3 µM of each primer and 12.5 µl
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 *w*AlbA and *w*AlbB 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 (≤ 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).

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

Results

Removing Wolbachia from infected mosquitoes by antibiotic treatments

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

CHIKV replication in Wolbachia-infected and Wolbachia-free mosquitoes

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

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

Variation of Wolbachia density following CHIKV infection

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

Viral and bacterial densities in midguts and salivary glands

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

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

When examining the two organs, we only considered *Wolbachia*-positive organs as the bacteria were not detectable in some of them. Thus, the mean number of *w*AlbA strain tends 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 day 2 pi to 0 at day 7 pi in the salivary glands (Figure 4B). In contrast, after only a slight reduction, the relative number of *w*AlbB strain remained stable at around $10^{0.5}$ *Wolbachia* per *actin* copies (Figure 4C).

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

Female survival after CHIKV infection

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

CHIKV load in females according to lifespan

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

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

Oviposition

Time to first egg laying

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

Number of eggs laid per mosquito

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

Time between first oviposition and mosquito death

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

Hatching characteristics

Proportion of mosquitoes with at least one egg hatched

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

Hatching rate per female

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

Vertical transmission efficiency

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

References

1. Ahantarig A, Trinachartvanit W, Kittayapong P (2008) Relative *Wolbachia* density of field-collected *Aedes albopictus* mosquitoes in Thailand. *Journal of Vector Ecology*, **33**, 173-177.
2. Berticat C, Rousset F, Raymond M, Berthomieu A, Weill M (2002) High *Wolbachia* density in insecticide-resistant mosquitoes. *Proceedings Biological Sciences*, **269**, 1413-1416.
3. Brownstein JS, Hett E, O'Neill SL (2003) The potential of virulent *Wolbachia* to modulate disease transmission by insects. *Journal of Invertebrate Pathology*, **84**, 24-29.
4. Coffinet T, Mourou JR, Pradines B *et al.* (2007) First record of *Aedes albopictus* in Gabon. *Journal of the American Mosquito Control Association*, **23**, 471-472.
5. Delatte H, Paupy C, Dehecq JS *et al.* (2008) *Aedes albopictus*, vector of chikungunya and dengue viruses in Reunion Island: biology and control. *Parasite*, **15**, 3-13.
6. Dhileepan K, Azuolas JK, Gibson CA (1996) Evidence of vertical transmission of Ross River and Sindbis viruses (Togaviridae: Alphavirus) by mosquitoes (Diptera: Culicidae) in southeastern Australia. *Journal of Medical Entomology*, **33**, 180-182.
7. Dobson SL, Bourtzis K, Braig HR *et al*. (1999) *Wolbachia* infections are distributed throughout insect somatic and germ line tissues. *Insect Biochemistry and Molecular Biology*, **29**, 153-160.
8. Dobson SL, Marsland EJ, Rattanadechakul W (2001) *Wolbachia*-induced cytoplasmic incompatibility in single- and superinfected *Aedes albopictus* (Diptera: Culicidae). *Journal of Medical Entomology*, **38**, 382-387.

9. Dubrulle M, Mousson L, Moutailler S, Vazeille M, Failloux AB (2009) Chikungunya virus and *Aedes* mosquitoes: saliva is infectious as soon as two days after oral infection. *PLoS ONE*, **4**, e5895.
10. Dutton TJ, Sinkins SP (2005) Filarial susceptibility and effects of *Wolbachia* in *Aedes pseudoscutellaris* mosquitoes. *Medical and Veterinary Entomology*, **19**, 60-65.
11. Fontenille D, Toto JC (2001) *Aedes (Stegomyia) albopictus* (Skuse), a potential new Dengue vector in southern Cameroon. *Emerging Infectious Diseases*, **7**, 1066-1067.
12. Fragkoudis R, Attarzadeh-Yazdi G, Nash AA, Fazakerley JK, Kohl A (2009) Advances in dissecting mosquito innate immune responses to arbovirus infection. *Journal of General Virology*, **90**, 2061-2072.
13. Fulhorst CF, Hardy JL, Eldridge BF, Presser SB, Reeves WC (1994) Natural vertical transmission of western equine encephalomyelitis virus in mosquitoes. *Science*, **263**, 676-678.
14. Haine ER (2008) Symbiont-mediated protection. *Proceedings. Biological Ssciences / The Royal Society*, **275**, 353-361.
15. Hardy JL, Houk EJ, Kramer LD, Reeves WC (1983) Intrinsic factors affecting vector competence of mosquitoes for arboviruses. *Annual Review of Entomology*, **28**, 229-262.
16. Hedges LM, Brownlie JC, O'Neill SL, Johnson KN (2008) *Wolbachia* and virus protection in insects. *Science*, **322**, 702.
17. Hertig M (1936) The rickettsia, *Wolbachia pipientis* and associated inclusions of the mosquito *Culex pipiens*. *Parasitology*, **28**, 453-486.

18. Hoffmann AA, Turelli M (1997) Cytoplasmic incompatibility in insects. In: Influential passengers: inherited microorganisms and Arthropods reproduction (ed. O'Neill SL, Hoffmann AA & Werren JH), Oxford University Press, Oxford.
19. Husrt GDD, Jiggins FM, Schulenberg JHG *et al*. (1999) Male-killing *Wolbachia* in two species of insect. *Proceedings of the Royal Society of London B*, **266**, 735-740.
20. Kambhampati S, Rai KS, Burgun SJ (1993) Unidirectional cytoplasmic incompatibility in the mosquito, *Aedes albopictus*. *Evolution*, **47**, 673-677.
21. Kittayapong P, Mongkalangoon P, Baimai V, O'Neill SL (2002) Host age effect and expression of cytoplasmic incompatibility in field populations of *Wolbachia*-superinfected *Aedes albopictus*. *Heredity*, **88**, 270-274.
22. Klasson L, Kambris Z, Cook PE, Walker T, Sinkins SP (2009) Horizontal gene transfer between *Wolbachia* and the mosquito *Aedes aegypti*. *BMC Genomics*, **10**, 33.
23. Kremer N, Voronin D, Charif D, Mavingui P, Mollereau B, Vavre F (2009) *Wolbachia* interferes with ferritin expression and iron metabolism in insects. *PLoS Pathogens*, **5**, e1000630.
24. Landmann F, Orsi GA, Loppin B, Sullivan W (2009) *Wolbachia*-mediated cytoplasmic incompatibility is associated with impaired histone deposition in the male pronucleus. *PLoS Pathogens*, **5**, e1000343
25. Lee JS, Lee KC, Kim KK *et al.* (2009) *Acinetobacter antiviralis* sp. nov., from Tobacco plant roots. *Journal of Microbiology and Biotechnology,* **19**, 250–256.
26. Lipsitch M, Nowak MA, Ebert D, May RM (1995) The population dynamics of vertically transmitted parasites. *Proceedings of the Royal Society of London*, **B260**, 321-327.

27. McGraw EA, Merritt DJ, Droller JN, O'Neill SL (2002) *Wolbachia* density and virulence attenuation after transfer into a novel host. *Proceedings of the National Academy of Sciences of the United States of America,* **99**, 2918-2923.

28. McMeniman CJ, Lane RV, Cass BN *et al*. (2009) Stable introduction of a life-shortening *Wolbachia* infection into the mosquito *Aedes aegypti*. *Science,* **323**, 141-144.

29. Moreira LA, Iturbe-Ormaetxe I, Jeffery JA *et al.* (2009) A Wolbachia symbiont in *Aedes aegypti* limits infection with dengue, Chikungunya, and Plasmodium. *Cell*, **139**, 1268-1278.

30. Mourya DT (1987) Absence of transovarial transmission of chikungunya virus in *Aedes aegypti* & *Ae. albopictus* mosquitoes. *Indian Journal of Medical Research*, **85**, 593-595.

31. Osborne SE, Leong YS, O'Neill SL, Johnson KN (2009) Variation in Antiviral Protection Mediated by Different *Wolbachia* Strains in *Drosophila simulans*. *PLoS Pathogens*, **5**, e1000656. doi:10.1371/journal.ppat.1000656

32. Raoult D, Drancourt M. (1991) Antimicrobial therapy of rickettsial diseases. *Antimicrobial Agents Chemotherapy,* **35**, 2457-2462.

33. Rousset F, Bouchon D, Pintureau B, Juchault P, Solignac M (1992) *Wolbachia* endosymbionts responsible for various alterations of sexuality in arthropods. *Proceedings Biological Sciences,* **250**, 91-98.

34. Ruang-Areerate T, Kittayapong P, McGraw EA, Baimai V, O'Neill SL (2004) *Wolbachia* replication and host cell division in *Aedes albopictus*. *Current Microbiology,* **49**, 10-12.

35. Sanders HR, Foy BD, Evans AM *et al.* (2005) Sindbis virus induces transport processes and alters expression of innate immunity pathway genes in the midgut of the disease vector, *Aedes aegypti*. *Insect Biochemistry and Molecular Biology*, **35**, 1293-1307.

36. Scholte EJ, Schaffner F (2007) Waiting for the tiger: establishment and spread of the *Aedes albopictus* mosquito in Europe. In: Emerging pests and vector-borne disease in Europe (ed. Takken W & Knols BGJ), Wageningen Academic Publishers, Wageningen.

37. Schuffenecker I, Iteman I, Michault A *et al.* (2006) Genome microevolution of Chikungunya viruses causing the Indian Ocean outbreak. *PLoS Medicine*, **3**, e263.

38. Sinkins SP, Gould F (2006) Gene drive systems for insect disease vectors. *Nature Reviews Genetics*, **7**, 427-435.

39. Smith CEG (1956) The history of dengue in tropical Asia and its probable relationship to the mosquito *Aedes aegypti*. *Journal of Tropical Medicine and Hygiene*, **59**, 243-251.

40. Souza-Neto JA, Sim S, Dimopoulos G (2009) An evolutionary conserved function of the JAK-STAT pathway in anti-dengue defense. *Proceedings of the National Academy of Sciences of the United States of America*, **106**, 17841-17846.

41. Sprenger D, Wuithiranyagool T (1986) The discovery and distribution of *Aedes albopictus* in Harris County, Texas. *Journal of the American Mosquito Control Association*, **2**, 217-219.

42. Stouthamer R, Breeuwer JAJ, Luck RF, Werren JH (1993) Molecular identification of microorganisms associated with parthenogenesis. *Nature*, **361**, 66-68.

589 43. Teixera L, Ferreira A, Ashburner M (2008) The bacterial symbiont *Wolbachia* induces
590 resistance to RNA viral infections in *Drosophila melanogaster*. *PLoS Biology*, **6**, e2.
591 44. Tortosa P, Courtiol A, Moutailler S, Failloux AB, Weill M (2008) Chikungunya-
592 *Wolbachia* interplay in *Aedes albopictus*. *Insect Molecular Biology*, **17**, 677-684.
593 45. Toto JC, Abaga S, Carnevale P, Simard F (2003) First report of the oriental mosquito
594 *Aedes albopictus* on the West African island of Bioko, Equatorial Guinea. *Medical*
595 *and Veterinary Entomology*, **17**, 343-346.
596 46. Tram U, Sullivan W (2002) Role of delayed nuclear envelope breakdown and mitosis
597 in *Wolbachia*-induced cytoplasmic incompatibility. *Science*, **296**, 1124-1126.
598 47. Turley AP, Moreira LA, O'Neill SL, McGraw EA (2009) *Wolbachia* infection reduces
599 blood-feeding success in the dengue fever mosquito, *Aedes aegypti*. *PLoS Neglected*
600 *Tropical Diseases*, **3**, e516.
601 48. Vazeille M, Mousson L, Failloux AB (2009) Failure to demonstrate experimental
602 vertical transmission of the epidemic strain of Chikungunya virus in *Aedes albopictus*
603 from La Réunion Island, Indian Ocean. *Memorias do Instituto Oswaldo Cruz,* **104**,
604 632-635.
605 49. Vazeille M, Moutailler S, Coudrier D *et al*. (2007) Two Chikungunya isolates from
606 the outbreak of La Reunion (Indian Ocean) exhibit different patterns of infection in
607 the mosquito, *Aedes albopictus*. *PLoS ONE*, **2**, e1168.
608 50. Werren JH (1997) Biology of *Wolbachia*. *Annual Review of Entomology*, **42**, 587-609.
609 51. Xi Z, Ramirez JL, Dimopoulos G (2008) The *Aedes aegypti* Toll pathway controls
610 Dengue virus infection. *PLoS Pathogens*, **4**, e10000098.

52. Zhou W, Rousset F, O'Neill SL (1998) Phylogeny and PCR based classification of *Wolbachia* strains using *wsp* gene sequences. *Proceedings of the Royal Society of London B*, **265**, 509-515.

53. Zouache K, Voronin D, Tran-Van V, Mousson L, Failloux AB, Mavingui P (2009a) Persistent *Wolbachia* and cultivable bacteria infection in the reproductive and somatic tissues of the mosquito vector *Aedes albopictus*. *PLoS ONE*, **4**, e6388.

54. Zouache K, Voronin D, Tran-Van V, Mavingui P (2009b) Composition of bacterial communities associated with natural and laboratory populations of *Asobara tabida* infected with *Wolbachia*. *Applied and Environmental Microbiology*, **75**, 3755-3764.

Acknowledgements

We are grateful to the DRASS in the Reunion for providing the ALPROV mosquito strain, to the French National Reference Center for Arboviruses for the E1-226V CHIKV strain, and to Mylène Weill from the "Institut des Sciences de l'Evolution de Montpellier" (ISEM) for providing the pQuantAlb plasmid. LM was supported by the Agence Nationale de la Recherche (ChikVendoM ANR-06-SEST07). We also wish to thank Marie Vazeille and Sara Moutailler for valuable discussions, François Rougeon for critical reading of the manuscript, and Michèle Bouloy for her constant support. We are grateful to Katherine Kean for correcting the manuscript. This work was funded by the ANR ChikVendoM drive, the "Fondation pour la Recherche sur la Biodiversité" (FRB, formerly IFB, CD-AOOI-07-012), and the Institut Pasteur (ACIP A-10-2009).

633 **Figure Legends**

634

635 **Figure 1.** *Wolbachia* densities, *w*AlbA (■) and *w*AlbB (♦), 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 *w*AlbA and *w*AlbB 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 *w*AlbA and *w*AlbB was given per *actin*
647 copies.

648

649 **Figure 3.** Variations of *w*AlbA and *w*AlbB 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 *w*AlbA and
654 *w*AlbB was given per *actin* copies. These low levels of *w*AlbA and *w*AlbB which are not
655 found in field-collected mosquitoes could be the consequence of laboratory rearing
656 conditions.

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

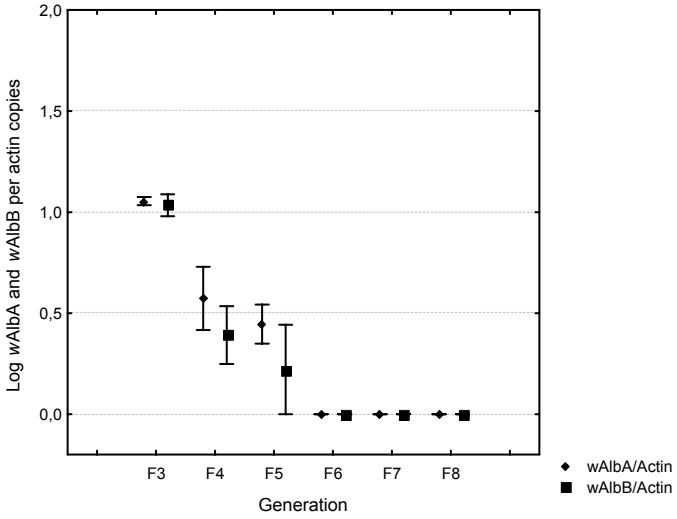
Figure 5. Survival of *Wolbachia*-free *Ae. albopictus* after exposure to an infectious or non-infectious blood-meal with CHIKV at a titer of $10^{7.5}$ PFU/mL. Dead mosquitoes were scored every day to estimate the female life duration following exposure to a blood-meal.

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

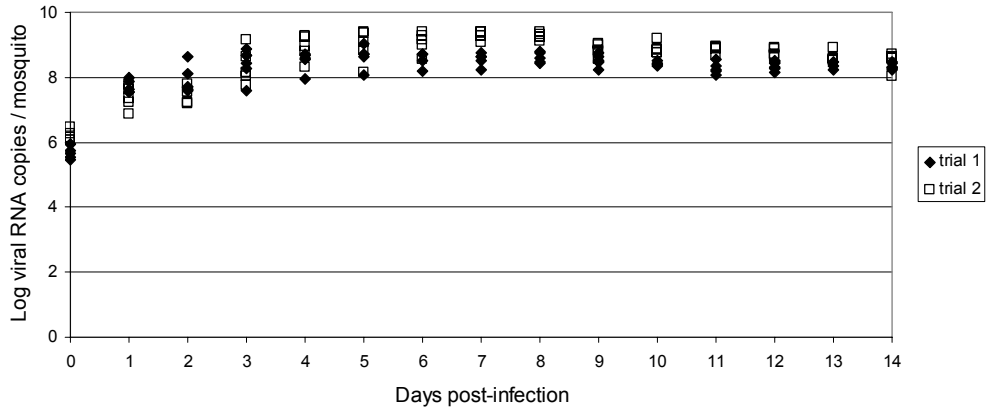
Table. Life duration, oviposition characteristics (delay to laying, number of laid eggs and time between first oviposition and female death) and hatching characteristics (proportion of females with at least one egg hatched and hatching rate per female) of *Wolbachia*-free *Ae. albopictus* from the Reunion 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*	2#	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)

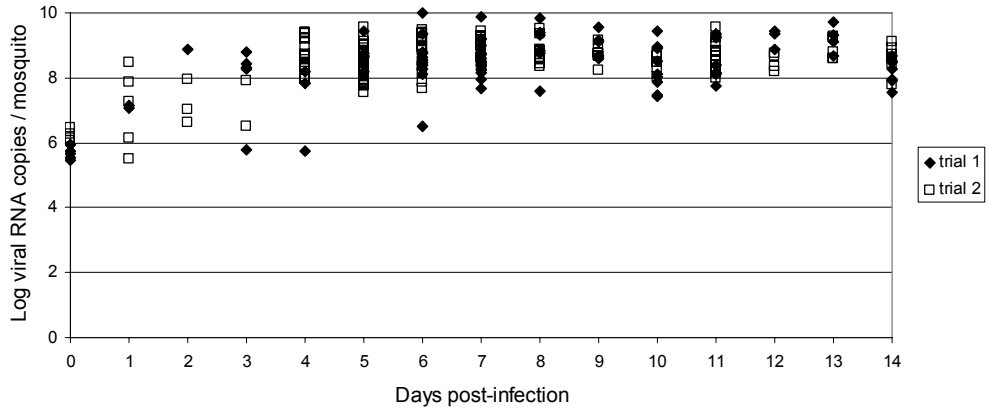
N, number of female analysed; SD, standard deviation; *, trial 1; #, trial 2.


2,0
1,5
1,0
0,5
0,0
Log wAlbA and wAlbB per actin copies
F3
F4
F5
F6
F7
F8
Generation
wAlbA/Actin
wAlbB/Actin

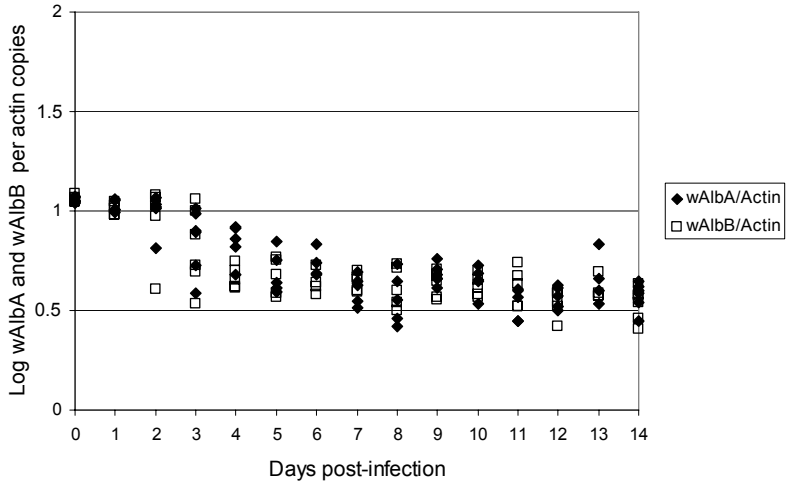
(A)


Log viral RNA copies / mosquito
Days post-infection
trial 1
trial 2

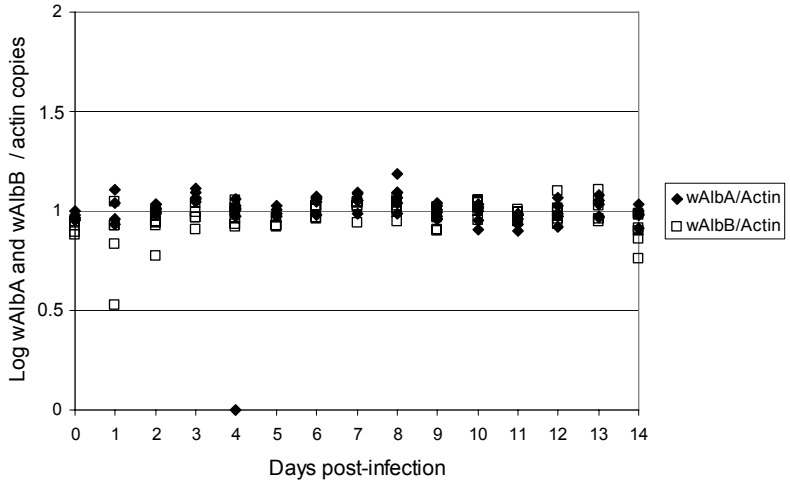
(B)


Log viral RNA copies / mosquito
Days post-infection
trial 1
trial 2

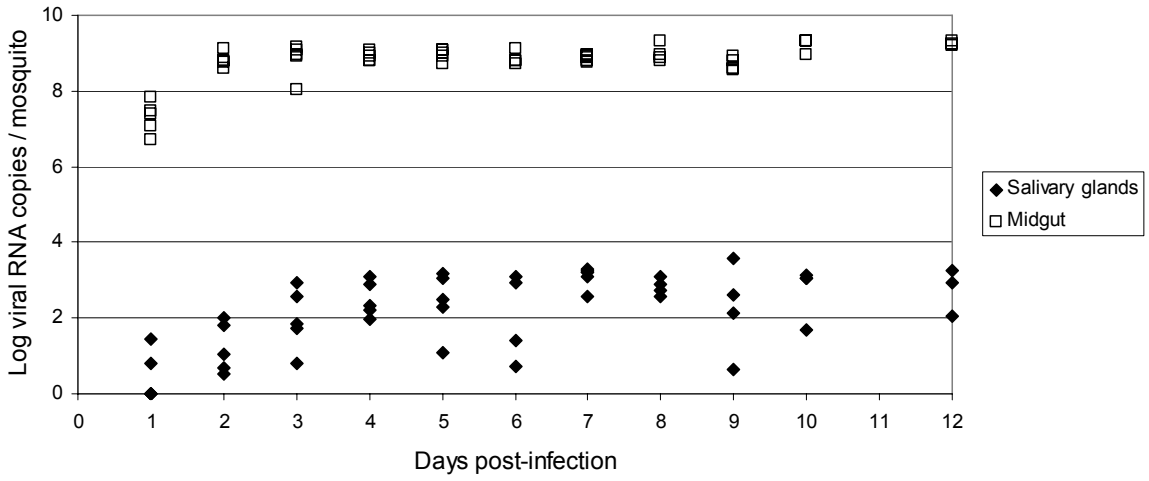
(A)


Log wAlbA and wAlbB per actin copies
2
1.5
1
0.5
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days post-infection
wAlbA/Actin
wAlbB/Actin

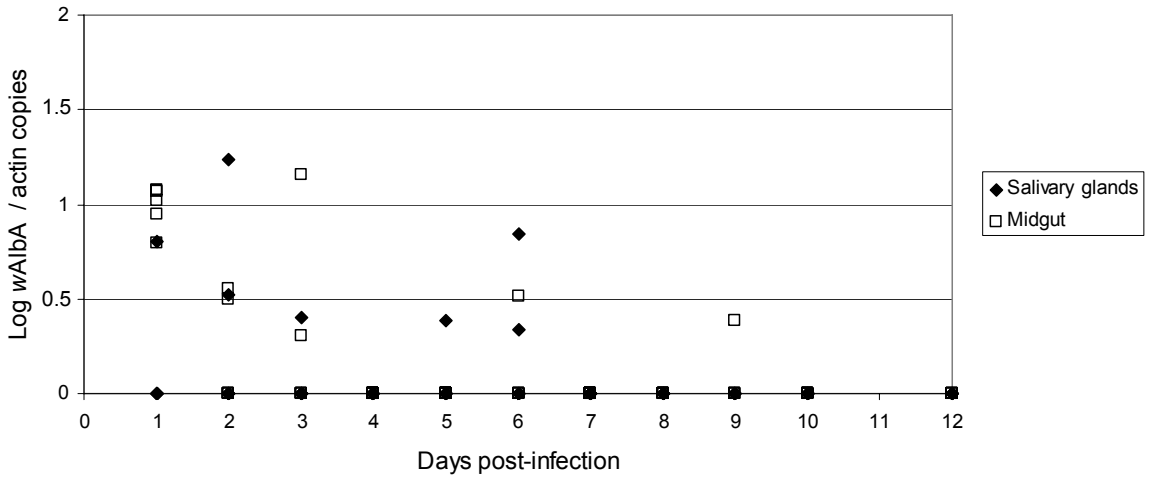
(B)


Log wAlbA and wAlbB / actin copies
2
1.5
1
0.5
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days post-infection
wAlbA/Actin
wAlbB/Actin

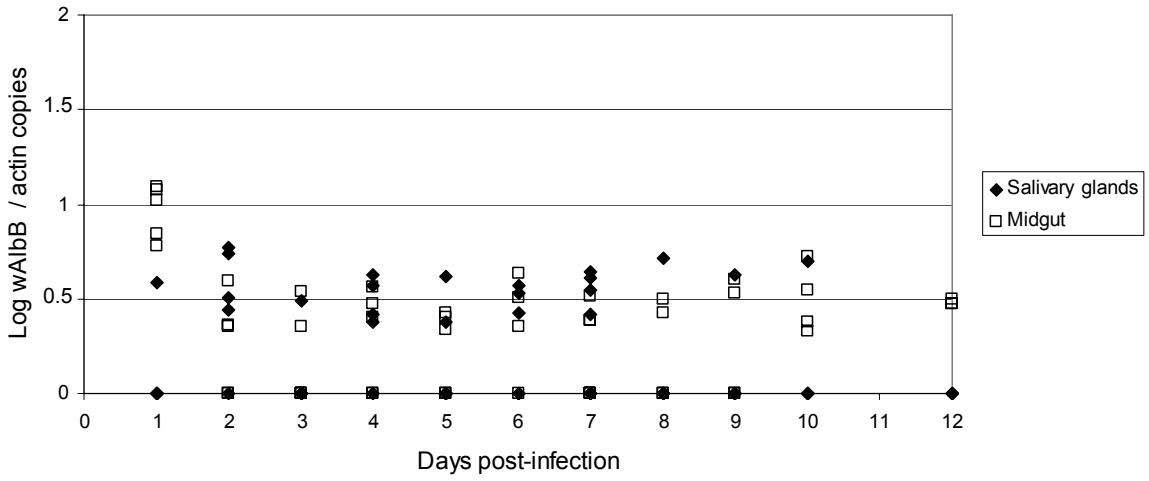
(A)


Log viral RNA copies / mosquito
Days post-infection
Salivary glands
Midgut

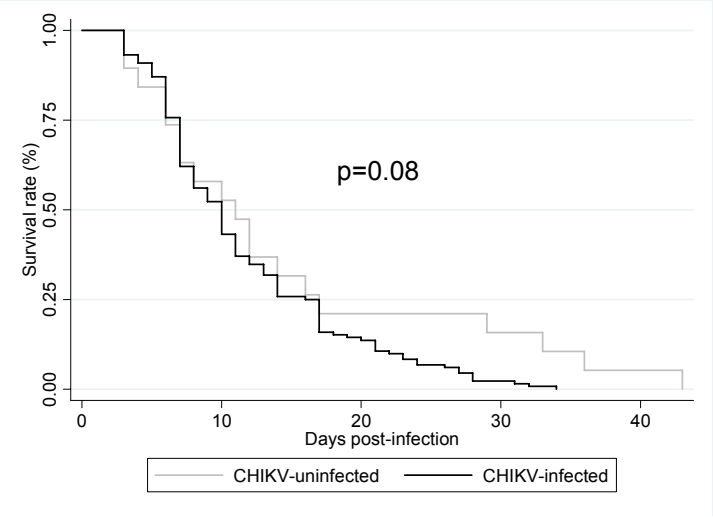
(B)


Log wAlbA / actin copies
Days post-infection
Salivary glands
Midgut

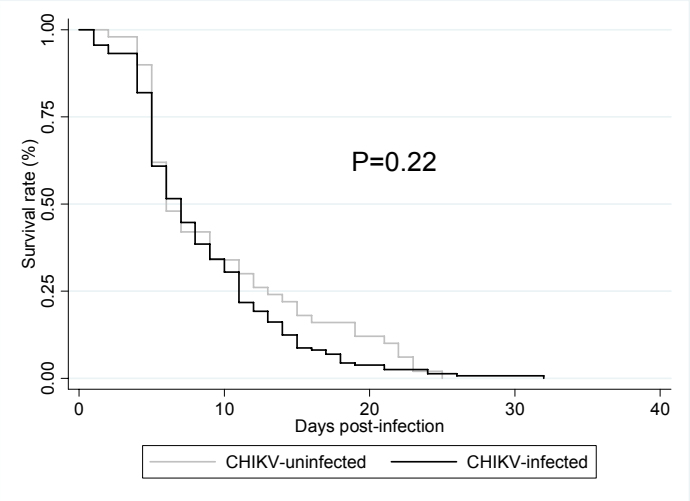
(C)


Log wAlbB / actin copies
Days post-infection
Salivary glands
Midgut

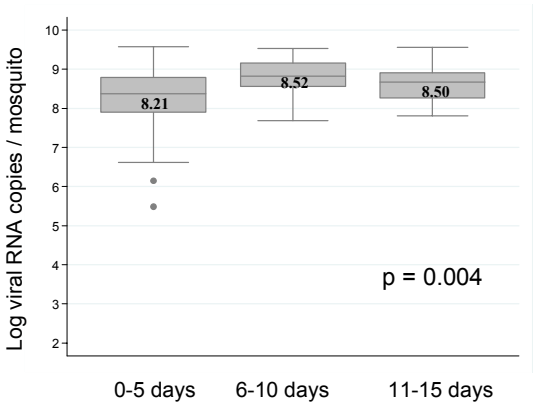
Trial 1



Trial 2



Trial 1



Trial 2