Wolbachia strain wAlbB maintains high density and dengue inhibition following introduction into a field population of Aedes aegypti

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Aedes aegypti mosquitoes carrying the wAlbB Wolbachia strain show a reduced capacity to transmit dengue virus. wAlbB has been introduced into wild Aedes aegypti populations in several field sites in Kuala Lumpur, Malaysia, where it has persisted at high frequency for more than 2 years and significantly reduced dengue incidence. Although these encouraging results indicate that wAlbB releases can be an effective dengue control strategy, the long-term success depends on wAlbB maintaining high population frequencies and virus transmission inhibition, and both could be compromised by Wolbachia–host coevolution in the field. Here, wAlbB-carrying Aedes aegypti collected from the field 20 months after the cessation of releases showed no reduction in Wolbachia density or tissue distribution changes compared to a wAlbB laboratory colony. The wAlbB strain continued to induce complete unidirectional cytoplasmic incompatibility, showed perfect maternal transmission under laboratory conditions, and retained its capacity to inhibit dengue. Additionally, a field-collected wAlbB line was challenged with Malaysian dengue patient blood, and showed significant blocking of virus dissemination to the salivary glands. These results indicate that wAlbB continues to inhibit currently circulating strains of dengue in field populations of Aedes aegypti, and provides additional support for the continued scale-up of Wolbachia wAlbB releases for dengue control.

This article is part of the theme issue ‘Novel control strategies for mosquito-borne diseases’.

1. Background

Releases of Aedes aegypti carrying the maternally inherited bacterial endosymbiont Wolbachia are being trialled in several countries as a novel arbovirus intervention [1–3]. Certain strains of Wolbachia possess attributes ideal for vector control; they have a capacity to invade mosquito populations while simultaneously reducing the transmission potential for important arboviruses, including dengue. The capacity of Wolbachia to spread through a population arises from a mating incompatibility (commonly known as cytoplasmic incompatibility, CI) between Wolbachia-carrying males and Wolbachia-free females, which renders the resulting progeny inviable, while Wolbachia-carrying females are able to reproduce successfully with both Wolbachia-carrying and Wolbachia-free males. Aedes aegypti carrying...
the wAlbB Wolbachia strain have been released in several sites in Kuala Lumpur, Malaysia, resulting in stable establishment in some areas. Residents of sites with high wAlbB frequencies experienced a decline in dengue incidence of approximately 40% compared to controls, although this is likely an underestimate of the true decrease in transmission given the potential for exposure outside of the release areas [1].

Levels of viral inhibition vary substantially between Wolbachia strains, ranging from no inhibition to complete blocking [4–6]. Different strains replicate to different levels within a host, and with a few exceptions, the level of viral blocking generally shows a positive correlation with Wolbachia intracellular density [6,7]. The distribution of Wolbachia in host tissues is also important for transmission blocking given evidence that viral inhibition is cell autonomous [8,9]. To achieve transmission in a mosquito, an arbovirus present in a bloodmeal must invade the midgut epithelium, disperse in the haemolymph, and eventually establish an infection in the salivary glands. The presence of Wolbachia in the somatic tissues of the midgut and salivary glands is, therefore, central to the transmission-blocking phenotype. The high-density strains wMelPop and wAu reach very high somatic densities and generate particularly strong transmission blocking [4,8], although high Wolbachia densities are also associated with virulence in the host, negatively affecting a range of life-history traits including fecundity, longevity and egg survival over extended periods of quiescence [4,10]. Higher density strains, therefore, have a lower invasion potential which can limit their use in field interventions. wMelPop-carrying Ae. aegypti were released in field sites in Australia and Vietnam and despite reaching high initial frequencies, the strain was lost once releases ceased [11]. wAlbB reaches intermediate densities in Ae. aegypti and has a relatively low impact on many aspects of host fitness [4,12], while providing significant inhibition of dengue transmission [4,13,14].

A variety of environmental and symbiont/host genetic factors influence the density and tissue distribution of Wolbachia. High larval breeding site temperatures, for example, cause dramatic reductions in the density of some strains [4,15,16], although wAlbB appears to be relatively heat stable [4,16]. Host factors are important in restricting Wolbachia tropism to the germline in native associations; wAlbB is largely restricted to the germline in its native host Aedes albopictus [17], whereas wAlbB in Ae. aegypti has a broad somatic distribution [4]. Interestingly, transfer of the wMel strain (native to Drosophila melanogaster) into Ae. albopictus results in high densities in midgut and salivary gland tissue [17] and strong viral inhibition [18]; thus restricted tissue tropism within a particular host species can be Wolbachia strain-specific. In addition to inter-species variation, intra-species differences may also play an important role. Experimental evolution of wMel-carrying Ae. aegypti generated differences in virus inhibition among wMel-carrying lineages which correlated with genetic changes in the host genome, although this was independent of Wolbachia density [19].

As with any vector control method, evolutionary responses have the potential to disrupt the long-term stability of a Wolbachia-based intervention. Since Wolbachia is maternally transmitted, the symbiosis is expected to evolve towards a more benign or even mutualistic association through adaptations of the host, the symbiont or both [20]. Given that virus transmission blocking is largely governed by Wolbachia densities in somatic tissues, and that high titre somatic Wolbachia can be virulent, natural selection may act to decrease densities in the midgut and salivary glands, leading to a reduction in overall viral inhibition. However, an eventual loss of viral inhibition is not necessarily the default evolutionary outcome. Virus inhibition may even be selected for; a recent study reported that a lineage of wMel-carrying Ae. aegypti that exhibited lower virus inhibition also had reduced relative fitness [19]. Importantly, the authors of this study managed to select for lower virus inhibition from field-collected mosquitoes through experimental evolution, while failing to select for stronger inhibition, indicating that lineages with strong inhibition persist at high frequency in the field. Furthermore, Wolbachia-mediated resistance to pathogenic insect-specific viruses may act as a source of selection to maintain the virus inhibition phenotype, although the magnitude of this selective pressure remains to be determined in wild mosquito populations. Consistent with a lack of strong selection on Wolbachia or host, the wMel strain has maintained both virus inhibition and deleterious fitness traits in Australian populations of Ae. aegypti nearly a decade after introduction [21,22].

Given the potential for host genotype effects on viral inhibition, and the possibility of host and symbiont evolution, long-term monitoring of field populations for phenotypic stability forms an important part of the routine surveillance of a Wolbachia intervention. The wAlbB-carrying Ae. aegypti strain used in field releases in Malaysia was originally transferred into an Ae. aegypti colony isolated from wild-caught mosquitoes in Kuala Lumpur in the 1960s, and showed complete CI induction, 100% maternal transmission and inhibition of dengue transmission [4]. After backcrossing to field-collected males to improve field performance particularly within the context of pesticide resistance, the wAlbB strain was released in a number of sites in urban Kuala Lumpur, including Mentari Court (a set of seven 18-floor apartment buildings), and Shah Alam (§7) a commercial/residential zone. Releases in Mentari Court ceased on the 5 March 2018, with wAlbB spreading rapidly and maintaining greater than 90% frequency 2 years later [1]. Releases in Shah Alam ceased on 20 April 2019, with wAlbB maintaining a frequency of greater than 84% more than a year later. Given the far greater genetic diversity of wild compared to laboratory populations, and a higher degree of mating competition and selection on mosquito fitness in the field, there may be faster evolution towards lower wAlbB densities in the field versus the laboratory. To assess whether the field population has undergone an accelerated rate of selection on density we examined a colony of wAlbB-carrying Ae. aegypti recently established from mosquitoes collected in Mentari Court, 20 months post cessation of field releases. Wolbachia density and tissue distribution, the stability of the DENV-2 dengue blocking phenotype, CI and maternal transmission rates are assessed and compared to a laboratory wAlbB colony. In addition to evaluating the post-release phenotypic stability of wAlbB, the competence of a wAlbB field strain established from the Shah Alam release site was evaluated through oral challenge with DENV-1 infected blood from a Malaysian dengue patient.

2. Results

(a) Wolbachia density and tissue distribution

The densities of Wolbachia in whole adult females reared from the F3 eggs of a wAlbB-carrying Ae. aegypti line established
Wolbachia density was determined at 5 and 10 days post adult eclosion by qPCR. Twenty-four females were analysed per group. Individual dots represent Wolbachia densities in individual females. A significant difference was found between wAlbB.MC and wAlbB.L densities at day 5 (2-sample t-test, p < 0.0001), although no difference was found at day 10 (p = 0.17). Boxplots show median and interquartile range.

Densities were also measured in the dissected ovary, midgut and salivary gland tissues from F4 females. wAlbB density in the ovaries was stable over time and between mosquito lines (figure 1). While at day-5 PE, there was no difference in wAlbB density between age and mosquito lines (figure 2b). While at day-5 PE, there was no difference in midgut wAlbB levels between the two lines, at day-10 PE the Wolbachia density was significantly higher in the wAlbB.MC line compared to wAlbB.L. Wolbachia density in salivary glands was similar between mosquito lines at all ages (figure 2c).

(b) Maternal transmission and cytoplasmic incompatibility

Rates of Wolbachia maternal transmission in the wAlbB.MC line were assessed in the progeny of crosses between wild-type (Wolbachia-free) males and wAlbB.MC females (i.e. in the absence of CI). All the G0 offspring were found to carry wAlbB out of 48 progeny assessed at the larval stage, indicating high rates of maternal transmission (binomial confidence intervals, 92.6–100%). Reciprocal crosses between the wAlbB.MC line and the Wolbachia-free wild-type line indicated the retention of full uni-directional CI, with no eggs hatching from crosses between wAlbB.MC males and wild-type females (table 1). No significant difference in hatch rate was observed between wAlbB.MC males and females, and wild-type males and wAlbB.MC females, indicating no loss in capacity to rescue CI.

(c) Dengue inhibition

To determine the dengue inhibition capacity of the wAlbB.MC line, females from the wAlbB.MC and wAlbB.L lines, and a Wolbachia-free wild-type strain recently established from field-caught mosquitoes from Kuala Lumpur were orally challenged with a DENV-2-spiked bloodmeal. Viral load in dissected salivary gland tissues was assessed by qRT-PCR 12 days post-challenge. Both the wAlbB.MC and wAlbB.L lines showed a large and significant reduction in the levels of virus in salivary glands compared to the wild-type control (figure 3). Although the wAlbB.MC line showed a lower mean viral load than the wAlbB.L line, the difference between the wAlbB-carrying lines was not statistically significant.

A second wAlbB-carrying field-derived line was established from mosquito larvae collected at the Shah Alam release site in Kuala Lumpur (hereon wAlbB.SA). To assess the capacity of wAlbB to block isolates of dengue virus currently circulating in Malaysia, females from the wAlbB.SA, wAlbB.L and wild-type lines were challenged with blood from DENV-1 infected patients. Individual female mosquitoes were dissected at 7 and 9 days post oral challenge, and midgut and salivary gland tissues were assessed for the abundance of viral RNA. Statistically significant reductions in the levels of DENV-1 RNA were detected in both the midgut and salivary gland tissues of both the wAlbB.SA and wAlbB.L lines relative to the Wolbachia-uninfected wild-type strain (figure 4). No significant differences in Wolbachia density were observed between the dengue challenged adults from the wAlbB.SA and wAlbB.L lines (electronic supplementary material, figure S1).

3. Discussion

wAlbB-carrying Ae. aegypti collected from a field site in Kuala Lumpur 20 months following the cessation of releases [1] did not show significant reductions in overall Wolbachia density or a diminished somatic density and tissue distribution compared to a laboratory colony [4], suggesting an absence of strong selection in the field. In fact, densities were found to be higher in the field line in whole bodies and midgut tissues for some time points. Further work will be needed to determine whether this difference is maintained with broader sampling and whether it is driven by host and/or symbiont genetic factors, or other variables such as the microbiota. Importantly, wAlbB in the field line continues to effectively inhibit the capacity for dengue virus to disseminate to and infect the salivary glands of females, thereby reducing transmission potential, with levels of viral inhibition similar to those observed in the laboratory wAlbB line and comparable to that observed in similar challenges performed in the ancestral line soon after generation [4]. Additionally, a wAlbB-carrying field-derived line strongly inhibited dissemination of dengue virus to the salivary glands when challenged with clinical isolates of DENV-1 infected blood recently collected from a hospitalized patient in Kuala Lumpur. These results indicate at least a medium-term stability of wAlbB density and dengue blocking in field populations of Ae. aegypti. The results from the clinical blood challenges are particularly encouraging given that estimates of virus blocking are often lower when patient blood is used compared to laboratory-prepared viremic bloodmeals [23,24], and that the clinical isolate was infected with DENV-1, a serotype that has
Figure 2. Wolbachia density in dissected tissues across mosquito lines and ages. Ovaries, midguts and salivary glands were dissected at days 5 and 10 post adult eclosion, and wAlbB density was determined by qPCR. Each of the five dots represents the Wolbachia density from pools of tissue from three individual female mosquitoes. No significant differences were observed in ovary density (line effect: $F_{1,16} = 0.97$, $p = 0.33$; age effect: $F_{1,16} = 0.02$, $p = 0.89$; line-by-age interaction: $F_{1,16} = 0.55$, $p = 0.47$) or salivary gland density (line effect: $F_{1,16} = 0.004$, $p = 0.95$; age effect: $F_{1,16} = 3.39$, $p = 0.08$; line-by-age interaction: $F_{1,16} = 0.002$, $p = 0.96$). A significantly higher Wolbachia density was observed in the midguts of the wAlbB.MC line at day 10 (line effect: $F_{1,16} = 26.77$, $p < 0.0001$; age effect: $F_{1,16} = 14.45$, $p = 0.0016$; line-by-age interaction: $F_{1,16} = 14.96$, $p = 0.0014$), but not at day 5 (pairwise t-test, $p = 0.6$). Boxplots show median and interquartile range. Five biological replicates were performed for each treatment.

Table 1. Hatch rates from reciprocal crosses between wAlbB.MC and wild-type Ae. aegypti. Percentages represent egg hatch rates and the total number of eggs assessed is shown in parentheses. No difference in hatch rate was observed between crosses of wAlbB.MC males and females, and wild-type males and wAlbB.MC females ($p > 0.8$, Fisher’s Exact test).

|           | male | female |
|-----------|------|--------|
| Wild type | 67%  | 63%    |
| wAlbB.MC  | 63%  | 63%    |

previously shown lower susceptibility to Wolbachia-mediated inhibition relative to other serotypes [13,24].

As high Wolbachia densities in somatic tissues are correlated with reduced fitness, selection may be expected to favour the evolution of genetic factors capable of limiting symbiont tropism to those tissues essential for maternal transmission and CI, i.e. the germline. While somatic Wolbachia do occur in many co-evolved native Wolbachia–host combinations [25], in some native associations, such as those found in Ae. albopictus [17] and Glossina morsitans [26], Wolbachia is largely restricted to the ovaries and testes of the host. Somatic tissue distribution following transfer into Ae. aegypti tends to be broad, with particularly high densities in salivary gland tissues [4]. The rate of evolution of host or Wolbachia factors that limit somatic densities is likely to depend on the fitness costs associated with the symbiosis, with higher costs resulting in stronger and more rapid selection for restricted tropism. Artificial transfer of the virulent wMelPop Wolbachia strain to Drosophila simulans, for example, resulted in high initial fitness costs through reductions in fecundity and egg hatch rates, which were partially attenuated after several generations [27]. This was accompanied by reductions in density, and suggests rapid selection for host and/or symbiont genotypes capable of suppressing Wolbachia over-replication. By contrast, however, severe fitness costs caused by wMelPop have persisted for over a decade in a transfected line of Ae. aegypti, suggesting stability of the deleterious phenotypes in this species [28].

Figure 3. DENV-2 inhibition in wAlbB-carrying Ae. aegypti lines. Females from the wAlbB.MC, wAlbB.L and wild-type lines were fed a bloodmeal spiked with DENV-2. After an incubation period of 12 days, salivary glands were dissected and the viral load was quantified by RT-qPCR. Ten, 20 and 27 females were analysed for the wild-type, wAlbB.MC and wAlbB.L lines, respectively. Black dots indicate salivary glands from individual mosquitoes. Red lines indicate median values. wAlbB.MC and wAlbB.L showed a significant reduction in viral titres ($p < 0.004$ for both comparisons, one-way ANOVA with Dunnett’s). There was no difference in viral load between the wAlbB.MC and wAlbB.L lines ($p = 0.88$, one-way ANOVA with Dunnett’s).
populations is uncertain given the average lifespan of wild mosquitoes is expected to be much shorter [29]. Furthermore, selection for reduced wMelPop densities in D. simulans may be particularly rapid as it is a native Wolbachia host; existing host factors capable of suppressing native strains may be active against novel associations, although these factors may require some adaptive optimization against divergent Wolbachia strains. When wAlbB was transferred into Culex quinquefasciatus, for example, it showed a limited somatic tissue distribution similar to the native wPip strain—with which it is closely related—while the more distantly related wAlbA strain which was also transferred reached high somatic densities [30]. The low fitness costs associated with wAlbB and the absence of native Wolbachia in Ae. aegypti suggests that restrictive host-factor evolution with this symbiont/host combination may be slow, perhaps requiring much longer evolutionary timescales than are relevant in the context of dengue control programmes.

The wAlbB strain was successfully established in several sites in urban Kuala Lumpur in 2018, and has maintained high frequencies since [1]. However, the persistence of wAlbB in wild Ae. aegypti populations is contingent on the maintenance of high rates of maternal transmission and CI given that this strain has some fitness costs [31]. The wAlbB maternal transmission rate was complete in the field line, and ovary densities were comparable to those found in the laboratory colony. Maintenance of high ovary density is consistent with evolutionary expectations, as infected females with reduced ovary densities and imperfect maternal transmission would suffer fitness costs in areas of high Wolbachia frequency resulting from mating incompatibility with Wolbachia-carrying males.

The wAlbB field line also displayed maintenance of full CI induction and rescue capacity. Evolutionary models predict that the stability of the CI phenotype may be compromised over time by the evolution and spread of host CI repressors [20,32,33]. Modelling suggests that CI repressors will arise disproportionately in males [20,33], driven by the fitness benefits of Wolbachia-carrying male compatibility with Wolbachia-free females. The existence of host CI repressors is supported by the lack of strong CI in some native Wolbachia/host associations, where the native host becomes fully susceptible to CI upon artificial transfer of a new symbiont strain, or conversely when the native strain induces stronger CI in a non-native host, as observed in several studies in Drosophila species [34–36]. Evolutionary models also predict that the symbiont genes underlying the CI phenotype will tend to lose function over time due to a lack of selection on CI levels [21,32]. Consistent with this, the recently identified CI genes often carry loss-of-function mutations [37–40]. However, the persistence of strong CI in many native associations (including in the mosquito species Ae. albopictus and C. quinquefasciatus), strongly suggests that loss of CI tends to occur over much longer evolutionary timescales, and would, therefore, be highly unlikely to impact on a vector control intervention.

4. Conclusion

There is more variability in Wolbachia density in wild mosquitoes than in laboratory colonies [1], associated with environmental variability in larval conditions including, for example, exposure to environmental antibiotics that can reduce Wolbachia density [41]. Thus, perfect maternal transmission and CI may not be maintained in wild mosquitoes; direct monitoring of field populations for phenotypic stability will be an ongoing component of the Wolbachia intervention.
The data presented here indicate, however, that wAlbB tested in the laboratory has not been attenuated in its invasion, population maintenance and dengue inhibition capacities following its presence for 20 months in a field Ae. aegypti population. These data support the sustainability of interventions using wAlbB to control dengue transmission. Long-term monitoring across diverse release sites will be required to fully evaluate the potential for Wolbachia to reduce the global burden of dengue.

5. Methods

(a) Mosquito strains and rearing

Two Wolbachia wAlbB-carrying Ae. aegypti lines were used in this study. The first is the original wAlbB-carrying line, wAlbB.L (Lab), generated as previously described [4] and maintained in controlled laboratory settings for approximately 5 years. A second line, named wAlbB.MC, was derived from field-collected eggs from a release area (Mentari Court, 3°04'N 101°36'E) in Kuala Lumpur, Malaysia, in December 2019. As previously described [1], releases of Wolbachia-carrying mosquitoes for vector control started in this area in October 2017 and ceased in March 2018. Ovitraps (plastic containers, 96 mm height, 67 mm diameter) with 150 ml water and a wooden ovipositor, were placed in apartment buildings in Mentari Court for a week. Eggs from several ovitraps were hatched in the laboratories of the Institute of Medical Research in Kuala Lumpur (KL); the obtained adults (F0) were morphologically identified and Ae. aegypti were reared in laboratory settings as previously described [1]. Progeny were sent as eggs to the Centre for Virus Research in Glasgow, UK. Mosquitoes used in this study ranged from F2 and F4. The wild-type Ae. aegypti were colonized from Wolbachia-negative mosquitoes collected in control areas of KL in February 2018 and maintained in the insectary of the Centre for Virus Research in Glasgow, UK, for more than 15 generations. Wolbachia status was confirmed in wild-type and wAlbB-carrying mosquitoes by PCR. Mosquito lines were maintained at standard insectary conditions: 27°C and 70% relative humidity with a 12-h light/dark cycle. Larvae were fed with tropical fish pellets (Tetramin, Tetra, Melle, Germany) and adults maintained with 5% sucrose solution ad libitum. Blood meals were provided by an artificial blood-feeding system (Hemotek, UK) using human blood (Scottish National Blood Transfusion Service, UK). Eggs were collected on a wet filter paper (Grade 1 filter paper, Whatman plc, GE Healthcare, UK), desiccated for 5 days and hatched in deionized water containing 1 g l⁻¹ bovine liver powder (MP Biomedicals, Santa Ana, California, USA).

(b) Wolbachia density in the wAlbB.MC and wAlbB.L lines

The wAlbB frequency in the wAlbB.MC line was found to be 100% in 48 screened individuals from the F2 generation and was measured by quantitative PCR (qPCR) as described below. Wolbachia density between wAlbB-carrying strains was compared 5 and 10 days PE. Whole-body gDNA was extracted from 24 females from the F3 using STE buffer (10 µM Tris HCL pH 8, 100 mm NaCl, 1 mm EDTA) with a 95°C denaturation for 10 min. Additionally, ovaries, salivary glands and midguts (five pools of three organs per replicate) were also dissected from 5-day- and 10-day-old F1 females using sterile forceps and needles in a drop of sterile PBS buffer, and immediately transferred in STE buffer for DNA extraction. qPCR analysis was performed using the relative quantification of the Wolbachia 16S ribosomal gene (16S_qPCR_F: GAAAGCCTGATCCAGCAAG/16S_qPCR_R: CGAGGTATGAC-CAGACTTCT), against the homothorax gene (HTH) as the reference gene [40]. 2 x SYBR-Green mastermix (Biotool, Houston, Texas, USA) with a BioRad CFX-96 real-time PCR detection system (Bio Rad, Hercules, California, USA) were used for the amplification reaction. The reaction was 95 °C for 5 min, 40× cycles of 95°C for 15 s and 60°C for 30 s, followed by a melt-curve analysis.

(c) Maternal transmission and cytoplasmic incompatibility

Rates of CI were assessed by performing reciprocal crosses between the wild-type and wAlbB.MC lines using 25 virgin females and males in each cross. Mosquitoes were allowed to mate for 3-5 days before blood-feeding. Eggs were desiccated for 5 days at standard insectary conditions (27°C and 70% relative humidity), counted and hatched in water containing 1 g l⁻¹ bovine liver powder. Larvae were counted at the L2-L3 stage, and the hatch rate percentage was measured. To assess whether the recaptured line conserved the complete maternal transmission demonstrated in the original line [4], 48 individual progeny resulting from the cross between wAlbB.MC females and wild-type males were tested at the larval stage for Wolbachia using qPCR as described above.

(d) Dengue challenges with blood spiked with DENV-2

Susceptibility to DENV-2 dengue virus was assessed using the New Guinea C strain (Public Health England). The virus was propagated in Ae. albopictus C6/36 cells, the supernatant was harvested, concentrated using Amicon Ultra-15 filters (Millipore, IRL) and titrated with fluorescent focus assay (FFA). The primary antibody for DENV was MAB8705 anti-dengue virus complex antibody (Millipore); the secondary antibody was the Goat anti-mouse Alexa Fluor 488, A-11001 (Thermo Scientific, Waltham, Massachusetts, USA). Fluorescent foci were counted by eye (from dilutions with less than 100 foci) and virus titres calculated and expressed as FFU ml⁻¹.

Seven-day-old females were offered an infectious bloodmeal consisting of human blood and virus suspension (1.4 x 10⁵ FFU ml⁻¹). Engorged females were selected, transferred in containers in a climatic chamber and maintained at 27°C, 70% relative humidity, 12-h light/dark cycle with 5% sugar solution. After 12 days, salivary glands were dissected in sterile PBS and scored for DENV infection using RT-qPCR. RNA was extracted with Trizol (Thermo Fisher) and diluted to 100 ng µl⁻¹. cDNA was synthesized with High Capacity cDNA reverse transcription kit (Thermo Fisher). Virus copies were quantified using primers amplifying the viral gene NS5 (NS5-F: ACAAGTGCAACACCTGCTCATCC/NS5-R: GCCCGAACATTGCTTCTCTC), and Ct values normalized to the Rp517 mosquito gene (Rp517 F: CAATTTTATTTCATTGTGGTGAT/ Rp517 R: CACTTCCAGGTCGCTTGAT).

(e) Collection of dengue patient blood

Blood from a dengue patient was obtained from the General Hospital Kuala Lumpur, Malaysia. Arrangements were made with the medical officer (MO) in charge of the dengue ward prior to collecting blood. The blood was collected on the same day as feeding. The MO in charge recruited the best candidate based on the pre-set inclusion and exclusion criteria (for information sheet and consent form, see electronic supplementary material, file 1). The patient was initially briefed about the project and the reason for drawing the blood. The patient had complete freedom to give or withdraw consent for the use of their blood. Upon receiving consent, 5 ml of blood was withdrawn and placed in EDTA tubes. Blood was kept on ice at all times and used in feeding experiments on the same day.

(f) Dengue challenges with patient blood infected with DENV-1

The experimental oral infection was conducted within an arthropod containment level 2 (ACL-2) insectarium. A mouse skin
membrane was used in conjunction with a Hemotek Feeding System (Accrington, UK) housed in an isolation glove box. Of dengue patient blood, 1.5 ml was introduced into each feeder. A total of 150 adult female mosquitoes from each group were used in the feeding experiment. Mosquitoes were 3–5 days old and were starved (of sugar solution) overnight. Mosquitoes were allowed to feed for approximately 30 min. The mosquitoes were placed at −20°C for 30 s to allow sorting. Unfed mosquitoes were discarded. Engorged mosquitoes were maintained at 27°C with a relative humidity of 80% (±10%). At each time-point (days 7 and 9), a subset of the living mosquitoes was dissected, with the isolation of midguts and salivary glands. The remaining carcasses were stored. Individual organs and carcasses were kept in tubes containing 100 µl MEM media. Samples were stored at −20°C prior to nucleic acid extraction.

(g) Nucleic acid extraction and quantitative PCR from patient blood and mosquitoes challenged with DENV-1

Total nucleic acids were extracted from dissected organs using an innuPREP DNA/RNA Mini Kit (Analytik Jena AG, Jena, Germany), enabling the isolation of both RNA and DNA for quantification of DENV and Wolbachia, respectively. The DENV strain from patient blood was initially confirmed using real-time reverse transcription PCR (RT-qPCR) with DENV 1–4 primer sets [42]. For each run, multiplex reactions were prepared by combining primer/probes for DENV 1 + 3 or DENV 2 + 4 in a single run. The PCR mixture contained 2 µl of extracted RNA, 5 µl of SensiFAST Probe No-ROX One Step Mix (Bioline, Taunton, MA), 0.4 µl of respective primer combinations (DENV 1 + 3 and/or DENV 2 + 4) (400 nM), 0.1 µl of the respective probe (400 nM), 0.1 µl reverse transcriptase, 0.2 µl Ribosafe RNase inhibitor and 1.8 µl of nuclease-free water. A RT-PCR was performed using the following programme: 45°C for 10 min, initial denaturation at 95°C for 2 min, followed by 40 cycles with 95°C for 5 s, and 60°C for 10 s. A DENV-1 specific primer set (DENV-1-F: ATCCAGTCCTCAATGATTCGA/rDENV-1-R: CAGGGTGTATGTCCAGTGGCCTTA/wAlbB1-Probe Cy5-TGTTGATCACTTG/GCTGTTAGCCCT-IowaBlackFQ) and Wolbachia-specific primer sets (wAlbB1-F CCTTACCTCCTGGCAACAA/wAlbB1-R GGATTGTCAGTGCCCTTA/wAlbB1-Probe Cy5-TGGTGACTCTGTGCGTCTAACCT-IowaBlackFQ) were used and Ct values were normalized to the RpS17 mosquito gene (RpS17-F: CACTCCCA GTTCCGTGGTAT/RpS17-R: GGACACTTCCGGCAGTAGT/GpS17-Probe: FAM-AGGAGGAAC GTGAGCGCAGAAGCA-IowaBlackFQ).

(h) Wolbachia density assessment in DENV-1 infected females

Wolbachia density was measured in the extracted DNA from the mosquito carcasses following the dissection of salivary glands and midguts by multiplex qPCR. A mastermix consisting of 2 µl of extracted DNA, 5 µl of PrimeTime Gene Expression Master Mix 2x, 0.375 µl of individual primer sets and 2 µl of nuclease-free water was used. Primers were Ae. aegypti-specific (aRpS6-F ATCAAGAACGGCGGTTCG/aRpS6-R CAGGTG-CAGGATCTCTCATGATTCGA/aRpS6-Probe HEX-AGTCCGGCAAGGAAGCCGAA-IowaBlackFQ) and Wolbachia-specific (wAlbB-F GCTGTTAGCCCTCAACAA/wAlbB-R GGATTGTCAGTGCCCTTGA/wAlbB-Probe Cy5-TGGTGACTCTGTGCGTCTAACCT-IowaBlackFQ). PCRs were performed on a LightCycler 480II system (Roche, Germany).

(i) Data analysis

All statistical analyses were conducted in the R software v. 3.2.3. Viral titre and Wolbachia density data were analysed with linear models after log10 transformation to meet the assumptions of normality and homoscedasticity. Post hoc pairwise comparisons were performed with the function glht using a correction for multiple testing (R package multcomp [43]).

Data accessibility. All raw data have been deposited in the University of Glasgow data repository, link http://dx.doi.org/10.5525/gla.researchdata.1044.

Authors’ contributions. N.A.A., J.M., M.V.M.-T. and T.H.A. carried out the insect rearing, the molecular laboratory work and the data analysis; G.M.R.K. and W.A.N. established the field colony; J.M., M.V.-M., T.H.A., A.A.H. and S.P.S. wrote the manuscript. All authors gave final approval for publication.

Competing interests. We declare we have no competing interests.

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