RESEARCH ARTICLE

Novel phenotype of *Wolbachia* strain wPip in *Aedes aegypti* challenges assumptions on mechanisms of *Wolbachia*-mediated dengue virus inhibition

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Abstract

The bacterial endosymbiont *Wolbachia* is a biocontrol tool that inhibits the ability of the *Aedes aegypti* mosquito to transmit positive-sense RNA viruses such as dengue and Zika. Growing evidence indicates that when *Wolbachia* strains wMel or wAlbB are introduced into local mosquito populations, human dengue incidence is reduced. Despite the success of this novel intervention, we still do not fully understand how *Wolbachia* protects mosquitoes from viral infection. Here, we demonstrate that the *Wolbachia* strain wPip does not inhibit virus infection in *Ae. aegypti*. We have leveraged this novel finding, and a panel of *Ae. aegypti* lines carrying virus-inhibitory (wMel and wAlbB) and non-inhibitory (wPip) strains in a common genetic background, to rigorously test a number of hypotheses about the mechanism of *Wolbachia*-mediated virus inhibition. We demonstrate that, contrary to previous suggestions, there is no association between a strain’s ability to inhibit dengue infection in the mosquito and either its typical density in the midgut or salivary glands, or the degree to which it elevates innate immune response pathways in the mosquito. These findings, and the experimental platform provided by this panel of genetically comparable mosquito lines, clear the way for future investigations to define how *Wolbachia* prevents *Ae. aegypti* from transmitting viruses.

Author summary

Dengue virus, transmitted by the *Aedes aegypti* mosquito, is one of the fastest-growing infectious diseases, causing an estimated 390 million human infections per year worldwide. Vaccines have limited efficacy and there are no approved therapeutics. This has driven the rise of novel vector control programs, in particular those that use the bacterium, *Wolbachia*, which prevents transmission of dengue and other human pathogenic viruses when stably introduced into *Ae. aegypti* populations. Although this is proving to
be a highly effective method, the details of how this biocontrol tool works are not well understood. Here we characterise a new Wolbachia strain, wPip, and find that Ae. aegypti carrying wPip are still able to transmit dengue similar to mosquitoes that do not carry Wolbachia. This finding has allowed us to begin a rigorous program of comparative studies to determine which features of a Wolbachia strain determine whether it is antiviral. Understanding these mechanisms will enable us to predict the risk of viral resistance arising against Wolbachia and facilitate preparation of second-generation field release lines.

Introduction

The Aedes aegypti mosquito is the primary vector for many human pathogenic viruses including dengue (DENV), Zika (ZIKV) and chikungunya (CHIKV). Global incidence of arthropod-borne viruses (arboviruses) such as these is increasing in response to urbanization in the tropics, as well as globalization and expansion of the geographical range of Ae. aegypti [1, 2]. Arboviruses like DENV are typically associated with acute, self-limiting febrile disease, although severe manifestations can occur, in some instances leading to death. There are currently no approved specific antiviral therapeutics for DENV, ZIKV or CHIKV and recently developed vaccines for DENV are suboptimal and controversial [3–6]. As such, treatment is supportive only and limiting virus transmission is largely dependent on vector control. Conventional vector control programs attempt to suppress mosquito populations by removing breeding sites, or using insecticide sprays targeting adult mosquito habitats. However, the increasing incidence of these diseases demonstrates that current control programs are failing and there is a need for novel efficacious and cost-effective alternatives.

A new generation of vector control approaches exploits the obligate endosymbiont, Wolbachia. Various strains of Wolbachia are naturally found in at least 40% of insect species [7]. Wolbachia is maternally transmitted, and some strains induce a phenomenon known as cytoplasmic incompatibility (CI) which provides a reproductive advantage to females that carry Wolbachia. Significantly, many Wolbachia strains can protect arthropod hosts from viral infection [8–11]. Wolbachia is not typically found in Ae. aegypti, but it can be stably transferred into these mosquitoes by microinjection. Some Wolbachia strains including wMel (derived from Drosophila melanogaster) and wAlbB (from Ae. albopictus), protect Ae. aegypti from infection by flaviviruses such as DENV and ZIKV, and alphaviruses including CHIKV [12–15]. Recent field trials have tested the utility of these mosquitoes as a biocontrol method. Aided by maternal transmission, as well as CI, short term releases of wMel- or wAlbB-carrying Ae. aegypti have resulted in rapid introgression into wild Ae. aegypti populations. Recent reports indicate this can significantly reduce DENV incidence [16–18].

Despite success of these field trials, we do not understand how Wolbachia inhibits arbovirus infection. Several hypotheses have been proposed, including competition between Wolbachia and viruses for metabolic resources and physical space within host cells [11, 19–21], modification of host gene expression, and immune priming in new hosts [11, 22–24]. Specifically, multiple studies have shown that Wolbachia induces changes in cellular cholesterol and lipid homeostasis [25–27], and DENV replication can in part be restored in Wolbachia-carrying Ae. aegypti cells or mosquitoes by chemical modulation of cholesterol content, or diet supplementation [25, 27]. Recent work has also shown that changes in host gene expression due to the presence of Wolbachia may be an important driver of the antiviral state, with the mosquito insulin receptor [28], and cadherin [29] identified as key targets.
Despite this information, how these effects are induced, and why they impact DENV, is largely unclear. Understanding the contributions of each of these mechanisms to arboviral inhibition in Wolbachia-carrying *Ae. aegypti* has proven to be complex, and it is likely that the antiviral syndrome that Wolbachia species induce in the host is multi-layered, with no single mechanism responsible for this phenotype [30, 31].

We and others have produced and characterized a series of *Ae. aegypti* lines transinfected with different Wolbachia strains (summarized in Table 1), including several strains from *Drosophila* species, and different mosquito species.

So far, all 7 of the Wolbachia-carrying *Ae. aegypti* lines tested have provided some level of protection against flaviviruses. Here, we characterize the vector competence of *Ae. aegypti* transinfected with *w* Pip (from *Culex quinquefasciatus*, supergroup B). Past data has suggested that removal of *w* Pip from its natural host by antibiotic treatment leads to an increase in the replication of DENV-related flavivirus West Nile virus (WNV), indicating that *w* Pip may be antiviral in this context [38]. We previously reported the generation of a *w* Pip-*Ae. aegypti* line [32], and that *w* Pip resides at a high density in *Ae. aegypti*–a feature widely regarded as important for *Wolbachia* to impart its antiviral effects [39–41]. However, we show here that *w* Pip does not restrict flavivirus replication or dissemination, and these mosquitoes transmit infectious virus. We leverage this finding to understand more about what makes a *Wolbachia* strain antiviral. We compare the density and tissue specificity of *w* Pip and antiviral strains *w* Mel and *w* AlbB, in *Ae. aegypti* backcrossed onto the common laboratory Rockefeller *Ae. aegypti* line and identify no link between strains that reside at high density in the midgut or salivary glands, and those that protect against DENV. Furthermore, we determine that commonly used proxies of immune activation of the host are not induced by all antiviral *Wolbachia* strains. Thus, our finding of a *Wolbachia* strain that does not inhibit DENV replication in *Ae. aegypti* has allowed us to isolate the antiviral effects of *Wolbachia* from the general symbiont effects for the first time in this mosquito species, enabling us to resolve conflicting reports regarding the mechanisms that drive *Wolbachia*-mediated virus inhibition.

### Results

**w** Pip does not block flavivirus replication in virus-injected *Ae. aegypti*

Past reports suggest *w* Pip is a good antiviral candidate for testing in *Ae. aegypti*, as it may provide viral protection to *Culex quinquefasciatus* [38], and it resides at high density in *Ae. aegypti* [32]. We assessed the vector competence of *w* Pip-*Ae. aegypti* by intrathoracic injection challenge and infectious blood meal, comparing DENV replication in this line to a matched *Wolbachia*-free line (tetracycline-treated *w* Pip-*Ae. aegypti*; *w* Pip.Tet). As a control, we included *w* Mel-*Ae. aegypti* (predominantly used by the World Mosquito Program as their field release line), and its matched *Wolbachia*-free line (*w* Mel.Tet).

Briefly, 60 seven-day old females were injected with 6.3 x 10⁵ TCID₅₀/ml of DENV-3, or a 10-fold dilution thereof. Total RNA was extracted from whole, surviving mosquitoes 7 days post infection. Absolute DENV-3 RNA copies were determined in each mosquito by qRT-PCR and

### Table 1. *Wolbachia* stains that have been introduced into *Ae. aegypti* to date.

| Host species | Wolbachia strain(s) | Wolbachia supergroup | References |
|--------------|---------------------|----------------------|------------|
| *D. melanogaster* | wMel, wMelCs, wMelPop | A | [15, 32–34] |
| *D. simulans* | wRi, wAu | A | [32, 34] |
| *Ae. albopictus* | wAlbA, wAlbB | A and B, respectively | [34–37] |
| *Cx. quinquefasciatus* | *w* Pip | B | [32] |
extrapolation to a standard curve. Consistent with previous reports, wMel restricted DENV replication by approximately 1 log₁₀ compared to its matched Tet control line, when injected with either concentration of virus (Fig 1A and 1B) [32, 42]. Notably, 37 of 49 injected wMel mosquitoes scored positive for DENV-3 infection (>1000 copies/mosquito, based on the Limit of Detection 95%) when injected with 6.3 x 10⁵ TCID₅₀/ml, compared to just 15 of 47 wMel mosquitoes when injected with 6.3 x 10⁴ TCID₅₀/ml (Fig 1A and 1B, in parenthesis above the bar charts),
consistent with better viral restriction occurring when the mosquitoes are challenged with a lower virus titre [32]. In stark contrast to the protection afforded by wMel, wPip did not inhibit either concentration of DENV-3 compared to the matched wPip.Tet control line. As well as failing to reduce the amount of virus in these mosquitoes, the number of infected wPip-mosquitoes also remained high and comparable to the matched wPip.Tet control line, demonstrating this Wolbachia strain does not provide antiviral protection when transinfected into Ae. aegypti.

Since wPip may inhibit WNV in its native host mosquito species, Cx. quinquefasciatus [38], we next examined whether wPip could inhibit WNV in an Ae. aegypti host. Ae. aegypti carrying wPip, wMel, or their respective Tet control lines, were injected with WNV strain Kunjin virus (KUNV) at 1.4 x 10^7 TCID50/ml or a 10-fold dilution thereof. Mosquitoes were collected 7-days post-infection and RNA was isolated from whole mosquitoes. Total KUNV RNA copies relative to mosquito host RPS17 RNA, was determined in each mosquito by qRT-PCR. Similar to previous reports, wMel provided substantial protection against KUNV, with 2 or 5log10 reductions in viral RNA copies measured in wMel-infected mosquitoes compared to wMel.Tet when injected with high and low virus concentrations, respectively (Fig 1C and 1D) [42]. However, as was observed for DENV-3 infections, wPip failed to provide protection against KUNV at either high or low concentrations of virus. Thus, wPip is not antiviral towards multiple flaviviruses in Ae. aegypti.

**wPip does not restrict DENV replication, dissemination or transmission in Ae. aegypti following an infectious blood meal**

Since the route of viral infection can affect the ability of a Wolbachia strain to inhibit flaviviruses [35], we next examined the impact of wPip on Ae. aegypti infection by DENV following an infectious blood meal. Female mosquitoes carrying wMel or wPip and their respective Tet control lines were fed a blood meal containing freshly harvested cell culture-derived DENV-3 (6.6 x 10^6 TCID50/mL diluted 1:1 in sheep blood). Mosquitoes were incubated for 15 days, then the body and head of the mosquito were collected separately as indicators of established infection and viral dissemination, respectively. No wMel-carrying mosquitoes that took a blood meal established a DENV-3 infection, compared to 71% of the matched Tet control cohort, and a >4log10 reduction in mean DENV-3 copies/body were observed in the presence of wMel (Fig 2A Bodies and Table 2). By contrast, 89% of wPip-transinfected mosquito bodies scored positive for DENV-3, similar to 97% of the matched Tet control cohort, with a slight, although significant, increase in the mean viral copies/body in wPip-carrying mosquitoes compared to its matched Tet control cohort. No wMel mosquitoes had viral RNA disseminated to their heads, compared to 72% of wMel.Tet mosquitoes (Fig 2A Heads and Table 2). Similar numbers of wPip- and wPip.Tet-carrying mosquitoes scored positive for disseminated infection (83% and 96%, respectively), and the mean DENV copies/head was slightly but significantly reduced in wPip-infected mosquitoes compared to wPip.Tet. Note that the slight increases and decreases in viral copy number observed for wPip-carrying mosquitoes compared to the Tet control line are likely to be due to biological variability, as they were not consistent across 3 independent experiments (S1 Fig). The overall trend clearly showed no difference between the vector competence of these two lines.

We further examined the saliva from a proportion of mosquitoes that were fed a virus-spiked blood meal to determine whether infectious virus could be transmitted by wPip-carrying mosquitoes. Saliva was collected from ~15 mosquitoes/line (donor mosquitoes) at 14-days post-infection. Each saliva sample was then injected into the thorax of 6 wMel.Tet recipient mosquitoes to assess the replication competence of the virus in a common host. Seven days later, injected mosquitoes were harvested, total RNA was extracted and qRT-PCR performed
Fig 2. wPip does not restrict DENV replication, dissemination or transmission in *Ae. aegypti* following an infectious blood meal. (A) Seven-day old female mosquitoes were fed a blood meal containing DENV-3 (6.6 × 10⁶ TCID₅₀/ml) and incubated for 15 days. DENV genome copies were determined by qRT-PCR for each body as a measure of infection, and for each head as a measure of viral dissemination. Data are the mean viral genome copies per mosquito body or head ± SEM, with individual data points overlaid, and are representative of 3 independent experiments. Statistical analyses were performed using a Mann-Whitney test where **p < 0.01, ***p < 0.001, ****p < 0.0001. Red line indicates LOD₉₅ of the qRT-PCR reaction (10³ DENV copies). Zero values have been plotted as 10⁰ (1) to allow visualization on the log₁₀ scale (B). At 14 d.p.i., saliva from 15 mosquitoes/line from (A) was collected (donor mosquitoes) and each saliva sample injected into 6 wMel.Tet mosquitoes (recipients) to determine whether the blood-fed mosquitoes were producing infectious virus. DENV genome copies in entire recipient mosquitoes were determined as in (A) and mosquitoes with DENV values above LOD₉₅ were scored positive. Columns represent mosquitoes from a single donor, where black indicates the % recipients infected with DENV from a single donor mosquito. White is uninfected. Number of recipients analyzed from each donor, and whether the donor body was positive (+) or negative (-) for DENV are indicated above the columns.

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to determine the number of recipients positive for virus infection (>10^3 DENV copies/mosquito). wMel caused a significant reduction in the number of donor mosquitoes that carried infectious DENV in their saliva, compared to its matched Tet control line (Fisher’s exact test \( p < 0.05 \)), but wPip did not (Fig 2B and Table 2).

These data consistently demonstrate that wPip does not provide \( \text{Ae. aegypti} \) mosquitoes with protection against DENV-3 infection or dissemination, and infectious virus is produced by mosquitoes that carry wPip.

**Differences in mosquito host genetics do not underlie wPip’s lack of antiviral activity**

Following microinjection of a new Wolbachia strain into \( \text{Ae. aegypti} \), an intense genetic bottleneck occurs as we select individual mosquitoes that carry Wolbachia. To determine whether the lack of antiviral activity observed for wPip was due to specific genetic features of the host mosquito selected during this process, we backcrossed our Wolbachia-carrying lines to the inbred laboratory mosquito line, Rockefeller, through six generations, placing each Wolbachia strain into the same nuclear genetic background. Intrathoracic injection of these lines with DENV-2 confirmed a lack of viral restriction by wPip (S2 Fig). Thus, the inability of wPip-\( \text{Ae. aegypti} \) to inhibit flaviviruses is consistent in different \( \text{Ae. aegypti} \) nuclear backgrounds.

**High Wolbachia density in \( \text{Ae. aegypti} \) salivary glands and midgut is not required for viral inhibition**

Our novel identification of a Wolbachia strain that does not appear to restrict flaviviruses in \( \text{Ae. aegypti} \), generated an opportunity to determine what features of Wolbachia are common to antiviral strains. Several reports have suggested that the ability of a Wolbachia strain to inhibit viruses is dependent on the density at which it resides in its host [39–41]. Our previous work demonstrated that wPip resides at similar or slightly higher levels than wMel in whole \( \text{Ae. aegypti} \) mosquitoes throughout the adult lifespan [32]. However, it is not known whether these strains reside differentially within specific tissues that may explain the disparity in their antiviral activity. To test this rigorously we used our Rockefeller \( \text{Ae. aegypti} \) lines carrying the antiviral Wolbachia strains wMel or wAlbB (classified as Wolbachia supergroup A and B strains, respectively), or wPip (from supergroup B), to compare the densities of Wolbachia in the salivary glands and midguts of female mosquitoes/line, 6–7 days post emergence. Wolbachia density was determined by amplifying the conserved Wolbachia 16S rRNA gene and normalising this to the \( \text{Ae. aegypti} \) host RPS17 gene. All 3 Wolbachia strains were found to reside at similar densities in whole mosquitoes (between 10 and 14 Wolbachia per host cell, Fig 3A). Although

### Table 2. Restriction of DENV-3 infection and dissemination by wPip.

| Line      | Body % DENV +\(^a\) (\(n = 87\)) | Head % DENV +\(^a\) (\(n = 87\)) | Saliva Donor mosquitoes with infectious saliva (\(n\)) |
|-----------|----------------------------------|----------------------------------|-----------------------------------------------------|
| wMel      | 0                                | 0                                | 0 (15)                                               |
| wMel.Tet  | 71                               | 72                               | 6 (14)                                               |
| wPip      | 89                               | 83                               | 3 (14)                                               |
| wPip.Tet  | 97                               | 96                               | 5 (13)                                               |

\(^a\) Calculated as a percentage of total engorged mosquitoes.

\((n)\) total mosquitoes examined.

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Fig 3. High Wolbachia density in *Ae. aegypti* salivary glands and midgut is not required for viral inhibition. (A–F) Density of Wolbachia within female mosquitoes was determined by qPCR following DNA extraction from whole mosquitoes, or from dissected tissues including salivary glands, head/thorax, midgut, abdomen (ovaries removed), or ovaries, as indicated (6–7 days post-emergence). Density is expressed as the mean ratio between the conserved Wolbachia 16S rRNA gene and the *Ae. aegypti* host RPS17 gene. Data are the mean and SEM of 24 mosquitoes.
mosquito salivary glands must become infected with virus in order for the mosquito to transmit DENV, we do not know whether this tissue contributes to virus inhibition by supporting high levels of Wolbachia. We tested whether antiviral Wolbachia strains localise in this tissue at a higher density than the non-inhibitory strain, wPip. Surprisingly, the mean relative wMel density was shown to be very low (1 Wolbachia per host cell), while wAlbB and wPip resided at substantially and significantly higher mean levels (11 and 13 Wolbachia per host cell; p<0.0001 Kruskal-Wallis test) (Fig 3B). Given this unexpected finding, we next examined whether the tissues surrounding the salivary glands may be contributing high Wolbachia densities to mediate the antiviral phenotype observed for wMel-carrying mosquitoes. To do this we separated the head/thorax from the abdomen of mosquitoes and determined the Wolbachia density. The densities of wMel, wAlbB and wPip in the head/thorax closely reflected what was observed in salivary glands alone (Fig 3C). It therefore appears that high levels of Wolbachia are not required in or around the salivary glands in order to provide an antiviral phenotype. Similarly, the findings for wPip demonstrate that high levels of Wolbachia can reside in this critical tissue and not impact virus inhibition.

We next used confocal laser scanning microscopy to examine whether wPip localises differently within the salivary gland tissue, to enable DENV replication. Salivary glands were dissected from female mosquitoes 6 days post emergence and stained by fluorescence in situ hybridization (FISH) using probes that detect the conserved Wolbachia 16S rRNA gene and DAPI to demarcate the salivary gland tissue. Slides were imaged as 3D z-stacks and 2D images were generated by Maximum Intensity Projection (MIP) using Fiji software. Images are representative of >8 salivary gland sets per mosquito line, from 2 independent experiments. Scale bar: 100 μm.

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Asterisks indicate significance compared to Ae. aegypti-wMel (Kruskal-Wallis test with Dunn’s correction; **p<0.01, ***p<0.001). G) Salivary glands were dissected from 6 female mosquitoes 6 days post emergence and stained by fluorescence in situ hybridization (FISH) using probes that detect the conserved Wolbachia 16S rRNA gene and DAPI to demarcate the salivary gland tissue. Slides were imaged as 3-dimensional z-stacks and 2D images generated by Maximum Intensity Projection (MIP) using Fiji software. Images are representative of >8 salivary gland sets per mosquito line, from 2 independent experiments. Scale bar: 100 μm.
density, then wPip, with wAlbB residing at the highest density (Fig 3E). Thus, the tissues immediately surrounding the midgut are not supporting high levels of wMel to supplement the lower Wolbachia densities observed in this tissue.

Analyses of ovaries dissected from each line identified that the high density of wMel observed in whole mosquitoes was largely driven by ovary-localization (Fig 3F). wMel was present in ovaries at 48 Wolbachia per host cell, significantly higher than in the ovaries of wAlbB- and wPip-carrying mosquitoes (9 and 13 Wolbachia per cell, respectively; p<0.0001 Kruskal-Wallis test). Interestingly, previously reported maternal transmission rates for Wolbachia strains wMel, wAlbB and wPip in Ae. aegypti are almost always 100% [15, 32, 36] suggesting that that the significantly lower levels of wAlbB and wPip in the ovaries are sufficient to support a high rate of maternal transmission.

Of note, wAlbB and wPip (both belonging to supergroup B and therefore more closely related to each other than to wMel) seem to display similar tissue distribution and densities in all the tissues examined here, while the profile of wMel is quite different. These findings indicate that a strict localization and density profile at the tissue level is not linked to the antiviral phenotype of Wolbachia strains.

Wolbachia-mediated antiviral protection is not dependent on elevated innate immune response pathways in Ae. aegypti

We next tested the hypothesis that Wolbachia infection in Ae. aegypti elevates expression of several innate immune pathway components, particularly in novel Wolbachia-host associations, to create an antiviral environment [11, 22–24]. To do this we used our panel of genetically comparable Rockefeller Ae. aegypti-Wolbachia lines, with the addition of wMelPop (also in the Rockefeller background)—a strongly antiviral Wolbachia strain that enhances expression of innate immune pathway components in Ae. aegypti [11, 22–24]. We assessed the expression levels of Toll pathway components (cecropin D, cecropin E, defensin C), known to control anti-DENV defences in mosquitoes [43], and previously reported to be upregulated by some Wolbachia strains in Ae. aegypti [11, 22–24]. We also assessed expression levels of C-type lectin (immune recognition molecule) and transferrin (regulation of oxidative stress through iron sequestration), other proteins involved in innate immunity and previously reported to be upregulated by some Wolbachia strains in Ae. aegypti [11, 22, 24].

Twenty-four female mosquitoes carrying wMel, wAlbB, wPip, wMelPop, or Rockefeller (no Wolbachia control) were collected 5-days post-emergence. Expression levels of immune molecules were measured by qPCR, and quantified relative to the mosquito gene RPS17. Mosquitoes carrying wMelPop had significantly elevated levels of each of these representative pathway components compared to the Rockefeller control (Kruskal-Wallis test, Fig 4A–4E). The magnitude of expression increase, relative to the Rockefeller control mosquitoes, varied between the transcripts examined with the smallest increase observed for C-type lectin (10-fold increase; Fig 4D) and the largest observed for defensin C (~250-fold; Fig 4C). In contrast, only very small or no increase in expression of these components was observed in Ae. aegypti carrying wMel, wAlbB or wPip, relative to the Rockefeller control. wMel did significantly increase expression of cecropin E and defensin C (Fig 4B, C), but these increases were very small in comparison to wMelPop (6- and 2.5-fold increases in the presence of wMel, compared to 180- and 270-fold increases in the presence of wMelPop) and were not observed for the other antiviral strain, wAlbB. Interestingly, C-type lectin, while significantly increased by wMelPop, was significantly decreased by all other strains relative to the Rockefeller control (Fig 4D). This is the first time Wolbachia-induced immune gene expression has been examined in genetically comparable Ae. aegypti lines, using a variety of Wolbachia strains that either inhibit or do not
inhibit DENV. Using this approach, we can state that elevated expression of these immune components is not consistently associated with a Wolbachia-mediated antiviral phenotype in *Ae. aegypti*.

**Discussion**

Dissecting the molecular mechanisms that underpin the inhibition of human pathogenic viruses by various Wolbachia strains will facilitate the continued success and longevity of Wolbachia-based biocontrol programs. This will provide a means to screen for the possible emergence of viral resistance prior to detecting an increase in human disease in *Ae. aegypti*-Wolbachia established areas. In addition, it will allow us to identify second-generation Wolbachia strains that may restrict viruses using different mechanisms.

*wPip* is the first Wolbachia strain that has been introduced into *Ae. aegypti* without providing antiviral protection towards flaviviruses. A recent study from Ant *et al.*, 2018, introduced *wAlbA* from *Ae. albopictus* into *Ae. aegypti*. This line carried *wAlbA* at a high density yet did not restrict DENV or ZIKV following intrathoracic viral injection [35]. However, when vector competence was examined following an infectious blood meal (DENV or ZIKV), viral infection, dissemination and transmission rates were significantly reduced [35]. We have previously shown that intrathoracic injection challenges with high virus concentrations can overwhelm...
Wolbachia-mediated inhibition [32]. This mode of infection may underestimate the ability of a Wolbachia strain to inhibit arboviruses unless injections are performed using a range of virus concentrations. These findings demonstrate the importance of rigorous assay design for vector competence analyses.

Our generation of a novel panel of genetically comparable Ae. aegypti lines carrying different Wolbachia strains, including one with a non-antiviral strain (wPip), has allowed us to begin rigorously testing a series of hypotheses of the mechanisms underlying viral restriction by Wolbachia. Until recently, it was widely accepted that inhibition of viruses in this context correlated with Wolbachia density. This dogma was based on a series of experiments using mosquito cell culture and Drosophila lines carrying a variety of Wolbachia strains, or titrations of a single Wolbachia strain by antibiotic treatment [39–41, 44]. However, here we demonstrate that antiviral phenotype is not dictated by the density at which it resides in either the whole body, salivary glands, the midgut or the tissues immediately surrounding these in the mosquito. This conclusion is supported by a recent publication from Flores et al. who determined that wMel inhibits DENV replication in mosquito abdomens better than wAlbB, despite wAlbB residing at higher density than wMel in the midgut and abdomen as shown here [37]. Furthermore, it is consistent with reports that wAlbA resides at substantially higher levels than wAlbB and wMel in the midgut and the salivary glands of Ae. aegypti, despite wAlbA showing a relatively limited ability to inhibit flaviviral replication [34, 35]. Amuzu and McGraw (2016), examined this in another way, determining that the relationship between DENV inhibition and wMel density within a single Ae. aegypti tissue sample is not linear [45]. This finding supports our conclusion that it is not simply the amount of Wolbachia present in a tissue that determines whether or not a Wolbachia strain is able to inhibit DENV. Studies into flavivirus infection in mosquitoes without Wolbachia indicate that tissues including the midgut, trachea and salivary glands evidently become infected following virus uptake and dissemination [46, 47]. Since we report relatively high levels of wPip (non-antiviral) in the abdomen and salivary glands, it will be interesting to characterise how DENV can infect tissues already occupied by Wolbachia.

If Wolbachia does not have to be present at high densities to impair viral replication e.g. in the midgut, then this also questions an existing hypothesis that Wolbachia impairs viral replication by competing for space within host cells [11, 20, 21]. Further work is needed to analyse the subcellular localisation of various Wolbachia strains to examine whether this may determine the antiviral activity of a strain.

Several studies have implied that Wolbachia may prime the host innate immune system, preventing arboviral establishment [11, 22–24]. The importance of the contribution of this mechanism has been clouded by the fact that expression levels of these pathway components seem to vary depending on how long the Wolbachia strain has resided in its host [22]. We selected a series of immune effectors previously shown to be upregulated by wMelPop, wMel or wAlbB in Ae. aegypti, relative to no-Wolbachia control lines. These included toll pathway components (cecropin D, E, and defensin C), C-type lectin (immune recognition molecule), and oxidative stress regulator transferrin [11, 22–24]. Using our panel of comparable Wolbachia-Rockefeller mosquito lines our findings definitively show that these pathways do not need to be primed at high levels by Wolbachia in order to restrict viral replication. While we have not exhaustively considered all innate immune pathways, this finding is consistent with observations from Rances et al., 2012, who identified upregulation of many immune genes only occurred in wMel- and wMelPop-Ae. aegypti where the endosymbiont was a newly acquired infection, but not in the original D. melanogaster host where both these strains are antiviral [22]. It is possible these strains may use overlapping mechanisms to inhibit viruses, but these data indicate that immune priming may be particular to wMelPop. The induction of these
pathways by wMelPop is of such a magnitude that they could be easily measured at the whole mosquito level, but refining the experiment to investigate expression levels in individual tissues may reveal smaller increases in expression levels by other Wolbachia strains.

We initially produced the wPip-Ae. aegypti line based on work in the native Culex mosquito host that showed removal of wPip led to enhanced levels of WNV [38]. Given that wPip does not protect against multiple flaviviruses in Ae. aegypti, our results show that there is likely to be a specific Wolbachia-host interaction that determines whether a Wolbachia strain creates an antiviral state in its host. This host-dependent context has previously been seen for wAlbB: this strain does not provide clear protection against flaviviruses in its native Ae. albopictus host [13], but effectively inhibits flaviviruses in Ae. aegypti [13, 34, 42].

While we are the first to stably introduce wPip into Ae. aegypti, wPip was previously introduced into Ae. albopictus: as a single infection, as a double infection with wMel [48], and as a triple infection with the natural wAlbAwAlbB Wolbachia strains [49]. These studies have shown that wPip alone in Ae. albopictus is not antiviral, while combining wPip with wMel (previously shown to have antiviral activity in Ae. albopictus) does inhibit arboviruses, and the addition of wPip to the natural wAlbA/wAlbB combination significantly reduces DENV and ZIKV replication. This complicated scenario may suggest that wPip modifies these hosts in different ways, such that effects in Ae. albopictus cannot strictly be extrapolated to Ae. aegypti. Although it should be noted that wPip has at least 5 genetically distinct groups and it is possible that these groups may differ in their antiviral activity [50].

We should also be open to the possibility that where a consistent antiviral phenotype is observed for one Wolbachia strain in different hosts, multiple mechanisms that are species-specific may contribute to the antiviral phenotype.

The importance of host context was recently shown in another way by Ford et al. (2019), who selected for wMel-Ae. aegypti that impart strong or weak antiviral effects towards DENV. The group showed that mosquitoes that were more antiviral expressed higher levels of the host gene cadherin [29]. It will be of interest in future studies to determine whether all antiviral Wolbachia strains induce specific changes in host gene expression in Ae. aegypti, that are not induced by wPip.

The issue of host context poses an interesting issue for selecting Wolbachia strains for novel Ae. aegypti lines—that is, how can we predict whether a new strain will induce an antiviral state? Notably, so far it seems that the magnitude of the antiviral effect of a Wolbachia strain measured in Drosophila spp. predicts how the strain will behave in Ae. aegypti (from the 5 strains examined) [10, 11, 15, 32, 34, 44, 51]. This has not been the case for mosquito-derived Wolbachia strains–wAlbA and wAlbB are not antiviral in their native host, but do provide protection in Ae. aegypti, while wPip is reported to be antiviral in Cx. quinquefasciatus, but not in Ae. aegypti [13, 35, 38]. Perhaps this is due to differences in the way these Wolbachia strains localise in each host. And/or, if multiple mechanisms contribute to viral inhibition, perhaps each host-Wolbachia combination has some or all of these mechanisms in play. The introduction of other novel Wolbachia strains into Ae. aegypti is required to determine if this trend holds.

In this study we have generated a unique and powerful tool: a panel of Ae. aegypti lines that differ only in the Wolbachia strain that they carry, including wPip-Ae. aegypti which does not restrict flavivirus replication or dissemination. This is an important finding as it identifies a refined negative control that isolates the antiviral effects of Wolbachia for studies trying to understand how Wolbachia affects its host. A Wolbachia-free control has been used in the past, but this control does not account for the many host effects Wolbachia can induce just by residing as an endosymbiont, that may not be responsible for creating an antiviral state [52, 53]. In this way, we have been able to re-examine two hypotheses of how Wolbachia inhibits
arboviruses, that have been subject to multiple conflicting reports—the importance of tissue-specific *Wolbachia* density in predicting the antiviral activity of a strain in *Ae. aegypti*, and the role of immune priming in inhibiting flaviviruses. The strength of this approach was recently shown by LePage *et al.* (2017) who compared the genomes of *Wolbachia* strains that do or do not induce CI to manipulate host reproduction, identifying the two genes responsible for inducing this phenotype [54]. We have now initiated a series to experiments to carefully dissect the mechanisms that drive *Wolbachia*-mediated viral inhibition.

**Methods**

**Mosquito rearing**

All *Ae. aegypti* mosquitoes were reared and maintained as described previously [32, 33, 55]. Briefly, adult mosquitoes were maintained at 26˚C, 65% relative humidity (RH) and a 12 h light/dark cycle in a climate-controlled room. Mosquitoes were blood fed on the arms of human volunteers (Monash University human ethics permit CF11/0766-2011000387). The *Wolbachia*-infected wMel, wAlbB and wPip lines as well as matched Tet-control lines (*Wolbachia* infected lines that have been cured of their infection by tetracycline treatment) used in these experiments have been described previously [15, 32, 37, 56]. Tet-control lines were backcrossed to their matched *Wolbachia*-carrying lines for a minimum of three generations to homogenise their genetic backgrounds.

To generate a panel of genetically comparable *Wolbachia*-carrying *Ae. aegypti* lines, we backcrossed females from the wMel, wAlbB, wMelPop and wPip to males of the inbred laboratory *Ae. aegypti* line, Rockefeller [57] (BEI resources), for six generations.

To exclude any influence of mosquito age on our experiments, age-controlled adults emerging within a 24 h window were used.

**Vector competence**

DENV-3 Cairns 08/09 strain (Genbank accession number: JN406515.1) and DENV-2 strain (originally isolated from a patient in Vietnam in 2010) were prepared by inoculation of C6/36 cells with a multiplicity of infection (MOI) of 0.1 and collection of culture supernatant 6–7 days later. KUNV stocks (Genbank accession number: MRM61C) were prepared by inoculation of C6/36 cells with a multiplicity of infection (MOI) of 1 and collection of culture supernatant 48 hours later. Virus concentrations were determined by TCID$_{50}$ as previously described [58] using monoclonal antibody 4G2 [59].

For feeding experiments with DENV-3 (Cairns 08/09) infected blood, 100 seven-day old age-controlled female mosquitoes were placed in 500 mL plastic containers (five containers per *Wolbachia* line, three containers per Tet line), starved for up to 24 h and allowed to feed on a 50:50 mixture of defibrinated sheep blood and tissue culture supernatant containing freshly harvested 6.6 x 10$^6$ TCID$_{50}$/mL of DENV-3. Feeding was done through a piece of desalted porcine intestine stretched over a water-jacketed membrane feeding apparatus pre-heated to 37˚C. Mosquitoes were left to feed in the dark for approximately 1–2 hours. Fully engorged mosquitoes were placed in 500 mL containers at a density of < 25/container, and incubated for 15 d at 26˚C with 65% RH and a 12 h light/dark cycle.

For adult microinjections, 60 six- or seven-day old age-controlled female mosquitoes were anesthetized by CO$_2$. Mosquitoes were injected intrathoracically with 69 nL of DENV (DENV-3 Cairns 08/09 strain at 6.3 x 10$^5$ or 6.3 x 10$^4$ TCID$_{50}$/ml, or DENV-2 Vietnam strain at 2.4 x 10$^5$) in RPMI media (Life Technologies) using a pulled-glass capillary and a handheld microinjector (Nanoject II, Drummond Scientific). For KUNV injections, 69 nL of 1.4 x 10$^7$ or 1.4 x 10$^6$ TCID$_{50}$/ml was injected per mosquito using the same method as described for DENV-3.
Injected mosquitoes were incubated for 7 days (15 mosquitoes/cup) at 26°C with 65% RH and a 12 h light/dark cycle.

To quantify DENV-3 or KUNV genomic copies, total RNA was isolated from mosquitoes (entire mosquitoes for injection experiments, or head and bodies separately for blood-fed mosquitoes) using the RNAeasy 96 QiAcube HT kit (Qiagen). DENV-3 RNA was amplified by qRT-PCR (LightCycler Multiplex RNA Virus Master, Roche), using primers to the conserved 3'UTR: Forward 5'-AAGGACTAGAGTGTAGAGGAGGACC; Reverse 5'-CGTTCTGTCGCCTGGATGTG; Probe 5'-HEX-AACAGCATATTGACGCTGGAGAGACCAGA-BHQ1-3' [60]; absolute copies were determined by extrapolation from an internal standard curve generated from plasmid DNA encoding the conserved 3'UTR sequence. Mosquito extracts with ≥1000 copies of DENV per body were scored positive, based on the LOD95 (limit of detection 95%) for DENV-3 with this primer set. KUNV RNA was amplified by using primers that span the 3' end of the conserved NS5 gene, and the 3'UTR: Forward 5'-AACCCCAAGTGAGAGAGAGG TGGA; Reverse 5'-TCAGGCTGCCACACAAAAA; Probe 5'-HEX-CGATGTTCCATACCTGGCAAACG-BHQ1-3' [61]. KUNV RNA copies were quantified relative to Ae. aegypti house-keeping gene RPS17 using the delta CT method (2^CT(reference)/ 2^CT(target)). KUNV RNA copies with a CT of < 33 were scored positive for infection. Note that all surviving mosquitoes were processed for virus injection experiments, while a maximum of 87 mosquitoes/line were collected and processed for blood feeding experiments.

Virus transmission

To determine whether Wolbachia-infected mosquitoes were capable of transmitting infectious virus, 15 blood-fed mosquitoes per line were collected at 14 days post blood feed (donor mosquitoes). The proboscis from each donor was inserted into a 10 μL pipette tip containing 10 μL 1:1 FBS:30% sucrose [62]. Legs and wings were removed to encourage the mosquitoes to spit. Pipette tips were collected 1 hour later and the virus solution ejected onto parafilm. The solution was drawn up into a pulled-glass capillary attached to a handheld microinjector (Nanoject III, Drummond Scientific) and 600 nL was injected into 6 seven-day old recipient wMel.Tet mosquitoes. Replicate recipient mosquitoes were stored in a single container for 7-days post injection. RNA was extracted from the whole bodies of all surviving mosquitoes, and qRT-PCR was performed as described above.

Wolbachia density and distribution

Relative Wolbachia density in wMel, wAlbB and wPip was determined in whole or dissected tissues from female mosquitoes at 6–7-days post emergence, using qPCR with primers to amplify a fragment of the conserved 16S rRNA gene (forward primer: 5’-GAGTGAAGAAGG CCTTGGG-3’, reverse primer: 5’-CACCGAGTTCAGAGGACTTC-3’, probe 5’-LC640-CTGTGAGTACGCTATTATCCT-IowaBlackRQ-3’) and the reference Ae. aegypti rps17 gene (forward primer: 5’-TCCGGTGTATCTCCATCAAGCT-3’, reverse primer: 5’-CAGGAGGACGTATTGTC-3’, probe 5’FAM-CAGGAGGAGGAACGTGA GCGAC-BHQ1-3’) [32]. Wolbachia densities were quantified relative to RPS17 using the delta CT method as previously (2^CT(reference)/ 2^CT(target)).

Salivary gland dissections and fluorescence in-situ hybridization (FISH) staining

Female mosquitoes were collected 6-days post emergence, knocked down at -20°C for 2 minutes, then kept in a petri dish on ice until dissection. Individuals were dissected on a microscope slide in a drop of PBS. Briefly, the head of the mosquito was sliced off using a dissection
needle, and the salivary glands popped out by gently squeezing the thorax with needle-tipped forceps. Salivary glands were gently transferred to a small droplet of PBS on poly-lysine-coated slides. Tissues were fixed in cold 4% paraformaldehyde in PBS for 15 minutes, rinsed 3 times in PBS, then permeabised in 100% ethanol for 5 minutes and air dried. Slides were incubated in hybridization buffer containing fluorescently labelled 16S rRNA probes (cross-reactive with all three Wolbachia strains) [11] overnight at 37˚C in a humidified chamber. Slides were washed in SSC buffers + 10 mM DTT, stained with DAPI, and mounted as described by Moreira et al., 2009 [11].

**Confocal microscopy**

Slides were imaged using a Nikon C1 Upright confocal microscope at 20X magnification (under oil) as 3-dimensional z-stacks with a step-size of 3 microns. Images were acquired with NIS-Elements software. Maximum Intensity Projection images and scale bars were generated in Fiji software (Version 1.52; National Institutes of Health). Note that the image of wPip salivary glands was produced by stitching together two images taken from the same sample, as the lobes spread too wide to image under a single field of view [63].

**Quantitative RT-PCR for immune gene targets**

RNA was extracted from 6-day old female mosquitoes (24 per line including Rockefeller, wMel, wAlbB, wPip) using the RNeasy 96 QIAcube HT kit (Qiagen). Samples were DNaseI treated and cDNA was generated from 8 μL of purified RNA/individual (~500 ng of RNA) using SuperScript III First-Strand Synthesis System (ThermoFisher). cDNA was diluted 2-fold with RNase-free water then expression levels of selected immune genes was determined by amplifying 1 μL of cDNA with primers for cecropin D (AAEL000598; forward 5’-GCTAGGTTCAAACGGAAGCAG, reverse 5’-TCCTACAACAAACCGGAGAG) [24], cecropin E (AAEL000611; forward 5’-TTGCACTCGGGTCTGCATC, reverse 5’-ACACGTGTTTCCGA CTCCTTC) [24], defensin C (AAEL003832-RA; forward 5’-GCTGAGTGGGTTCGGTGTA, reverse 5’-GGCGGGACATAGCTCCTC) [22], C-type lectin (AAEL005641; forward 5’- GTCTCGGGTGCAATACACT, reverse 5’-CCCTATCGTTCCACTCCT) [24] or Transferrin (AAEL0015458; forward 5’-TCAAGATCTGAAGGCCCAAAC, reverse 5’-GTCTTGACC TTCTCCAGACA) [24]. Expression levels were normalized to the Ae. aegypti house-keeping gene RPS17 (forward 5’- TCCGGTGGGATCTCCATGAGCT, reverse 5’-CATTCCGCCAG GTATGGTGT) using the delta CT method (2^ΔCT (reference)/ 2^ΔCT (target)).

**Ethics statement**

Blood feeding by volunteers (Monash University human ethics permit no CF11/0766-2011000387) for this study was approved by the Monash University Human Research Ethics Committee (MUHREC). All adult volunteers provided informed written consent; no child participants were involved in the study.

**Supporting information**

S1 Fig. wPip does not restrict DENV dissemination in Ae. aegypti following an infectious blood meal. Seven-day old female mosquitoes were fed a blood meal containing DENV-3 (2.09 x 10^7 TCID_{50}/ml rep. 1 and 3.39 x 10^6 TCID_{50}/ml rep. 2) and incubated for 14 days. DENV genome copies were determined by qRT-PCR for each head as a measure of viral dissemination. Data are the mean viral genome copies per mosquito head ± SEM, with individual data points overlaid, and were generated from 2 independent experiments (rep. 1 and rep. 2).
Statistical analyses were performed using a Mann-Whitney test where *p < 0.05,
**p < 0.001, ****p < 0.0001. Red line indicates LOD$_{95}$ of the qRT-PCR reaction. Zero values
have been plotted as 10$^0$ (1) to allow visualization on the log$_{10}$ scale. Numbers in parentheses
above the bars indicates the number of mosquito heads scored positive for DENV per total
engorged mosquitoes. Fisher’s exact test indicates significantly more mosquitoes show disseminated
virus in the wMel.Tet cohort compared to the wMel cohort (p<0.00001 rep. 1 and
rep. 2), but no significant difference in the number of mosquitoes showing disseminated virus
between wPip and wPip.Tet cohorts (p>0.05 rep. 1 and rep. 2).

S2 Fig. Differences in mosquito host genetics do not underlie wPip’s lack of antiviral activ-
ity. DENV-2 (isolated from a patient in Vietnam in 2010) [37] was injected into the thorax of
7-day old female mosquitoes at 2.4 x10$^5$ TCID$_{50}$/ml. RNA was extracted from whole mosquito
bodies 7-days post infection and virus replication was quantified by qRT-PCR. Data are the
mean number of DENV genome copies per mosquito ± SEM with individual data points over-
laid. Asterisks indicate significance compared to Rockefeller (no Wolbachia control) (Kruskal-
Wallis test with Dunn’s correction; ****p<0.0001).

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