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# A *Wolbachia* Symbiont in *Aedes aegypti* Limits Infection with Dengue, Chikungunya, and *Plasmodium*

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## SUMMARY

*Wolbachia* are maternally inherited intracellular bacterial symbionts that are estimated to infect more than 60% of all insect species. While *Wolbachia* is commonly found in many mosquitoes it is absent from the species that are considered to be of major importance for the transmission of human pathogens. The successful introduction of a life-shortening strain of *Wolbachia* into the dengue vector *Aedes aegypti* that halves adult lifespan has recently been reported. Here we show that this same *Wolbachia* infection also directly inhibits the ability of a range of pathogens to infect this mosquito species. The effect is *Wolbachia* strain specific and relates to *Wolbachia* priming of the mosquito innate immune system and potentially competition for limiting cellular resources required for pathogen replication. We suggest that this *Wolbachia*-mediated pathogen interference may work synergistically with the life-shortening strategy proposed previously to provide a powerful approach for the control of insect transmitted diseases.

## INTRODUCTION

Vector-borne diseases such as malaria, leishmaniasis and dengue, cause a tremendous impact on human mortality and morbidity worldwide. With increasing human movement (Adams and Kapan, 2009) and global warming effects (Barclay, 2008; Pachauri and Reisinger, 2007) re-emerging arboviral pathogens (Gould and Solomon, 2008) such as dengue (DENV) and Chikungunya (CHIKV) viruses are becoming an increasing threat (Ng and Ojcius, 2009; Staples et al., 2009).

Dengue is currently the most significant arboviral disease afflicting tropical and sub-tropical communities worldwide with an estimated global annual incidence of 50 million cases leading to tens of thousands of deaths (WHO, 2009). Between 2004 and 2007, unprecedented outbreaks of CHIKV occurred in islands of the western Indian Ocean, the Indian subcontinent and Europe (Staples et al., 2009). *Aedes aegypti* is considered the primary vector of both DENV and CHIKV. As effective vaccines do not exist for these viruses, prevention of human cases relies almost exclusively on control of the mosquito vectors. Despite some recent successes utilizing copepods as larval biological control agents (Kay and Vu, 2005), most control approaches are proving neither effective nor sustainable, and dengue is increasing in its geographic distribution, frequency of outbreaks and severity of disease (Kyle and Harris, 2008).

A new approach to dengue control has recently been proposed that targets mosquito longevity rather than abundance, through the introduction of a life-shortening strain of the bacterium *Wolbachia pipientis* into *A. aegypti* populations (Brownstein et al., 2003; Cook et al., 2008; Rasgon et al., 2003; Sinkins and O'Neill, 2000). *Wolbachia* is an intracellular inherited bacterium, predicted to naturally infect more than 60% of all insect species worldwide (Hilgenboecker et al., 2008; Jeyaprakash and Hoy, 2000) that is able to invade host populations through either the induction of a number of reproductive parasitism traits (O'Neill et al., 1997) or by positively influencing host fitness (Brownlie et al., 2009; Dobson et al., 2004; Kremer et al., 2009; Weeks et al., 2007).

Since the extrinsic incubation period of viruses and parasites within the mosquito vector is long relative to insect lifespan, *Wolbachia* infections that can invade mosquito populations and reduce adult *A. aegypti* lifespan are predicted to reduce pathogen transmission without eliminating the mosquito population (Brownstein et al., 2003; Rasgon et al., 2003; Sinkins and O'Neill, 2000). The successful introduction of the life-shortening *w*MelPop-CLA strain of *Wolbachia* from *Drosophila melanogaster*

**Table 1. Effect of *Wolbachia* on DENV-2 Infection**

| | | | PGYP1.out | (+ Wolb) | PGYP1.out.tet | (– Wolb) | Cairns3 | (– Wolb) |
|---|---|---|---|---|---|---|---|---|
| Experiment | Log DENV-2 per mL | Days post-infection | % body infection (n) | % disseminated infection (n) | % body infection (n) | % disseminated infection (n) | % body infection (n) | % disseminated infection (n) |
| 1 | 6.3 | 7 | 0 (25) | 0 (25) | NA[a] | NA | 64 (25) | 12 (25) |
| | | 14 | 0 (27) | 0 (27) | NA | NA | 57 (30) | 23 (30) |
| 2 | 6.0 | 7 | 0 (40) | 0 (40) | 100 (30) | 10 (30) | 95 (40) | 5 (40) |
| | | 14 | 0 (40) | 0 (40) | 97 (30) | 37 (30) | 95 (40) | 20 (40) |
| 3 | 5.3 | 7 | 0 (40) | 0 (40) | 30 (40) | 23 (40) | 50 (40) | 13 (40) |
| | | 14 | 0 (40) | 0 (40) | 48 (40) | 43 (40) | 73 (40) | 33 (40) |
| 4 | 7.8 | 7 | 5 (40) | 3 (40) | 78 (40) | 63 (40) | 63 (40) | 45 (40) |
| | | 14 | 8 (40) | 5 (40) | 70 (40) | 65 (40) | 75 (40) | 70 (40) |

*A. aegypti* were orally infected with fresh DENV-2 and viral load determined by cell culture ELISA.
[a] This mosquito line was unavailable for experiment 1

into *A. aegypti* has recently been reported (McMeniman et al., 2009). Mosquitoes carrying this *Wolbachia* strain show around a 50% reduction in adult female lifespan compared to uninfected mosquitoes.

Recent studies in *Drosophila* have shown that *Wolbachia* infections can protect against RNA viruses (Hedges et al., 2008; Teixeira et al., 2008). The *Wolbachia* strains *w*MelCS and *w*MelPop that infect *D. melanogaster* were found to delay mortality in flies when they were infected with a range of pathogenic viruses including *Drosophila* C virus, Flock House virus and Cricket paralysis virus. It is currently unknown how general this anti-viral effect is in terms of both the diversity of *Wolbachia* strains that can confer protection and the range of viruses that are affected by it although recent studies in *Drosophila* indicate that the effect appears limited to certain *Wolbachia* strains (Osborne et al., 2009).

Given that the *w*MelPop strain of *Wolbachia* provides protection against RNA viruses in *Drosophila*, and that the derived *w*MelPop-CLA strain of *Wolbachia* was introduced into the mosquito *A. aegypti* to shorten lifespan, we hypothesized that *w*MelPop-CLA infected mosquitoes may have altered vector competence for arboviruses. To test this hypothesis we exposed *Wolbachia*-infected and -uninfected mosquitoes to two important human pathogens; dengue and Chikungunya viruses. We also tested the same mosquito strains with the avian malaria parasite, *Plasmodium gallinaceum.*

## RESULTS

### *Wolbachia* and Dengue Virus

We tested the effect of *Wolbachia* on vector competence in two mosquito genetic backgrounds: the original inbred PGYP1 line, stably transinfected with *w*MelPop-CLA (McMeniman et al., 2009) and the same strain after five generations of backcrossing to the F1 progeny of wild-caught *A. aegypti*, collected in Cairns, Australia (PGYP1.out). These mosquito strains were compared to tetracycline-treated counterparts that were genetically identical but lacked the *Wolbachia* infection, named PGYP1.tet and PGYP1.out.tet, respectively. In addition, a wild-type strain of *A. aegypti* established from field-collected material in Cairns, Australia (Cairns3) was used as an additional negative control.

Mosquitoes were fed an artificial blood meal spiked with DENV-2 in four independent experiments to examine possible interactions with *Wolbachia*. The presence of DENV-2 in whole mosquito bodies was examined 7 and 14 days post exposure using a cell culture enzyme immunoassay (CCEIA) (Knox et al., 2003). In three separate experiments no *Wolbachia*-infected mosquitoes (PGYP1.out) tested positive for DENV-2, but DENV-2 infection rates in *Wolbachia*-uninfected mosquitoes (PGYP1.out.tet and Cairns3) ranged from 30%–100% (Table 1, Exp 1–3). Body viral infection rates in PGYP1.out.tet mosquitoes ranged from 30%–100% after 7 days and 48%–97% after 14 days, while the body viral infection rates in Cairns3 ranged from 50%–95% after 7 days and 57%–95% after 14 days. The disseminated viral infection rates measured through the presence of virus in mosquito legs in tetracycline-treated *A. aegypti* ranged from 10%–23% and 37%–43% after 7 and 14 days, respectively. Disseminated infections in the *Wolbachia*-free wild-type Cairns3 strain of *A. aegypti* ranged from 5%–13% and 20%–33% after 7 and 14 days, respectively (Table 1) ($p < 0.001$, chi-square). In one experiment (Table 1, Exp 4) when mosquitoes were fed the highest titer ($10^{7.8}$ Logs) of DENV-2 a small number of *Wolbachia*-infected mosquitoes tested positive for DENV-2 at both 7 and14 days post infection (5 and 8%, respectively) but this was significantly fewer than *Wolbachia* uninfected controls (63%–78% and 70%–75%, respectively) ($p < 0.001$, chi-square).

To provide a more conservative test of *Wolbachia*-mediated interference, mosquitoes were intrathoracically injected with DENV-2. These experiments circumvented the midgut barrier to infection (Woodring et al., 1996) and allowed for the delivery of a repeatable inoculating dose (around 2,750 infectious particles/mosquito) of DENV-2 that produced consistent high-titer infections in control mosquitoes. Accumulation of genomic (+RNA) and anti-genomic (-RNA) RNA strands was assessed at 5 and 14 days post injection by quantitative real time PCR using DENV-2 specific primers (Richardson et al., 2006). At both time points, the amount of DENV-2 RNA present was reduced by up to 4 logs in both the PGYP1 and PGYP1.out *Wolbachia*-infected mosquito strains compared to their paired tetracycline treated counterparts (Figure 1, and Table S1 available online). Furthermore, when mosquito saliva collected from mosquitoes

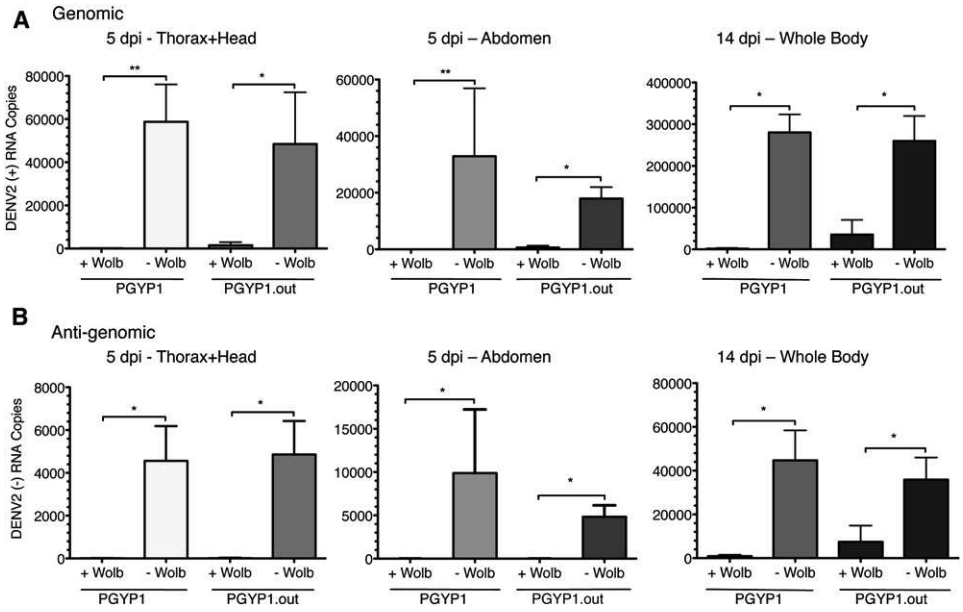


**Figure 1. Quantitative PCR Analysis of Dengue Virus in Mosquitoes**

Two strains of *Wolbachia*-harbouring (+ Wolb) *A. aegypti* mosquitoes (PGYP1 and PGYP1.out) and their tetracycline treated counterparts (- Wolb) (PGYP1.tet and PGYP1.out.tet) were intrathoracically injected with DENV-2 and the virus RNA was estimated by real-time PCR.

(A) Genomic RNA (+RNA) in thorax and head 5 days post-infection (dpi), abdomen 5 dpi and whole mosquito 14 dpi.

(B) Anti-genomic RNA (-RNA) in thorax and head 5 dpi, abdomen 5 dpi and whole mosquito 14 dpi. Bars represent grand means ± SEM calculated across four independent replicate experiments. *$p < 0.05$ by Mann-Whitney U test. See also Table S1.

14 days postinjection was tested for the presence of infectious virus by CCEIA, none of the *Wolbachia*-infected mosquitoes samples tested positive for virus (data not shown). A dramatic reduction in viral protein synthesis was also observed by immunofluorescent microscopy (IFA) (Figures 2A–2F) and western blot analysis (Figure S1).

Double immunofluorescent staining of paraffin sections of *Wolbachia*-uninfected control mosquitoes 14 days post-injection showed DENV-2 infection predominantly in mosquito fat body, ommatidia (Figures 2A–2F) and nervous system. DENV-2 was not detected in any of these tissues in *Wolbachia*-infected mosquitoes (PGYP1 and PGYP1.out) whereas *Wolbachia* was clearly visible in the fat tissue, ommatidia (Figure 2), brain, ovaries, and malpighian tubules. Only in a few rare individuals was DENV-2 detected in patches of fat tissue in PGYP1.out mosquitoes. However in these cases *Wolbachia* and DENV-2 were not co-localized in the same cells and DENV-2 was only seen in occasional patches of cells that were not infected with *Wolbachia* (Figure 2G). The presence of DENV-2 in some injected PGYP1.out mosquitoes was also confirmed by western blot (Figure S1A).

### *Wolbachia* and Chikungunya Virus

We then went on to determine if the virus interference phenotype would extend to the alphavirus CHIKV. The virus strain used in the experiments contained the Ala to Val mutation in the membrane fusion glycoprotein E1 gene (E1-A226V), which has been linked to increased infectivity in *A. albopictus* (Tsetsarkin et al., 2007). An Australian population of *A. aegypti* was recently shown to also be a highly efficient laboratory vector of this virus strain (van den Hurk et al., 2009).

Mosquitoes were exposed to a blood/virus mixture containing $10^{6.4}$ $CCID_{50}$/ml of CHIKV, and at various time points postexposure, mosquitoes were processed to quantify the number of viral RNA copies using qPCR and CHIKV-specific primers and probes (van den Hurk et al., 2009). Immediately after feeding, the number of CHIKV genomic (+ RNA) RNA copies in the body and head were comparable for all three lines, suggesting that they imbibed similar amounts of virus (Table 2). The median number of copies decreased in all three lines on day 2, prior to it increasing in the PGYP1.out.tet and Cairns3 mosquitoes to its highest level at day 14-postexposure. Day 14 infection rates were 87% and 79% for the PGYP1.out.tet and Cairns3 controls and 17% for the *Wolbachia* infected PGYP1.out line ($p < 0.001$, chi-square).

In all three mosquito groups, CHIKV RNA was detected in the legs and wings immediately after feeding (Table 2). This may represent either direct contact between the legs and/or wings and the blood/virus mixture or a rupture of the mesenteron, which released virus directly in the hemolymph (Turell, 1988). After day 0, CHIKV was not detected in the legs and wings of any PGYP1.out (+ Wolb) mosquitoes. In contrast, on all days postexposure, virus was detected in the legs and wings of PGYP.out.tet and Cairns3 control mosquitoes (- Wolb) and by day 14, the virus was detected in the legs and wings of 100% and 90%, respectively, of mosquitoes that had positive bodies and heads ($p = 0.125$, chi-square).

### *Wolbachia* and *Plasmodium*

Considering that the viral interference effect appeared robust for two unrelated arboviruses we then tested the effect on the protozoan parasite *P. gallinaceum*. While not a human pathogen,

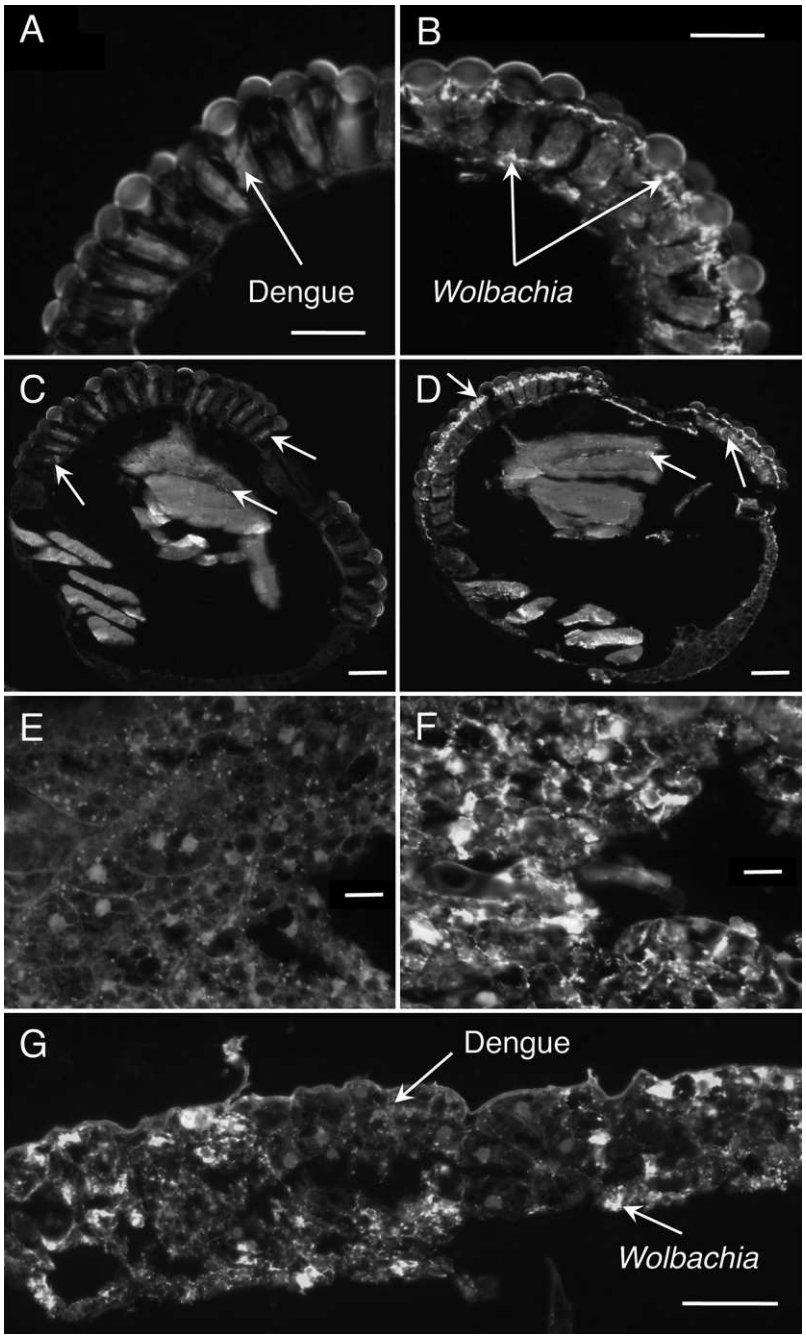


**Figure 2. Localization of *Wolbachia* and Dengue Virus in *A. aegypti* Mosquitoes**

Double immunofluorescence staining of mosquito sections showing the localization of dengue virus (red) and *Wolbachia* (green). Sections were probed simultaneously with polyclonal anti-wsp antibody (*Wolbachia*) and monoclonal anti-DENV antibody 4G4, followed by Alexa 488 (green) and Alexa 594 (red) conjugated antibodies, respectively. DNA (blue) is stained with DAPI. In panels (A, B, E, F, and G), the red, green and blue channels are merged. C and D show only red and green channels merged. (A, C, E) PGYP1.tet (- Wolb) mosquitoes, 14 days post-DENV-2 thoracic injection. Dengue virus is visible in ommatidia cells (A and C) and fat tissue (E). (B, D, F) PGYP1 mosquitoes (+ Wolb), 14 days post-DENV-2 thoracic injection. *Wolbachia* can be seen in ommatidia cells and brain (B and D) and fat tissue (F). In contrast no dengue virus was detected. (G) Cellular exclusion of DENV-2 by *Wolbachia.* The presence of both *Wolbachia* and DENV-2 was observed at very low frequency in a small number of *Wolbachia*-infected outcrossed mosquitoes, 14 days post-DENV-2 injection. Dengue is only apparent in cells lacking *Wolbachia* however. Scale bars: A-D, G: 50 μm; E, F: 20 μm. See also Figure S1.

this species of malaria parasite is known to be able to infect *A. aegypti* mosquitoes in the laboratory. Since no species of anopheline mosquitoes are known to carry natural *Wolbachia* infections and stable *Wolbachia* transinfected lines have yet to be generated, we considered the *A. aegypti – P. gallinaceum* model system most appropriate for testing effects on malaria parasites. *Wolbachia*-infected and uninfected *A. aegypti* mosquitoes (PGYP1.out and PGYP1.out.tet strains) as well as a susceptible strain of *Aedes fluviatilis* were fed in parallel on *P. gallinaceum* infected chickens. *A. fluviatilis* has a broad geographical distribution in Latin America and has been used in the laboratory as a safe avian malaria (*P. gallinaceum*) model vector, as it does not naturally transmit DENV or yellow fever virus (Tason de Camargo and Krettli, 1981).

Seven days post-feeding on infected chickens, mosquito midguts were dissected and presence and number of *Plasmodium* oocysts assessed. The *w*MelPop-CLA infection significantly reduced ($p < 0.0001$, chi-square) the frequency of mosquitoes in the population infected with at least a single oocyst and so potentially able to transmit *Plasmodium* (Figure 3A) from 74% (tet controls) down to 42%. The presence of *Wolbachia* also reduced the number of oocysts present in mosquitoes that were infected with the parasite (Figures 3A and 3B). To quantify the difference in parasite loads, 15 days after infection mosquitoes were collected and the DNA was extracted. The relative abundance of *Plasmodium* genomic DNA was measured by qPCR of the 18S ssu rRNA gene (Schneider and Shahabuddin, 2000) and normalized to the mosquito Actin gene. The results showed the same pattern of interference as observed from oocyst count data. In PGYP1.out mosquitoes *Plasmodium* genomic DNA was 26-fold less abundant than in PGYP1.out.tet lines (Figure 3C). Immunofluorescence analysis using an anti-CSP (*Plasmodium* circumsporozoite protein) monoclonal antibody showed the presence of mature oocysts in both mosquito species (Figure S2B), but very rarely in *Wolbachia*-infected mosquitoes.

When we incubated the mosquito sections with an anti-*Wolbachia* (wsp) antibody we serendipitously discovered

**Table 2. Effect of *Wolbachia* on CHIKV infection**

| | PGYP1.out | | | | PGYP1.out.tet | | | | Cairns3 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Days post-infection | Infected (%) | Disseminated (%) | Median copies in Bodies/ Heads (N) | Percentiles (25 - 75%) | Infected (%) | Disseminated (%) | Median copies in Bodies/ Heads (N) | Percentiles (25 and 75%) | Infected (%) | Disseminated (%) | Median copies in Bodies/ Heads (N) | Percentiles (25 and 75%) |
| 0 | 100 | 20 | 10.2 (10) n.s. | 10.0-10.4 | 100 | 20 | 10.2 (10) | 9.8-10.6 | 100 | 10 | 10.0 (10)n.s. | 9.8-10.5 |
| 2 | 80 | 0 | 9.1 (8)n.s. | 8.5-9.4 | 50 | 30 | 9.6 (5) | 9.3-10.2 | 70 | 40 | 9.5 (7)n.s. | 9.2-9.8 |
| 4 | 20 | 0 | 7.8 (2)* | 7.3-8.2 | 60 | 60 | 10.4 (6) | 9.7-10.8 | 50 | 30 | 10.0 (5)n.s. | 9.6-11.7 |
| 7 | 10 | 0 | 7.3 (1)n.a. | n.a. | 100 | 100 | 11.1 (10) | 10.8-11.3 | 100 | 90 | 10.39 (10)* | 8.4-10.8 |
| 10 | 0 | 0 | (0)n.a. | n.a. | 60 | 60 | 10.8 (6) | 10.6-10.9 | 90 | 90 | 10.6 (10) n.s. | 10.4-11.3 |
| 14 | 17 | 0 | 7.7 (3)** | 6.7-8.0 | 85 | 100 | 11.8 (26) | 10.9-11.9 | 80 | 90 | 11.3 (23)* | 10.3-11.6 |

*A. aegypti* were orally infected with fresh CHIKV and viral load ($Log_{10}$) determined by quantitative RT-PCR in bodies and heads or wings/ legs (for viral dissemination). Median copy number is based only on mosquitoes that were positive for virus. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by Mann Whitney-U tests for the comparisons of PGYP1.out and Cairns3 each against PGYP1.out.tet. n.s., nonsignificant; n.a., not applicable.

a *Wolbachia* infection in *A. fluviatilis* mosquitoes indicating that this species of mosquito was naturally infected with *Wolbachia*. PCR using *Wolbachia* general *wsp* primers (Braig et al., 1998; Zhou et al., 1998) amplified a fragment from all *A. fluviatilis* tested. Sequence of the amplified DNA indicated that this *Wolbachia* strain (named *w*Flu) belongs to the *Wolbachia* B supergroup and is distantly related to *w*MelPop-CLA. qPCR analysis revealed that the density of *w*Flu in *A. fluviatilis* is about 20-fold lower than the density of *w*MelPop-CLA in *A. aegypti* (Figure S3). We then examined the tissue localization of *Wolbachia* in both mosquito species and whereas *w*MelPop-CLA is distributed throughout most tissues of the mosquito including the fat body, anterior midgut, muscle, nervous tissue, malpighian tubules and ovaries, *w*Flu is present only in ovaries, malpighian tubules and less frequently in the head, but absent from ommatidia (Figures 4 and S3).

### Immunity Genes

To examine whether resistance of *Wolbachia* infected mosquitoes to pathogen infection may be related to stimulation or priming of the mosquito innate immune system, we quantified the expression of a sample of immune genes. It was recently demonstrated that some immune genes are differentially regulated in *A. aegypti* mosquitoes infected with dengue virus (Xi et al., 2008). Interestingly, regulation of the immune pathway genes in these mosquitoes was also stimulated by their natural gut microbiota and rearing mosquitoes aseptically, and so depleting their bacterial flora, resulted in a 2-fold increase of dengue virus in the midgut (Xi et al., 2008). We chose a subset of the genes that were shown to be upregulated upon dengue virus infection to assess the effect of *Wolbachia* infection on the mosquito immune system.

The expression levels of eleven immune pathway genes in the *w*MelPop-CLA infected PGYP1.out and its uninfected control line were compared for two independently reared cohorts of mosquitoes (Figure 5). In each of the experiments four genes encoding representatives of the immune effector molecules cecropin, defensin, thio-ester containing proteins (TEP) and C-type lectins were significantly upregulated in the presence of *w*MelPop-CLA, whereas FREP18 (fibrinogen-related protein 18) levels remained unchanged (Figures 5A and 5B). In contrast, while a statistically significant ($p < 0.05$) differential mRNA expression between mosquitoes with and without *Wolbachia* was observed for a subset of the genes from the Toll, IMD and Jak/STAT signaling pathways (Figure 5C Experiment 1- Rel 1A and SOCS36E; Figure 5D Experiment 2 – IMD and Rel 2) these differences were inconsistent across the two experiments, suggesting that the variation between cohorts was greater than any differences induced by *Wolbachia*. In addition, in these cases the fold-change of mRNA expression was low (below 2-fold), whereas the effector genes were induced as much as 100-fold by the presence of *Wolbachia* (Figure 5A and 5B). These results indicate that the presence of *w*MelPop-CLA in mosquitoes stimulates expression of at least some immune effector genes, although a clear stimulation of the classical innate immune signaling pathways was not repeatably identified.

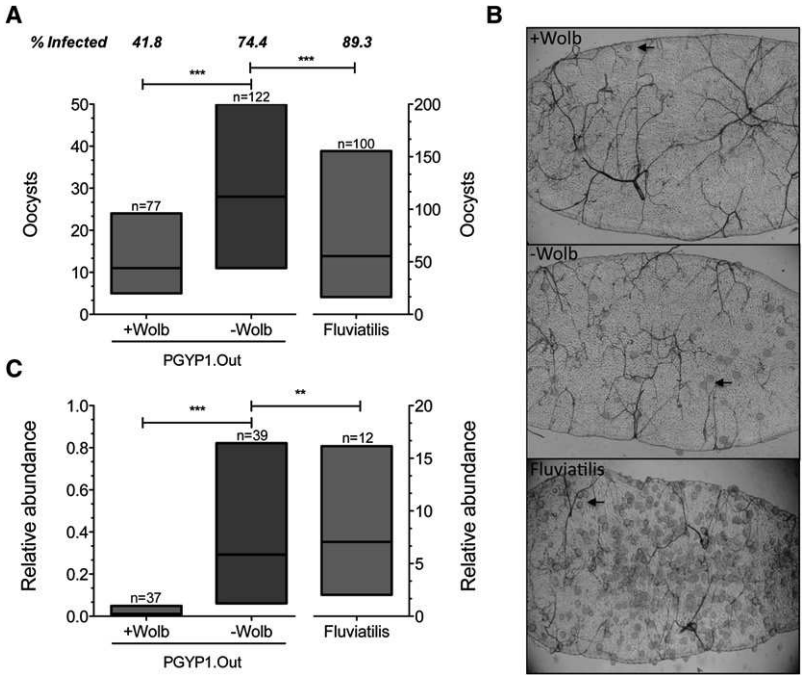


**Figure 3. *Plasmodium gallinaceum* Detection in *Aedes* spp. Mosquitoes**

*A. aegypti* and *A. fluviatilis* mosquitoes were fed on *P. gallinaceum* infected chickens and parasite was detected.

(A) Box plots of median numbers and 25 (bar below median) and 75 (above median) percentiles of oocyst intensities, 7 days postinfection in *w*MelPop-CLA infected (PGYP1.out, +Wolb) or uninfected (PGYP1.out.tet, -Wolb) *A. aegypti* and in *A. fluviatilis* mosquitoes (***$p < 0.0001$ by Mann-Whitney U test). Percentage of mosquitoes containing at least one oocyst is shown above the graph.

(B) Mercurochrome staining of mosquito midguts showing *P. gallinaceum* oocysts (arrows) in *w*MelPop (+Wolb) infected and uninfected (-Wolb) and in *A. fluviatilis* mosquitoes, seven days post-infection (100× magnification).

(C) Quantitative PCR analysis 15 days after infection showing the relative abundance of *Plasmodium* 18S ssu rRNA sequences in comparison to Actin gene (**$p < 0.005$, ***$p < 0.0001$ by Mann-Whitney U test). See also Figure S2.

## DISCUSSION

The finding that the *w*MelPop-CLA *Wolbachia* infection reduces the ability of two distantly related arboviruses and a malaria parasite from establishing productive infections in the mosquito shows that the pathogen interference effect of the *w*MelPop-CLA strain is general and fundamental. The mechanism is currently unclear although our data suggest that immune effector genes are upregulated in the mosquito in the presence of the *w*MelPop-CLA strain and are likely to play a role, even though key components of the currently accepted signaling pathways for these effectors do not seem to be transcriptionally modulated by *Wolbachia*.

It is also possible that the observed interference effect might relate to competition for key host cell components. This is supported by the observation that DENV-2 infection was only observed in *Wolbachia* infected mosquitoes in cells that lacked the *Wolbachia* infection (Figure 2G). It is known, for example, that insects need to obtain cholesterol and other fatty acids from their diet (Blitzer et al., 2005) and that *Wolbachia* and related bacteria also lack the biosynthetic capability to synthesize cholesterol and need to obtain it from the host insect (Lin and Rikihisa, 2003; Wu et al., 2004). In addition cholesterol is known to be a key fatty acid required for successful Flavivirus and Alphavirus replication that must be obtained from the host cell (Lu et al., 1999; Mackenzie et al., 2007). Similarly *Plasmodium* is also known to depend on host lipids in the mosquito stage (Atella et al., 2009). The relative importance of innate immune effectors and alternative mechanisms such as competition for critical host cell components such as cholesterol remains to be determined.

One critical paradox relating to *Wolbachia* infection and reductions in mosquito vector competence is the observation that some mosquito species that naturally carry *Wolbachia* infections such as *A. albopictus* are known to be competent vectors for a range of pathogens including DENV and CHIKV. Our fortuitous discovery that our control mosquito species *A. fluviatillis* supports very high oocyst loads, despite the presence of a naturally occurring *Wolbachia* strain, allowed us to provide an explanation for this observation. It is known that different *Wolbachia* strains commonly display quite variable somatic tissue distribution and densities (Dobson et al., 1999; Dutton and Sinkins, 2004; Miller and Riegler, 2006). Our analysis of *A. fluviatilis* shows that the *w*Flu *Wolbachia* strain has a very restricted tissue tropism in its host mosquito (Figures 2, 5, and S3) and overall *Wolbachia* densities are much lower (20-fold) than the *w*MelPop-CLA infection in *A. aegypti* (Figure S3A). This likely explains why no *Plasmodium* interference effect is observed in this species and may explain why *A. albopictus* is known to be a competent vector for arboviruses while being naturally infected with *Wolbachia*. The *w*MelPop *Wolbachia* strain in *Drosophila* is known to be unusual in that it grows to high densities (Min and Benzer, 1997) and has broad tissue tropism in *A. aegypti* (Figures 2 and 5), which may underpin its ability to so effectively interfere with both virus replication and parasite development.

Our results have significant implications for any future control measure based on the use of life-shortening *Wolbachia*. Currently the *w*MelPop-CLA infection has been demonstrated to reduce average mosquito lifespan by approximately 50% in the laboratory (McMeniman et al., 2009) that while sufficient to greatly reduce transmission could still allow some mosquitoes to live longer than the extrinsic incubation period for most human pathogens. The observed interference effect reported here appears sufficiently strong that it would act in conjunction with the life-shortening effect to control arbovirus transmission. Life-shortening effects on mosquitoes would become secondary and only act on individuals that might escape the direct viral

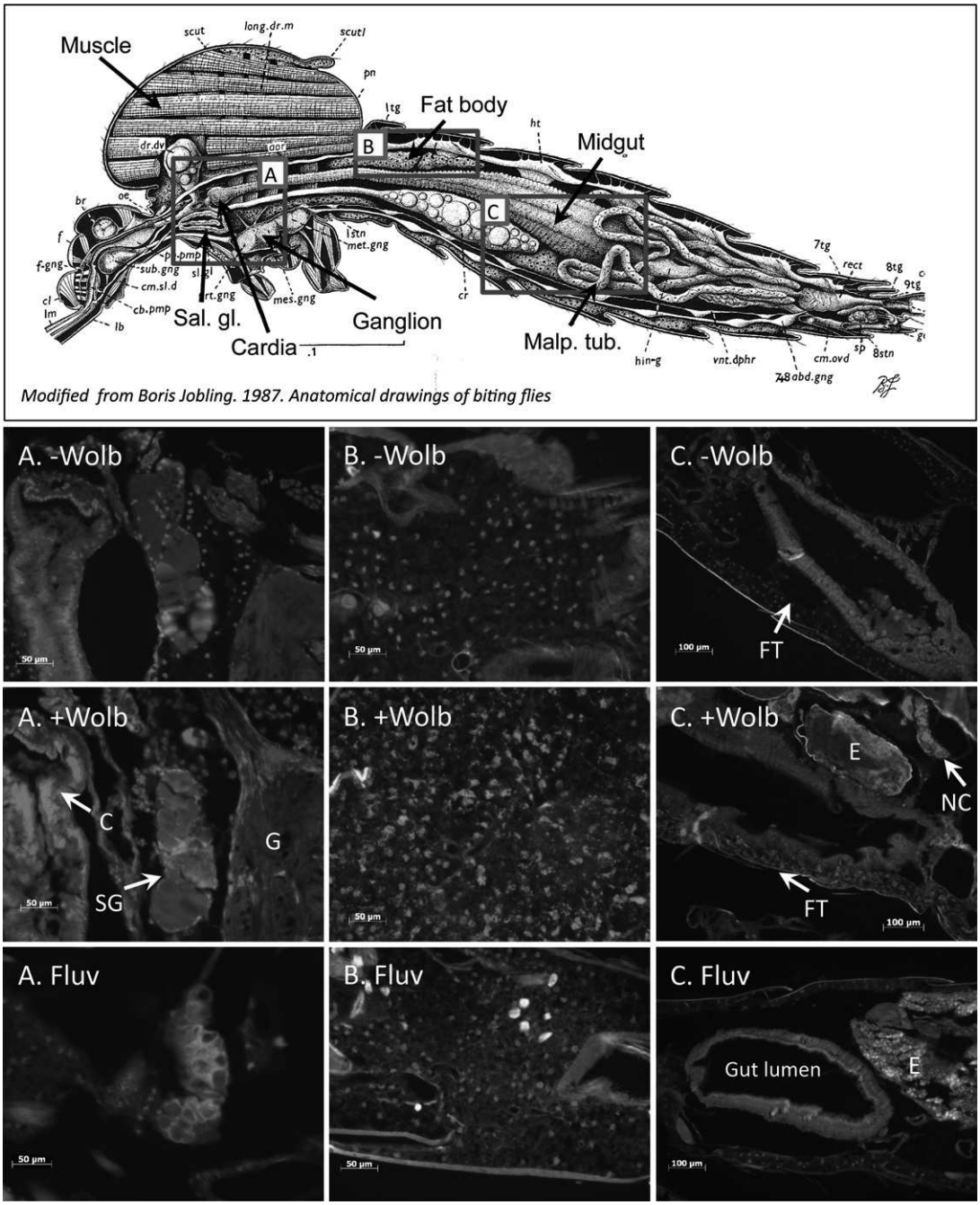


**Figure 4. *Wolbachia* Distribution in *Aedes* spp. Mosquitoes**

Fluorescence in situ hybridization showing the localization of *Wolbachia* (in red) in different tissues of *A. aegypti* and in *A. fluviatilis* mosquitoes. Sections were hybridized with two *Wolbachia* specific 16S rRNA probes labeled with rhodamine. DNA is stained with DAPI (blue). A green filter is used to provide contrast. The top diagram has been adapted from (Jobling, 1987).

(A) Anterior part of the digestive system, showing the salivary glands (SG) and the cardia (C), together with the thoracic ganglion (G) of uninfected *A. aegypti* (-Wolb), PGYP1.out (+Wolb) and *A. fluviatilis* mosquitoes. Panels B). Fat tissue showing the presence of *w*MelPop-CLA in PGYP1.out (+ Wolb) mosquitoes but absence of the bacteria in PGYP1.out.tet (-Wolb) and in *A. fluviatilis*. C) *w*MelPop-CLA is present in the fat tissue (FT) surrounding the gut in PGYP1.out

interference effect. The combined action of two distinct *Wolbachia* effects, lifespan reduction and virus interference, should also reduce the risk of resistance formation to the strategy.

The inhibitory effect of *w*MelPop-CLA *Wolbachia* against *P. gallinaceum* is a promising step toward the possible application of this strategy to control human malaria. *Anopheles*

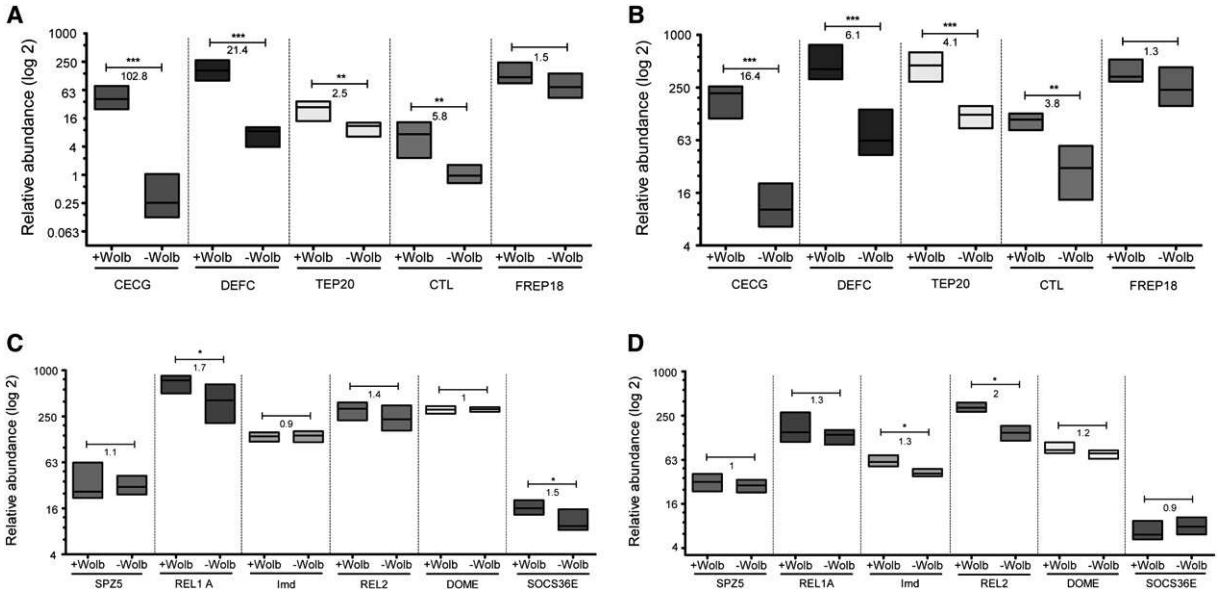


**Figure 5. Immune Gene Regulation in Response to *Wolbachia* Infection**

RT-qPCR analysis of mRNA expression from selected immune genes of PGYP1.out and PGYP1.out.tet mosquitoes. Graphs show the target gene to housekeeping gene ratio for the genes indicated from the immune pathways. Box plots of median numbers and 25 (bar below median) and 75 (above median) percentiles of 10 individual mosquitoes from a single cohort. Results from two independently reared cohorts are shown (cohort 1 [A and C]; cohort 2 [B and D]). Statistically significant medians by Mann Whitney-U test (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$) are indicated. Fold-change for the gene is shown above the box plots.

mosquitoes, as *A. aegypti*, are not naturally infected with *Wolbachia*, therefore transinfection experiments into these vector species are crucial to determine whether *Wolbachia* can also block human malaria in anopheline vectors. As the bacterium is able to establish somatic infection in the human malaria vector *Anopheles gambiae* (Jin et al., 2009), it is likely that the same interference effect may also be present when a stable infection of *Wolbachia* becomes available.

## EXPERIMENTAL PROCEDURES

### Mosquitoes

Five different *A. aegypti* lines were used including the original inbred *w*Mel-Pop-CLA infected line (PGYP1) and its tetracycline-cured counterpart PGYP1.tet (McMeniman et al., 2009). A genetically diverse line derived from PGYP1, named PGYP1.out was generated by backcrossing PGYP1 for three generations to F1 males of 52 independent field-collected isofemale lines from Cairns, Australia. Two further generations of backcrossing were conducted with F2 field-collected material (wild-type from Cairns, Australia). The backcrossing scheme used here is expected to replace 96.9% of the original inbred genotype. A tetracycline-cured counterpart (PGYP1.out.tet, -Wolb) was generated by antibiotic treatment of backcrossed adults, followed by two generations of recovery and re-colonization with gut bacteria as previously described (McMeniman et al., 2009). A genetically diverse wild-type line was also generated at the same time from field-collected material sourced from 245 ovitraps across seven suburbs of Cairns, Australia in late 2008 and named Cairns3. For the malaria experiments, a susceptible *A. fluviatilis* strain (Rodrigues et al., 2008) was used in parallel with PGYP1.out (+ Wolb) and PGYP1.out.tet (-Wolb) *A. aegypti* mosquitoes.

Insects were kept in a controlled environment insectary at 25°C, ~80% RH, 12 hr light regime. Larvae were maintained with fish food pellets (Tetramin, Tetra) and adults were offered 10% sucrose solution, *ad libitum*. Adult females were bloodfed on human volunteers (UQ human ethics approval 2007001379; QIMR approval P361) for egg production. Three- to five-day-old female mosquitoes were used for the DENV and malaria infection experiments. Seven day old females were used for the CHIKV experiments.

### Viruses

#### *Dengue virus*

Dengue virus serotype 2 (DENV-2) (92T) was isolated from human serum collected from a patient from Townsville, Australia, in 1992. Virus stocks were passaged five times in *Aedes albopictus* cell line (C6/36) grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), penicillin (100 μg/ml), streptomycin (100 μg/ml), and 1× glutamax (Invitrogen), and maintained at 28°C.

Supernatants from infected cell lines (C6/36) were collected 5 days after infection, separated into 0.5 ml aliquots, and frozen at −80°C. Virus used in microinjection experiments was obtained from thawed stocks of above and had a titer of $10^{7.6}$ $CCID_{50}$ per ml. To prepare the DENV-2 for oral feeding, the frozen virus stock was passaged once more through C6/36 cells and the supernatant was harvested at 5 d and then mixed with blood to formulate a bloodmeal for feeding. Virus solution with higher titer ($10^{8.85}$ $CCID_{50}$/ml) was obtained by harvesting the viral supernatant and the intracellular virus from cell lysates.

#### *Chikungunya Virus*

CHIKV strain 06113879, isolated from a viremic traveler returning from Mauritius to Victoria, Australia in 2006 was provided by the Victorian Infectious Diseases Research Laboratory, Melbourne, Australia. Cultures were grown at 37°C in Vero (African green monkey kidney) cells for 4 days before the supernatant was harvested and frozen at −80°C. This CHIKV stock was passaged once more in Vero cells and the virus was concentrated from 1.8 L of infected culture supernatant via ultracentrifugation at 10 000 *g* for 17 hr at 4°C. Pelleted virus was resuspended in 20 ml of Opti-MEM® reduced serum medium (GIBCO BRL®, Invitrogen, California) supplemented with 10% FCS before aliquots of the prepared virus were frozen at −80°C. The stock concentration had a final viral titer of $10^{8.0}$ $CCID_{50}$/ml.

### Exposure of Mosquitoes to Viruses

#### *Intrathoracic Injection with DENV-2*

Female mosquitoes were briefly anesthetized with $CO_2$ and placed on a glass plate over ice. Insects were handled with forceps under a dissecting scope and injected into their thorax (pleural membrane) with a pulled glass capillary and a handheld microinjector (Nanoject II, Drummond Sci.). 69 *n*l of DENV-2 stock was injected into each mosquito, which corresponds to approx. 2,750 virus particles/ mosquito. (See Supplemental Experimental Procedures for details).

#### *Oral Feeding with DENV-2 and CHIKV*

Mosquitoes were starved for 24 hr and then transferred to 1L or 2.5L plastic feeding containers. Prior to feeding, DENV-2 was harvested from C6/36 cell culture supernatant and diluted 1:5 in defibrinated sheep's blood. For the CHIKV experiments, frozen aliquots of stock virus were rapidly thawed, and diluted in washed defibrinated sheep blood and 1% sucrose. Blood-virus mixtures were maintained at 37°C for 1 hr and 4 hr for DENV-2 and CHIKV, respectively, using membrane feeders (Rutledge et al., 1964) and covered with a porcine intestine as the membrane. After feeding, mosquitoes were anesthetized using $CO_2$ and partially or non-engorged mosquitoes were discarded (see Supplemental Experimental Procedures for details).

### Cell Culture Enzyme Immunoassays

Titration of DENV-2 and CHIKV stocks and blood/virus mixtures was performed using a CCEIA method (Broom et al., 1998). For DENV-2, C6/36 cell monolayers (60%–90% confluent) in 96-well plates were inoculated with 50 μl/well of virus dilutions and plates were incubated at 28°C with 5% $CO_2$ for 5 d. Cell monolayers were then fixed and examined for DENV-2 antigens using a cocktail of flavivirus cross-reactive monoclonal antibodies (4G4 and 4G2) (Clark et al., 2007; Gentry et al., 1982). For CHIKV, all titrations were performed in Vero cells, which were incubated at 37°C with 5% $CO_2$. After 7 days, plates were examined for cytopathic effect (CPE), which was confirmed using the CCEIA and the broadly reactive alphavirus monoclonal antibody, B10 (Broom et al., 1998).

#### *Plasmodium gallinaceum*

Two to three day-old White Leghorn chickens were infected through intra-peritoneal or intradermal injection of *Plasmodium gallinaceum* 8A strain parasitized blood (Rodrigues et al., 2008). Parasitemia was determined every other day through Giemsa-stained blood smears. Ten microscopic fields were examined under immersion oil to count one hundred red blood cells and determine the ratio of infected cells. Presence of gametocytes and rising parasitemia was ensured in order to enhance the chance of mosquito infection. Before infection mosquitoes were deprived of sugar solution overnight and on the next morning chickens were placed on top of the cages and mosquitoes were fed for about 45 min. Only bloodfed female mosquitoes were kept for further observations. Four independent experiments were performed with independent cohorts. Seven days after bloodfeeding mosquitoes had their midguts dissected in 1 PBS and after staining the midguts with 0.2% Mercurochrome solution oocysts were counted under a microscope (DIC, 100X). Fifteen days after infection mosquitoes were collected and DNA was extracted (QIAGEN Blood & Tissue kit) for *Plasmodium* detection, using around 1 ng of genomic DNA in quantitative PCR reactions. Primers for the *Plasmodium spp.* 18S ssu rRNA gene (Schneider and Shahabuddin, 2000) were used for parasite amplification and *A. aegypti* Actin was used as a host control gene (primer sequences in Supplemental data). Analyses were performed with qGENE (Joehanes and Nelson, 2008) and Mann Whitney-U tests (STATISTICA V8, StatSoft, Inc.) to compare relative abundance between lines.

### Quantitative DENV PCR Analysis

Individual frozen mosquito bodies or body parts were placed into 2 ml screw cap vials with a glass bead (2 mm diameter, Sigma-Aldrich). 200 μl of Trizol (Invitrogen) was added and the sample homogenized for 150 s using a Mini BeadBeater (Biospec Products). 40 μl of chloroform was added to each tube and samples were thoroughly vortexed for 10 s. Tubes were centrifuged for 15 min at 14,000 *g* and 4°C and the supernatant containing the RNA was transferred to new tubes. RNA was precipitated with 40 μl of isopropanol, washed with 200 μl of 70% ethanol and resuspended in 25 μl of RNase-free milli-Q water. Samples were maintained at −80°C until further analysis (See Supplemental Experimental Procedures for details).

### CHIKV RT-qPCR Analysis

Individual frozen mosquito bodies and heads or legs and wings were homogenized for 3 min in 1 ml of Opti-MEM® reduced serum medium using glass beads and a mechanical homogenizer (Spex Industries, Edison, NJ). The supernatant from each sample was removed for potential virus isolation and stored at −80°C. The remaining mosquito pellet from each sample was resuspended in 200 μl of Opti-MEM® reduced serum medium and TRIzol® LS reagent (Invitrogen) and homogenized again as described above. After incubation at room temperature for 5 min and addition of 40 μl of chloroform, the entire homogenate for each sample was then vortexed for 15 s and transferred to a pre-spun Phase Lock Gel Heavy tube (5 Prime, GmbH, Germany). The lysed contents of each tube were allowed to settle for 5 min and organic and aqueous phases were separated by centrifugation at 16,000 *g* for 10 min. Aqueous phases were recovered from each tube before total RNA was extracted using a modification of the RNeasy Mini Kit protocol (QIAGEN, Australia) and on column-DNase treatment. RNA was eluted with 30 μl of RNase-free $H_2O$ and a final centrifugation step for 1 min. RNA samples were stored at −80°C prior to analysis by RT-qPCR. RNA standards were produced for the relative quantification of CHIKV RNA copy numbers normalized to RNA levels of the *A. aegypti* house-keeping gene RpS17 (See Supplemental Experimental Procedures for details).

### Immune Genes

PGYP1.out and PGYP1.out.tet mosquitoes were analyzed by RT-qPCR for a selection of immune genes. Two biologically independent cohorts of 10 sugar-fed, 5-6 day old, female mosquitoes were collected and analyzed from each mosquito line. Total RNA was extracted from whole mosquitoes using TRI REAGENT (Molecular Research Center, Inc.) or RiboZol (AMRESCO). The RNA samples were DNase treated (Promega) and reverse transcribed using random primers and SuperScript III Reverse Transcriptase (Invitrogen). Quantitative PCR was carried out as per Platinum SYBR Green protocol (Invitrogen). Primer sequences for REL1, REL2, CECG and DEFC were obtained elsewhere (Xi et al., 2008) and the other primers were designed using gene sequences obtained from VectorBase (see Supplemental data). The temperature profile of the qPCR was 95°C for 2 min, 50°C for 2 min and 40 cycles of 95°C for 10 s, 60°C for 10 s and 72°C for 20 s. The house-keeping gene RpS17 (Cook et al., 2006) was used to normalize expression. Target gene to house-keeping gene ratios were obtained for each biological replicate using QGene 4.2 (Joehanes and Nelson, 2008). Treatment effects on the expression ratios were examined using Mann Whitney-U tests in STATISTICA V8 (StatSoft, Inc.) and fold change was calculated by the REST method (Pfaffl et al., 2002).

### Immunofluorescence

Following the removal of legs and wings, 14 dpi mosquitoes were fixed overnight at 4°C in 4% (w/v) paraformaldehyde in PBS, containing 0.5% (v/v) Triton X-100. Fixed mosquitoes were dehydrated in an ethanol series of 50%–100% ethanol, followed by two toluene treatments and then infiltrated with paraffin wax (Paraplast-Xtra, McCormick Scientific) at 60°C. Paraffin-embedded mosquitoes were sectioned using a rotary microtome to obtain 8 μm sections that were adhered to superfrost plus slides (Menzel-Gläser). Slides were dried, de-paraffinated in 100% xylene, rehydrated in an ethanol series and washed in PBS-T before being blocked overnight in 2% (w/v) bovine serum albumin (BSA) in PBS-T at 4°C. Sections were then incubated simultaneously for 1 hr with anti-rabbit wsp (1:100) and anti-dengue (1:10) 4G4 or anti-*Plasmodium* CSP (Krettli et al., 1988) antibodies (1:100) (both monoclonal, developed in mouse), in blocking solution. Tissue sections were washed twice with PBS-T and then incubated simultaneously with Alexa-conjugated secondary antibodies (Alexa-488 developed in rabbit or Alexa-594, developed in mice, respectively, Molecular Probes, Invitrogen) diluted 1:1000 each in blocking solution for 1 hr at room temperature. After two washes in PBS-T, the slides were incubated in DAPI for 10 min, rinsed in PBS-T and then mounted using an antifading reagent (ProLong, Invitrogen). Immunostaining was analyzed with a Zeiss Axio Imager II epifluorescence microscope equipped with an

Axiocam camera, using the same exposure conditions for each filter channel. Photos are representative of at least 10 mosquitoes of each treatment.

### Fluorescence In Situ Hybridization

Paraffin-embedded mosquitoes were sectioned and de-paraffinated as described above. Sections were then dehydrated in an ethanol series and hybridized overnight at 37°C in a hybridization buffer containing 4X SSC, 50% formamide, 250 mg/ml dextran sulfate, 250 μg/ml poly(A), 250 μg/ml tRNA, 250 μg/ml salmon sperm DNA, 100 mM DTT and 0.5× Denhardt's solution and 200 *n*g of *Wolbachia* specific 16S rRNAprobes (W2: 5′- CTTCTGTGAGTACCGTCATTATC-3′ and W3: 5′-AACCGACCCT ATCCCTTCGAATA-3′) labeled at the 3′ end with rhodamine. Both probes are 100% homologous to both *w*MelPop and *w*Flu. Following overnight hybridizations, sections were washed twice in 1X SSC containing 10 mM DTT and twice in 0.5X SSC containing 10 mM DTT for 15 min each at 55°C, followed by a 10 min wash at 0.5× SSC containing 10 mM DTT and 1 μg/ml DAPI. Slides were briefly rinsed in water, mounted using an antifading reagent (ProLong, Invitrogen) and observed and photographed as described above.

## SUPPLEMENTAL DATA

Supplemental Data include three figures, one table, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)01500-1.

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