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Selection on *Aedes aegypti* alters *Wolbachia*-mediated dengue virus blocking and fitness

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The dengue, Zika and chikungunya viruses are transmitted by the mosquito *Aedes aegypti* and pose a substantial threat to global public health. Current vaccines and mosquito control strategies have limited efficacy, so novel interventions are needed[1,2]. *Wolbachia* are bacteria that inhabit insect cells and have been found to reduce viral infection—a phenotype that is referred to as viral 'blocking'[3]. Although not naturally found in *A. aegypti*[4], *Wolbachia* were stably introduced into this mosquito in 2011[4,5] and were shown to reduce the transmission potential of dengue, Zika and chikungunya[6,7]. Subsequent field trials showed *Wolbachia's* ability to spread through *A. aegypti* populations and reduce the local incidence of dengue fever[8]. Despite these successes, the evolutionary stability of viral blocking is unknown. Here, we utilized artificial selection to reveal genetic variation in the mosquito that affects *Wolbachia*-mediated dengue blocking. We found that mosquitoes exhibiting weaker blocking also have reduced fitness, suggesting the potential for natural selection to maintain blocking. We also identified *A. aegypti* genes that affect blocking strength, shedding light on a possible mechanism for the trait. These results will inform the use of *Wolbachia* as biocontrol agents against mosquito-borne viruses and direct further research into measuring and improving their efficacy.

The long-term efficacy of *Wolbachia* strains as biocontrol agents against mosquito-borne viruses, including dengue, Zika and chikungunya, will depend on the ongoing stability of their anti-viral properties[9]. Since *Wolbachia* strains were recently introduced into *Aedes aegypti* for biocontrol purposes, interactions between these two species are likely to undergo a period of co-adaptation—a trend commonly observed when microorganisms infect new hosts in nature[10]. Specifically, there is a concern that viral blocking may evolve to be less effective in *A. aegypti* over time, as it can be weaker in natively infected hosts[11–14].

Predicting the long-term stability of *Wolbachia*-mediated blocking is challenging given several key knowledge gaps. First, for natural selection to occur, there must be genetic variation in blocking, the level of which is unknown. Second, while there is evidence of many contributing factors to blocking, including resource competition and host immune modulation[14–18], the mechanism remains largely elusive. There is also conflicting evidence for whether *Wolbachia* density predicts blocking strength[9,12,13,19]. Third, the relationship between host fitness and the strength of *Wolbachia*-mediated viral blocking is unknown. Comparisons suggest that *Wolbachia* strains with stronger blocking confer higher fitness costs[13], so selection may favour reduced blocking[9]. Here, we investigate each of these knowledge gaps by selecting for high and low dengue blocking in *A. aegypti* (Fig. 1), performing whole-genome resequencing and genome-wide association studies, and measuring the impacts on mosquito fitness. We also included a control treatment where mosquitoes were selected randomly. Each selection regime was repeated on three independent populations.

In total, four rounds of selection were completed on all nine populations. On average, 200 offspring were taken from six selected mosquitoes per population per generation. This was done to impose the strongest selection pressure possible while ensuring that enough mosquitoes were reared for selection in the subsequent generation. We observed a significant divergence in viral load between selection treatments (Fig. 2a; mixed-effects model: treatment: $\chi^2 = 9.8$; degrees of freedom (d.f.) = 5; $P = 0.0073$). The viral load was significantly higher in populations selected for low blocking than those selected for high blocking and those randomly passaged (post-hoc Tukey's test: low versus high: $P = 0.0017$; low versus random: $P = 0.0005$). There was no difference between the random and high-blocking populations (post-hoc Tukey's test: random versus high: $P = 0.94$), both of which were also similar to the ancestral population (Fig. 2a). We found that the density of *Wolbachia* per mosquito body did not explain this variation in viral load (*Wolbachia* density: $\chi^2 = 1.36$; d.f. = 6; $P = 0.24$), nor was there an interaction between *Wolbachia* density and selection treatment (treatment × *Wolbachia* density: $\chi^2 = 0.25$; d.f. = 8; $P = 0.88$). Thus, *Wolbachia* density did not underpin the variation for blocking in our evolved populations.

To confirm that the response to selection was *Wolbachia* mediated, we compared the viral load of each evolved population in the presence and absence of *Wolbachia*. *Wolbachia*$^+$ populations showed the same pattern as they did immediately after selection (Fig. 2b; *Wolbachia*$^+$ mixed-effects model: treatment: $\chi^2 = 13$; d.f. = 5; false discovery rate (FDR)-corrected for multiple comparisons $P = 0.003$; post-hoc Tukey's test: high versus random: $P = 0.95$; random versus low: $P < 1 \times 10^{-5}$; high versus low: $P = 2.14 \times 10^{-5}$). In subpopulations where *Wolbachia* were removed, these differences disappeared (Fig. 2b; *Wolbachia*$^-$ mixed-effects model: treatment: $\chi^2 = 1.01$; d.f. = 5; FDR-corrected for multiple comparisons $P = 0.603$), confirming that the divergence between treatments was *Wolbachia* mediated. These data also show that the *Wolbachia*-mediated blocking in the low-blocking populations was on average 55% of that of the randomly passaged populations (a 45% loss in blocking ability compared with the control). In contrast, the reduction in viral load

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due to *Wolbachia* in the high-blocking populations was on average 99% of that of the randomly passaged populations.

These data reveal variation in the strength of *Wolbachia*-mediated virus blocking, and that the ancestral, high- and random-blocking populations exhibited similar levels of *Wolbachia*-mediated blocking. One potential explanation is that high-blocking genotypes are naturally selected for, existing at such high frequencies in the ancestral and random populations that they cannot be substantially increased by artificial selection[20]. Consistent with this hypothesis, the average realized heritability for the low-blocking populations was 0.35 ± 0.15, while it was 0.11 ± 0.23 for the high-blocking populations (Supplementary File 1). This explanation suggests that high-blocking genotypes have a fitness advantage over low-blocking genotypes.

To test whether high-blocking genotypes have an inherent fitness advantage, we estimated mosquito fitness in the absence of dengue infection as the intrinsic rate of natural increase (r). We did this using a discrete-time approximation to a population-scale model populated by life-history data collected in triplicate for each evolved population[21]. This included the median pupation time, adult sex ratio, female adult daily survival, and size and timing of egg clutches over three blood meals. We hatched populations in three experimental batches for logistical purposes, with each treatment equally represented in each batch, to be controlled for statistically. We ran the model assuming 92% survival from larvae to adulthood[22].

We found a significant negative relationship between the average viral load per infected mosquito and the average fitness (r) of uninfected mosquitoes taken from the same population (Fig. 3; mixed-effects regression: $\log_{10}$[copies of dengue virus per mosquito]: $\chi^2 = 9.04$; d.f. = 5; $P = 0.0026$). This relationship held across variation in fitness due to experimental batch. This indicates that high-blocking genotypes may have an inherent fitness advantage over low-blocking genotypes, supporting our hypothesis that high blocking is selected for.

To investigate whether the divergence in blocking was determined by genetic changes in *A. aegypti* and/or *Wolbachia*, we sequenced pools of 90 individuals from the ancestral and evolved populations, and compared the treatments for significantly differentiated single-nucleotide polymorphisms (SNPs) with a generalized linear model. To assess genome-wide significance, we estimated empirical significance thresholds based on exhaustive permutation of our experimental data. We found no SNPs in the *Wolbachia* genome that were significantly differentiated between the selection treatments (Supplementary Fig. 1). By comparing *A. aegypti* genomes from low- and high-blocking populations, we found a highly significant peak of differentiated SNPs on chromosome 1 (Fig. 4a). The most significant SNPs were found within the introns of two genes: *AAEL004389* (transcribing an α-mannosidase 2a enzyme) and *AAEL023845* (transcribing a cadherin protein) (Supplementary File 21). When comparing the low-blocking populations with the

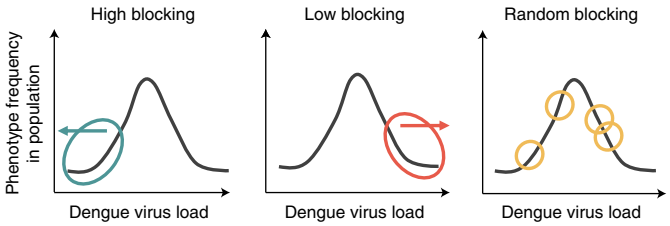

Fig. 1 | Illustration of the selection treatments. We selected for low and high values of *Wolbachia*-mediated dengue blocking alongside a control treatment where mosquitoes were selected at random. Each treatment consisted of three independent replicate populations randomly generated from the same ancestral population of *Wolbachia*-infected *A. aegypti* (*w*Mel.F strain) that represented the genetic diversity in Queensland, Australia. Circles represent the population of females whose eggs were selected based on their infection load to seed the subsequent generation of breeding. Arrows indicate the direction of selection. Blue represents selection for high blocking, red for low blocking; and yellow represents random selection.

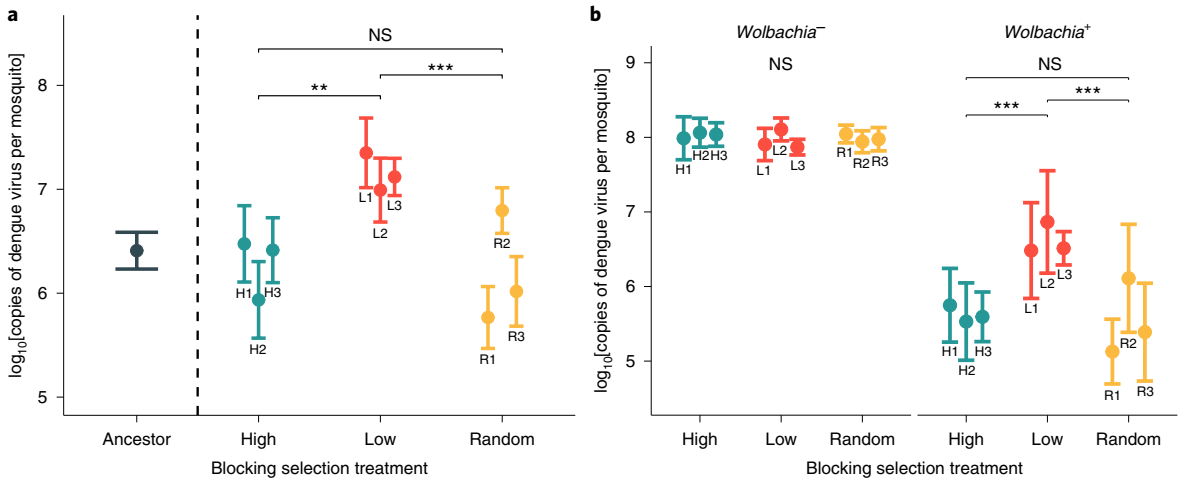

Fig. 2 | Evolution of *Wolbachia*-mediated blocking of the dengue virus in *A. aegypti*. All mosquitoes were injected with the same dose of virus. **a**, $\log_{10}$[copies of dengue virus per mosquito] in the high-, low- and random-blocking treatments after four rounds of selection (mixed-effects model: treatment: $\chi^2 = 9.8$; d.f. = 5; $P = 0.0073$; post-hoc Tukey's test: low versus high: $P = 0.0017$; low versus random: $P = 0.0005$; random versus high: not significant (NS; $P = 0.94$); *Wolbachia* density: $\chi^2 = 1.36$; d.f. = 6; $P = 0.24$; treatment × *Wolbachia* density: $\chi^2 = 0.25$; d.f. = 8; $P = 0.88$). **b**, $\log_{10}$[copies of dengue virus per mosquito] in the evolved populations either treated with the antibiotic tetracycline to remove *Wolbachia* (*Wolbachia*$^-$) or not (*Wolbachia*$^+$). These mosquitoes were all tested four generations after the selection experiment (*Wolbachia*$^-$ mixed-effects model: treatment: $\chi^2 = 1.01$; d.f. = 5; $P = 0.603$; FDR-corrected for multiple comparisons $P = 0.603$; *Wolbachia*$^+$ mixed-effects model: treatment: $\chi^2 = 13$; d.f. = 5; $P = 0.0015$; FDR-corrected for multiple comparisons $P = 0.003$; post-hoc Tukey's test: high versus random: $P = 0.95$; random versus low: $P < 1 \times 10^{-5}$; high versus low: $P = 2.14 \times 10^{-5}$). Sample sizes and further statistical information are listed in Supplementary Table 1. Replicate population IDs are noted under each error bar. Error bars represent 2 s.e. $^*P < 0.01$; $^{**}P < 0.001$; $^{***}P < 0.0001$.

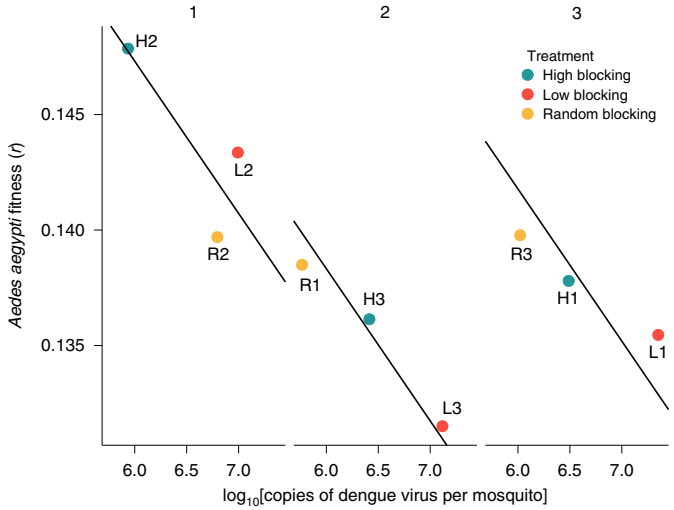

Fig. 3 | Relationship between the fitness of *Wolbachia*-infected mosquitoes and their ability to block dengue virus across the evolved mosquito populations. The intrinsic rate of natural increase (r) for each *A. aegypti* population was measured in the absence of dengue infection. This was calculated from an age-structured Leslie matrix model that combined different fitness measures that were collected empirically. Life-history data were collected in triplicate for each replicate population, and populations were analysed in batches (1–3) for logistical purposes. Replicate populations were assigned to batches randomly (mixed-effects regression: $\log_{10}$[copies of dengue virus per mosquito]: $\chi^2 = 9.04$; d.f. = 5; $P = 0.0026$). Experimental batch and treatment were included as random factors to control for non-independence. Data are presented by batch, which was the largest random source of variation. The colour of each point represents the selection treatment. Replicate population IDs are noted next to each point. Black lines fitted across treatments within each batch show the relationship between fitness and dengue virus load. Sample sizes and further statistical information are listed in Supplementary Table 1.

randomly passaged populations (Supplementary Fig. 2a), many of the SNPs within the cadherin gene were also significant; however, none of those within the α-mannosidase 2a gene were identified (Supplementary File 31). This suggests that cadherin gene may be more important for blocking across all populations.

The intronic location of the SNPs within the cadherin and α-mannosidase 2a genes suggest that they could have an effect on gene expression or splicing. We measured the expression of cadherin and α-mannosidase 2a in the mosquitoes that had been sequenced. We found that the expression of cadherin was significantly lower in mosquitoes with low *Wolbachia*-mediated dengue blocking compared with those with high blocking, including populations from both the high and random selection treatments (Fig. 4b; mixed-effects model: treatment: d.f. = 5; $\chi^2 = 19.07$; $P = 7.228 \times 10^{-5}$; post-hoc Tukey's test: low versus high: $P < 0.001$; low versus random: $P < 0.001$). Gene expression was measured relative to the expression of the housekeeping gene transcribing ribosomal protein S17 (*AAEL004175*, referred to here as *RPS17*). Gene expression was also slightly higher in the high- than the random-blocking populations (random versus high: $P = 0.042$). In contrast, we found no significant difference in α-mannosidase 2a gene expression between these populations (Fig. 4c; mixed-effects model: treatment: d.f. = 5; $\chi^2 = 2.49$; $P = 0.29$). This was consistent with our finding that the SNPs in α-mannosidase 2a were not significantly differentiated between the random and low populations, and so are not necessary for the difference in blocking strength. Therefore, these phenotypic data corroborate our genotypic data.

We investigated the allele frequencies of the most differentiated SNP within cadherin (position 238,777,216 on chromosome 1; Fig. 4d), and found that thymine was at a high frequency in the high-blocking populations, but also in the ancestral and random-blocking populations. This supports our hypothesis that genotypes associated with high blocking are maintained at a high frequency due to natural selection. Its fixation in almost all high- and random-blocking populations may explain why blocking could not be increased in the high populations relative to the random populations. The *A. aegypti* reference genome (Liverpool AGWG-AaegL5) also has a thymine at this position.

Cadherins are glycoproteins involved in cell–cell adhesion, cytoskeleton organization and the transduction of intracellular signals[23]. They are not members of classical innate immune pathways (Toll, Imd, RNA interference or the JAK/STAT pathway); however, dengue virus has been found to bind cadherins and could use them to either enter/exit or move within cells[24]. Functional annotations for cadherin from OrthoDB include secondary metabolite biosynthesis, transport and catabolism, and intracellular trafficking, secretion and vesicular transport. Flaviviruses rely heavily on the host cytoskeleton for cell entry and exit, movement through the cell, and remodelling of the endoplasmic reticulum as sites of replication[25]. At the same time, *Wolbachia* are known to manipulate the host cytoskeleton to ensure their localization, and thus transmission during mitosis and meiosis[25,26]. Moreover, *Wolbachia* have previously been shown to alter the expression of genes involved in cell–cell adhesion[27]. It is therefore possible that cadherin could affect how *Wolbachia* manipulates the host cytoskeleton, and thus the ability of viruses to enter and exit cells, move through cells, replicate, and recruit resources to the site of replication. Consistent with this, we identified that many of the less significant SNPs occurred within genes that were also related to the cytoskeleton, cell–cell adhesion and signal transduction (Supplementary File 2). The variation in these genes could also contribute to the variation that we see in blocking ability.

Together, our results have significant implications for the use of *Wolbachia* as a biocontrol agent in *A. aegypti* mosquitoes. Published data on blocking stability following release trials show that blocking is so far maintained at levels similar to the original populations[28]. Our results suggest that this outcome is probably due to the maintenance of blocking by natural selection, rather than a lack of genetic variation. It will therefore be important to investigate how this genetic variation will differ across mosquito populations around the globe, and whether the relationship with fitness seen here is generalizable. Our results also identify candidate *A. aegypti* genes that may help elucidate the mechanism of *Wolbachia*-mediated virus blocking and lead to methods to evaluate and improve its efficacy.

Further investigation into these genes will be needed to understand their functional roles against different serotypes of the dengue virus, against other viruses (such as Zika and chikungunya) and within other *Wolbachia* and *A. aegypti* genetic backgrounds, as well as their ecological relevance (including their impact on the rate of pathogen transmission (R_0)[29]). Moreover, the data within this paper were collected at one time point during infection; it will be important to see how variation in this gene impacts infection over a wider time frame. We will be utilizing CRISPR gene editing tools to investigate these questions.

Methods

Ethics. Experimental work was carried out at Monash University in Melbourne. The Monash University Human Research Ethics Committee gave ethical approval for human volunteers to provide blood meals to mosquitoes not infected with dengue virus (permit: CF11/0766-2011000387). One volunteer was involved throughout the study and provided written consent.

Mosquitoes. We used a population of *A. aegypti* that were infected with the *w*Mel.F strain of *Wolbachia*[5,30] and had been maintained in the laboratory for

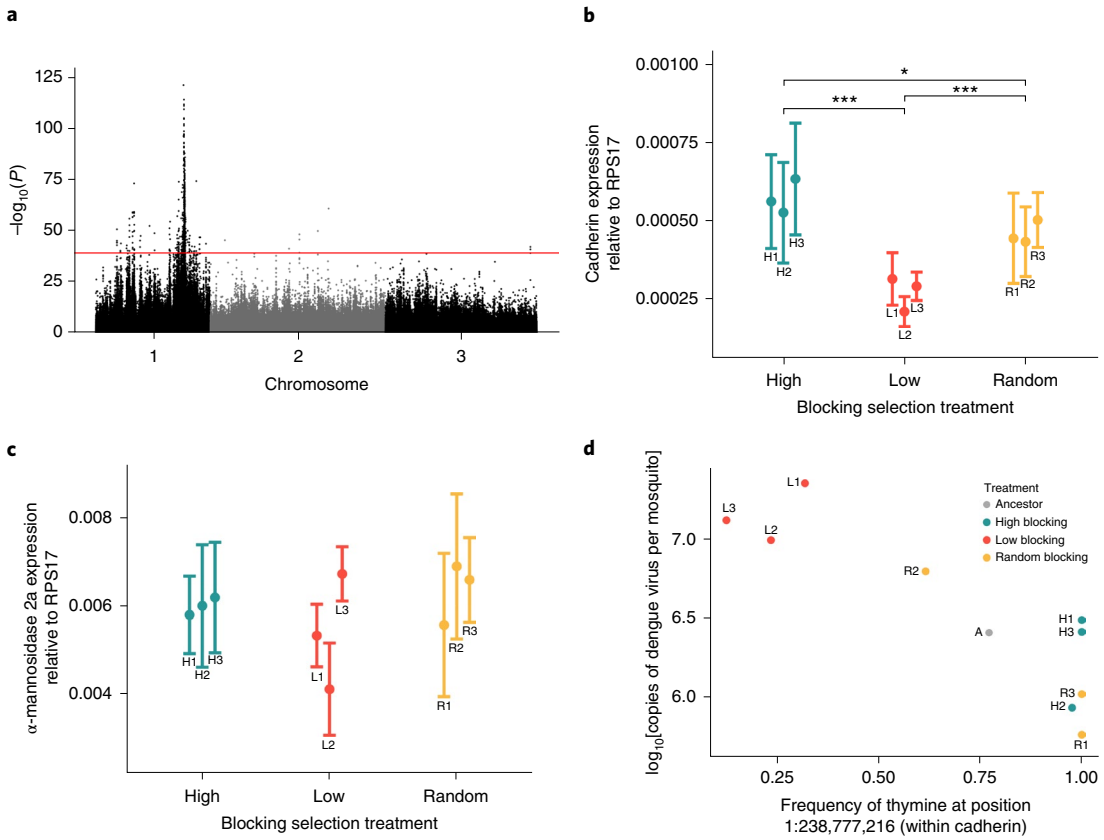

Fig. 4 | Genetic variation in *A. aegypti* associated with *Wolbachia*-mediated dengue virus blocking. a, Manhattan plot showing SNPs in the *A. aegypti* genome that were differentiated between the high- and low-blocking populations. The SNPs were analysed with a generalized linear model, and genome-wide significance was estimated using empirical significance thresholds based on exhaustive permutation of our experimental data. The red line is the 5% FDR threshold based on all possible permutations (39.3). **b**, Expression of cadherin relative to the housekeeping gene *RPS17* in the body (mixed-effects model: treatment: d.f. = 5; $\chi^2 = 19.07$; $P = 7.228 \times 10^{-5}$; post-hoc Tukey's test: low versus high: $P < 0.001$; low versus random: $P < 0.001$; random versus high: $P = 0.042$). **c**, Expression of α-mannosidase 2a relative to *RPS17* in the body (mixed-effects model: treatment: d.f. = 5; $\chi^2 = 2.49$; $P = 0.29$). **d**, Frequencies of thymine at position 238,777,216 on *A. aegypti* chromosome 1 plotted against the average dengue load for each population. This is the most significant SNP from the high versus low comparison within the cadherin gene. Replicate population IDs are noted under each error bar. Sample sizes and further statistical information are listed in Supplementary Table 1. Error bars represent 2 s.e. $^*P < 0.01$; $^{**}P < 0.001$; $^{***}P < 0.0001$.

33 generations. Every three generations, they were outcrossed with *Wolbachia*-free mosquitoes collected from Queensland, Australia, to maintain standing genetic variation[9,30]. Mosquitoes collected for outcrossing were replaced every six generations. During outcrossing, ~30% of males from the laboratory population were replaced with males from the collected population.

Dengue virus. We used dengue virus serotype 3, isolated from Cairns[31,32]. Virus was grown within C6/36 *Aedes albopictus* cells following standard methods[9]. Cells were grown to 80% confluency at 26 °C in T175 tissue culture flasks containing 25 ml RPMI 1640 media (Life Technologies) supplemented with 10% foetal bovine serum (Life Technologies), 2% HEPES (Sigma–Aldrich) and 1% Glutamax (Life Technologies). The media was then replaced with 25 ml RPMI supplemented with 2% foetal bovine serum, 2% HEPES and 1% Glutamax, and 20 µl virus was added. After 7 d, cells were scraped off and the suspension was centrifuged at 3,200*g* for 15 min at 4 °C. The supernatant was frozen in single-use aliquots at −80 °C, and all experiments were conducted using these aliquots. Virus titres were measured from a thawed aliquot by: (1) mixing 20 µl with 200 µl of TRIzol (Invitrogen); (2) extracting the RNA following the manufacturer's protocol and treating with DNAse I (Sigma–Aldrich); and (3) quantifying dengue virus RNA using quantitative reverse-transcription PCR (RT-qPCR) (see 'Dengue virus quantification'). Three independent extractions were performed and two replicates of each extraction were measured to generate an average value of 1.80×10^6 genomic copies of the dengue virus per ml.

Dengue virus quantification. Dengue virus was quantified via RT-qPCR using the LightCycler 480 (Roche). We used the TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher Scientific) in a total reaction volume of 10 µl, following the manufacturer's instructions[9]. The list of primers and probes is given in Supplementary Table 2. The temperature profile used was: 50 °C for 10 min; 95 °C for 20 s; 35 cycles of 95 °C for 3 s; 60 °C for 30 s; 72 °C for 1 s; and 40 °C for 10 s. Data were analysed using absolute quantification where the dengue virus copy number per sample was calculated from a reference curve. This reference curve was made up from known quantities of the genomic region of the virus that the primers amplify. This genomic region had previously been cloned into the pGEM-T plasmid (Promega) and transformed into *Escherichia coli*[33]. After growing *E. coli* in liquid Luria broth (LB) overnight at 37 °C, we extracted the plasmid using the PureYield Plasmid Midiprep System kit (Promega) and linearized it by restriction digest. We then purified the plasmid using phenol-chloroform extraction, resuspended in 20 µl of UltraPure distilled water (Invitrogen) and quantified it by Qubit. A dilution series of 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 copies of the genomic fragment was created and frozen as single-use aliquots. All assays measuring viral load used these aliquots, and three replicates of the dilution series were run on every 96-well plate to create a reference curve for dengue virus quantification.

***Wolbachia* quantification.** *Wolbachia* density was quantified as the number of *Wolbachia* genome copies (by amplifying the gene *WD0513*) relative to the number of mosquito genome copies (by amplifying the gene *RPS17*) via multiplex qPCR on the LightCycler 480 (ref. [34]), using the equation $\frac{2^{RPS17}}{2^{TM513}}$ (ref. [9]). We used the 2× LightCycler 480 Probes Master mix (Roche) in a total reaction volume of 10 µl. The list of primers and probes is given in Supplementary Table 2. The temperature profile of the qPCR was: 95 °C for 5 min; 35 cycles of 95 °C for 10 s; 60 °C for 15 s; 72 °C for 1 s; and 40 °C for 10 s. Basic relative quantification was used with mosquito genome copies as the reference, and *Wolbachia* genome copies were used as the target.

Selection experiment. We selected for low and high *Wolbachia*-mediated dengue blocking alongside a control treatment where mosquitoes were selected at random (Fig. 1). Each treatment included three independent populations generated from an ancestral population of mosquitoes using a random number generator[35]. For

each generation, eggs were hatched in trays (30 cm × 40 cm × 8 cm) containing 2 l of autoclaved reverse osmosis water to achieve 150–200 larvae per tray. Larvae were fed ground TetraMin tablets and reared under controlled conditions of temperature (26 ± 2 °C), relative humidity (~70%) and photoperiod (12 h:12 h light:dark). After pupation, pupae were placed within 30 cm × 30 cm × 30 cm cages in cups containing autoclaved reverse osmosis water for eclosion to achieve ~450 mosquitoes per cage. Mosquitoes were fed 10% sucrose water from dental wicks. When mosquitoes were 5–7 d old, each population was allowed to blood-feed on a human volunteer in a random order. Females that fed were separated into cups enclosed with mesh that contained moist filter paper to provide an oviposition site. Mosquitoes were fed 10% sucrose water from cotton wool.

After 4 d, eggs were collected, numbered and dried following a standard protocol for short-term egg storage[36]. The number of each set of eggs was written on the cups of the corresponding female. Egg collection was done before infection with dengue to prevent vertical transmission of the virus[37]. Between 40 and 70 females from each of the high- and low-blocking populations were anaesthetized with CO_2, injected with 69 nl of the dengue virus stock (equalling ~124 genomic copies of dengue; see 'Dengue virus') and returned to their numbered cups. Virus was delivered at a speed of 46 nl s^{-1} into the thorax using a pulled glass capillary needle and a manual microinjector (Nanoject II; Drummond Scientific). This controlled the infection dose by removing the variation that would have resulted from oral feeding, to ensure successful artificial selection. This method also ensured a sufficient number of infected mosquitoes to select between.

At 7 d post-infection, females were anaesthetized with CO_2, placed into individual wells of 96-well plates containing 50 µl extraction buffer and homogenized with a 3-mm glass bead. The extraction buffer was made up of squash buffer (10 mM Tris (pH 8.2), 1 mM EDTA and 50 mM NaCl)[38] with proteinase K at a concentration of 12.5 µl ml^{-1} (Bioline). Samples were then incubated for 5 min at 56 °C and 5 min at 95 °C. We then measured the viral load per mosquito using RT-qPCR (see 'Dengue virus quantification'). This method was used for rapid phenotype determination of a large number of samples. Mosquitoes were then ranked in order: (1) from the lowest viral load in the high-blocking populations; (2) from the highest viral load in the low-blocking populations; and (3) using a random number generator in the random population. Eggs from each mosquito were hatched into separate cups of autoclaved reverse osmosis water. The next day, larvae were taken from cups in rank order until ~200 larvae were collected for each replicate population. On average, offspring were taken from six mosquitoes per replicate population per generation. This was done to impose the strongest selection pressure possible while ensuring that enough mosquitoes would be reared for selection in the subsequent generation. At this point, the protocol was repeated. In total, four rounds of selection were completed.

Realized heritability for *Wolbachia*-mediated dengue blocking in *A. aegypti* was calculated for the high and low selection populations using the virus quantification data collected during three rounds of the selection experiment. The final generation was not included since the method of RNA extraction differed (see 'Generation 4'). Realized heritability was calculated by dividing the response to selection by the selection differential:

$$\text{Realized heritability} = \frac{\text{average first generation} - \text{average second generation}}{\text{average first generation} - \text{average selected parents}}$$

Generation 4. After four rounds of selection, mosquitoes from each population were reared and injected with the dengue virus as above (see 'Selection experiment'). After 7 d, ~30 mosquitoes from each population were dissected to separate the ovaries and the bodies. Ovaries contain disproportionately high densities of *Wolbachia*[28] and so were removed to increase the sensitivity. Dissections were performed in 1× phosphate buffered saline on a glass slide under a microscope. Dissecting needles were soaked in 80% ethanol between each dissection, and needles were changed between each population. Bodies were placed into 1.5-ml tubes containing 200 µl TRIzol, homogenized with a 3-mm glass bead, and stored at −80 °C until used.

RNA was extracted from the TRIzol following the manufacturer's protocols, and resuspended in 25 µl of UltraPure distilled water. Each sample was treated with DNAse 1, and virus quantification was carried out by RT-qPCR (see 'Dengue virus quantification'). The expression of cadherin and α-mannosidase 2a was measured relative to the expression of *RPS17*. We carried out complementary DNA synthesis on the remaining RNA samples, and measured gene expression on the LightCycler 480 (Roche) using the primers listed in Supplementary Table 2 and using SYBR Green I (Invitrogen) following the manufacturer's protocol to a final volume of 10 µl. Gene expression relative to *RPS17* was calculated using the equation $\frac{2^{\text{reference}}}{2^{\text{target}}}$. DNA was also extracted from the TRIzol following the manufacturer's protocols and resuspended in 25 µl of UltraPure distilled water. *Wolbachia* densities were measured using qPCR (see '*Wolbachia* quantification').

***Wolbachia* removal.** We treated subpopulations of each evolved population with tetracycline for two generations. Each generation, 10% sucrose water containing tetracycline (1.25 mg ml^{-1} tetracycline at pH 7 with unbuffered Tris; Sigma–Aldrich) was given to the mosquitoes[39] via dental wicks and replaced every 2 d. Control subpopulations were kept separately and fed 10% sucrose without tetracycline. Each subpopulation was initiated from 400 mosquitoes each. Since the microbiome can have important roles in mosquito resistance to arboviruses[34], we reared all subpopulations for a third generation with no antibiotic treatment, to allow microbiota recovery. We reared larvae following the same protocol as in the selection experiment (see 'Selection experiment'). We transferred 100 ml of the larval rearing water from each control subpopulation to the corresponding antibiotic-treated subpopulation to re-introduce the resident microbiota, as is standard procedure[40]. This water was checked for egg and larval contamination.

The following generation of mosquitoes (now four generations since the selection experiment) were reared and injected with virus as in the selection experiment (see 'Selection experiment') and collected in 1.5-ml tubes containing 200 µl TRIzol reagent after 7 d of infection. These samples were each homogenized with a 3-mm glass bead and stored at −80 °C. RNA and DNA extraction was carried out as above (see 'Generation 4'); the dengue viral load was quantified by RT-qPCR (see 'Dengue virus quantification') and *Wolbachia* clearance was confirmed by qPCR (see '*Wolbachia* quantification').

We calculated the average blocking strength in the low and high selection treatments relative to the randomly passaged treatment using the equations $\frac{L-L_W}{R-R_W}$ and $\frac{H-H_W}{R-R_W}$, respectively. Here, L, H and R indicate the average viral load per mosquito in the low-, high- and random-blocking populations not infected with *Wolbachia*. L_W, H_W and R_W indicate the average viral load per mosquito in the low-, high- and random-blocking populations infected with *Wolbachia*.

Genomic analysis. DNA was extracted from 90 individual mosquitoes from the ancestral population and each evolved population at generation 4. We extracted DNA from the TRIzol reagent (Invitrogen) using a modified version of the manufacturer's protocol with additional washing steps using phenol, chloroform and isoamylalcohol. DNA was sequenced using an Illumina HiSeq 3000 with 150-base pair paired-end reads.

FastQC version 0.11.4 was used with default settings to check the quality of the raw reads. To minimize false positives, Trimmomatic version 0.36 was used to trim the 3′ ends if the quality was <20, and the reads were discarded if trimming resulted in reads that were <50 base pairs in length (<0.5%). We mapped the resulting reads to the *Wolbachia* genome (AE017196.1) and the *A. aegypti* assembly (Liverpool AGWG-AaegL5) using BWA MEM 2.2.1, and checked for quality using Qualimap version 2.2.1. Indel realignment was completed using GATK version 3.8.0. Duplicates were removed using Picard version 2.17.8, and reads with poor mapping quality were removed using SAMtools 1.6 and filtering via hex flags: -q 20 (only include reads with a mapping quality of ≥ 20); -f 0×002 (only include reads with all of the flags mapped in a proper pair); -F 0×004 (only include reads with none of the flags unmapped); and -F 0×008 (only include reads with none of the flags mate unmapped). For *A. aegypti*, ~10% of reads were PCR duplicates (615,305,021) and ~58% of reads failed mapping quality filters (3,655,353,869). For *Wolbachia*, ~20% of reads were PCR duplicates (40,689,700) and ~7% of reads failed mapping quality (14,427,429). The quality was checked using Qualimap.

SNPs were called using PoPoolation2 based on a minimum coverage of 20 and a maximum coverage of 200 for *A. aegypti*. For *Wolbachia*, this was done based on a minimum coverage of 20 and a maximum coverage of 2,000, apart from the ancestral and H2 populations, which were based on a maximum coverage of 750 and 2,500, respectively. Coverage was ~46 for *A. aegypti* and ~1,288 for *Wolbachia* after duplicate and low-quality mapping were removed (~51 and ~1,754 before, respectively). We ran an alternative method to call variants to cross-check the output from the above method. Variants were called for each sample using the GATK HaplotypeCaller tool (gatk-4.0.8.1) using default settings except for ploidy, which was set to 10. A multi-sample variant file was then created merging the vcf files using the 'bcftools merge' command. SNPs from the original analysis were retained if at least one population had the same SNP called by GATK.

We identified SNPs that were significantly differentiated between treatments (see 'Statistics') and annotated them with gene information using gene transfer format files and bedtools intersect (bedtools version 2.25). Annotation files were downloaded from VectorBase (AaegL5.1) for *A. aegypti* and the National Center for Biotechnology Information (AE017196.1) for *Wolbachia*. Information on *A. aegypti* gene function was collected by searching VectorBase gene IDs on OrthoDB[41].

Mosquito life-history data. Life-history data were collected from each evolved replicate population from the high-, low- and random-blocking selection treatments, with three technical replicate populations each (27 populations in total). We collected the median time to pupation, adult sex ratio, female adult daily survival over three blood meals, and size and timing of egg clutches over three blood meals. These data were used to estimate mosquito fitness (see 'Model development for the estimation of mosquito fitness (*r*)' and 'Model parameterization from group-level life-history data').

Eggs from mosquitoes at generation 4 of the selection experiment were submerged into autoclaved reverse osmosis water in a vacuum chamber for 40 min. This reduced oxygen environment induced synchronous hatching within each population, reducing within-population variation in our data. We hatched populations in three batches for logistical purposes, with each treatment equally represented in each batch. Larvae were then separated from unhatched eggs and kept in uncrowded conditions (trays of ~200 larvae in 2 l of autoclaved reverse osmosis water). They had constant access to excess ground TetraMin tablets to

avoid environmentally induced fluctuations in *Wolbachia* density and mosquito fitness[42–44]. The numbers of larvae that pupated each day were recorded, and pupae were moved into cups of water within one 30 cm × 30 cm × 30 cm cage per replicate population (27 cages in total). Larvae and mosquitoes were reared under controlled conditions of temperature (26 ± 2 °C), relative humidity (~70%) and photoperiod (12 h:12 h, light:dark).

Once pupae emerged as adult mosquitoes, we counted the number of males and females, to determine the sex ratio, and transferred 60–80 females and 40 males per replicate into 20 cm × 20 cm × 30 cm cages for mating. Adult mosquitoes were fed 10% sucrose water from dental wicks. When mosquitoes were 5–7 d post-emergence, populations were allowed to blood-feed on a human volunteer for 15 min in a random order. The next day, we placed cups containing filter paper and autoclaved reverse osmosis water into each cage to allow for female oviposition. We changed these cups after 5 and 8 d, and counted the number of eggs laid per cage at each time interval. We repeated this twice, resulting in three blood meals and six egg collections. Following blood meals, we removed females that did not feed (and thus would not lay eggs) to estimate the eggs laid per female. Concurrently, we measured female daily mortality, which involved removing dead mosquitoes each day and censoring mosquitoes that were removed because they did not feed[45]. Accidental deaths and escapees were also censored.

Eggs were counted using an adapted version of a previously determined protocol[46]. We used a high-resolution colour scanner to take images of egg papers, and created a reference curve of manually counted eggs and the total area of each image (pixels^2) that was black, using ImageJ (ImageJ bundled with Java 1.8.0_172) (Supplementary Fig. 3). We used an Epson V39 flatbed scanner with 4,800 × 4,800 dpi. To ensure the highest accuracy, we used a paintbrush to separate overlapping eggs, and we scanned each filter paper while it was still moist, such that the eggs did not desiccate and change shape. Before using the 'analyze: particles' function in ImageJ[46], we converted the image to an 8-bit (black and white) image and set a threshold using the 'image:adjust:threshold' function to include only pixels with values between 0 and 95 when calculating the total particle area. This threshold was chosen as it best captured the eggs whilst excluding debris and visual artefacts.

Model development for the estimation of mosquito fitness (*r*). We measured life-history traits for each of our evolved populations of mosquitoes in triplicate (see 'Mosquito life-history data') and we used a discrete-time approximation to a population-scale model developed by Ohm et al.[21] to estimate mosquito population growth rates that would result if a mosquito population behaved identically to our evolved populations (see 'Model parameterization from group-level life-history data'). Population growth rates were modelled as the intrinsic rate of natural increase (r), which is considered a good estimate of fitness for organisms with overlapping generations, such as mosquitoes[47]. Briefly, the discrete-time approximation to the model described in Ohm et al.[21] can be written as:

$$\mathbf{n}_t + 1 = L\mathbf{n}_t \tag{1}$$

where $\mathbf{n}_t$ is a vector that describes the number of mosquitoes in a particular age class at time t, and L is a Leslie matrix given by:

$$L = \begin{bmatrix} 0 & 0 & \dots & 0 & 0 & \dots & b_i & b_{i+1} & \dots & b_{\alpha-1} & b_\alpha \\ e^{-\delta_E} & 0 & \dots & 0 & 0 & \dots & 0 & 0 & \dots & 0 & 0 \\ 0 & e^{-\delta_E} & \dots & 0 & 0 & \dots & 0 & 0 & \dots & 0 & 0 \\ \vdots & \vdots & \ddots & \vdots & \vdots & \vdots & \vdots & \vdots & \ddots & \vdots & \vdots \\ 0 & 0 & \dots & e^{-\delta_L} & 0 & \dots & 0 & 0 & \dots & 0 & 0 \\ 0 & 0 & \dots & 0 & e^{-\delta_L} & \dots & 0 & 0 & \dots & 0 & 0 \\ \vdots & \vdots & \ddots & \vdots & \vdots & \ddots & \vdots & \vdots & \ddots & \vdots & \vdots \\ 0 & 0 & \dots & 0 & 0 & \dots & S_i & 0 & \dots & 0 & 0 \\ 0 & 0 & \dots & 0 & 0 & \dots & 0 & S_{i+1} & \dots & 0 & 0 \\ \vdots & \vdots & \ddots & \vdots & \vdots & \ddots & \vdots & \vdots & \ddots & \vdots & \vdots \\ 0 & 0 & \dots & 0 & 0 & \dots & 0 & 0 & \dots & S_\alpha & 0 \end{bmatrix}$$

The matrix is a square matrix with dimensions equal to the length of the longest observed mosquito lifespan in our data, including the number of days as an egg (τ_E), larva (τ_L) and adult (α), or $\tau_E + \tau_L + \alpha$. δ_E and δ_L are the through-stage egg and larval mortality rates, which are converted to daily survival rates by the relationship described by Ohm et al.[21] and given here, where S_E is the egg through-stage survivorship and S_L is the larvae through-stage survivorship:

$$S_E = e^{-\delta_E \tau_E} \tag{2}$$

$$S_L = e^{-\delta_L \tau_L} \tag{3}$$

Age classes in the matrix begin with the first day of the mosquito egg stage (E) and end at the observed day of death for the last mosquito alive in the population described by the matrix. For adults, i denotes the time in days since eclosion and is an integer in the set of integers from 0 to the last day an adult mosquito in each experimental group was observed to be alive, or $i \in \{0, 1, \dots \alpha\}$, where α is the last day an individual was observed to be alive in the experimental population. The egg production rate, b_i, is dependent on age since pupal eclosion as observed empirically, and is taken as the average number of eggs mosquitoes in that treatment group laid per day multiplied by the sex ratio. Our model assumes constant daily mortality for juvenile stages, but uses variable values for daily adult mortality based on the values measured in our experiments (see 'Model parameterization from group-level life-history data').

Model parameterization from group-level life-history data. We constructed life tables for mosquitoes in each treatment from the life-history data (see 'Mosquito life-history data'). We recorded the number of mosquitoes that died each day in each of our treatments, to estimate daily mortality. For juvenile mortality rates, death in L1 and L2 stages of mosquito development are difficult to observe and prone to error because of unknown rates of cannibalism in these stages. We assumed 92% juvenile survival (egg to pupation) for mosquitoes that took 8 d to develop from egg to adulthood based on field data[22]. The model assumes density independence (that is, survival to the adult stage does not depend on the density of eggs or larvae). We used an egg development time of $\tau_E = 1$ d. *A. aegypti* eggs can remain in the egg stage for variable amounts of time, but we kept this constant between all treatment groups and chose the shortest possible development time. We parameterized the larval development time from development times observed in our experiments, with the median time for larval development as τ_L. The egg production rates (b_i) were also taken from the mean observed number of eggs laid by mosquitoes in our treatment groups each day.

We constructed a unique Leslie matrix for each technical replicate population (27 in total; see 'Mosquito life-history data') to estimate a unique value of the asymptotic population growth rate. The asymptotic growth rate is an estimate of fitness that combines fecundity and survivorship, but it should be noted that the estimate we calculated is constrained by the assumption of unlimited resources under laboratory conditions and density independence. The asymptotic growth rate for the population model in equation (1) was obtained by calculating the

right leading eigen value using the 'eigen' function in the R statistical environment (version 3.5.0). The asymptotic growth rate is equivalent to the intrinsic rate of natural increase, *r*.

Statistics. All statistical analyses were performed in R version 3.2.2 (http://www.r-project.org/) and are listed in Supplementary Table 1, along with sample sizes. Mixed-effects models were fit by maximum likelihood and statistically compared using a likelihood ratio test. Model terms were removed if they were not significant. We analysed $\log_{10}$[copies of the dengue virus per mosquito] using a mixed-effects model that included treatment, *Wolbachia* density and their interaction as fixed effects and replicate population as a nested random factor. We measured the effect of *Wolbachia* on $\log_{10}$[copies of the dengue virus per mosquito] using one mixed-effects model in the presence of *Wolbachia* and another in the absence of *Wolbachia*, and we corrected *P* values for multiple comparisons using the Benjamini–Hochberg FDR method. Both models included treatment as the fixed effect and replicate population as a nested random factor. We measured gene expression with mixed-effects models, including treatment as a fixed effect and replicate population as a nested random factor.

We generated a reference curve for the automated counting of eggs using a linear regression model with the total particle area (pixels^2) of an image that was black as the response and the number of eggs as the explanatory variable. We analysed the relationship between mosquito fitness and $\log_{10}$[copies of the dengue virus per mosquito] using a mixed-effects regression model including fitness as the response, viral load as the fixed effect, and experimental batch and treatment as random effects. Each point represents the average per replicate population.

We tested for differences in allele frequency between treatments using generalized linear models that were applied to replicate level major and minor allele counts. We fitted these single-SNP models using the glm() function in R and assumed a binomial error structure. To aid interpretation, we conducted these analyses in a pairwise fashion, analysing differentiation between all possible pairs of treatments (that is, high versus low, high versus random and low versus random. To assess the genome-wide significance of these models, and to account for the *P* value inflation that occurs in single-SNP analyses of evolve and resequence data, we estimated an empirical significance threshold based on exhaustive permutation of our experimental data[48]. We estimated a permutation-based *P* value threshold that corresponded to a genome-wide FDR of 5% by re-running our genome scan on all possible permutations of our pairwise contrasts between high and low, high and random and low and random. In each case, there were nine possible unique permutations excluding the observed arrangement of the six replicate populations. For each permuted dataset, we refitted our linear model to all SNPs and estimated the number of significant SNPs.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Raw data are deposited in the Dryad online repository (https://doi.org/10.5061/dryad.6vv10h0) and sequence data are available via the European Nucleotide Archive (accession number: PRJEB33044).

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Author contributions

S.A.F. and E.A.M. conceived and designed the experiments and wrote the manuscript. S.A.F. planned and performed the experiments, including mosquito rearing and infection, selection, RNA and DNA collection and analysis, antibiotic treatment, and life-history measurements. L.T.S. measured candidate gene expression. S.A.F. performed statistical analysis and interpreted the data. S.L.A., A.S., S.F.C. and I.A. collaboratively processed and statistically analysed the genomic data. S.A.F. interpreted the results. J.R.O. and S.A.F. designed the Leslie matrix models to assess mosquito fitness. J.R.O. ran the models. S.A.F. statistically analysed and interpreted the data.

Competing interests

The authors declare no competing interests.

Additional information

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	ImageJ Mains, J. W., Mercer, D. R. & Dobson, S. L. Digital image analysis to estimate numbers of Aedes eggs oviposited in containers. J Am Mosq Control Assoc 24, 496-501, doi:10.2987/5740.1 (2008).
Data analysis	All statistical analyses were performed in R version 3.2.2 (http://www.r-project.org/) For genomic analysis: Trimmomatic version 0.36, qualimap version 2.2.1, GATK version 3.8.0, picard version 2.17.8 & samtools 1.6

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Sample size	Sample sizes were as large as was physically possible given the size and replication of the selection experiment. The size was sufficient to demonstrate shifts in phenotype and to detect underlying SNPs in a few generations of selection.
Data exclusions	Mosquitoes were removed from the antibiotic treatment in fig.2b if Wolbachia was detected by qPCR (incomplete curing of Wolbachia)
Replication	Three independent lines represented the major treatments in the selection experiments: high, low and random. Their behavior with respect to one another demonstrated repeatability. Mosquito fitness measures were also carried out on three independent groups.
Randomization	Mosquitoes were assigned to treatments and replicate lines using a random number generator. Individual females were also chosen for participation in the random lines using a random number generator. Mosquito populations were fed and processed in an order assigned by a random number generator. Samples were randomized on plates with respect to treatment for assessment of Wolbachia and dengue virus loads.
Blinding	Researchers were not blinded.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology
☒	☐ Animals and other organisms
☐	☒ Human research participants
☒	☐ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Human research participants

Policy information about studies involving human research participants

Population characteristics	As the humans were themselves not being studied (no data were collected on them or their blood), none of these issues apply. They only blood fed populations of our mosquitoes on their arms.
Recruitment	Volunteers from a graduate student population.
Ethics oversight	Experimental work was carried out at Monash University, Melbourne. The Monash University Human Research Ethics Committee gave ethical approval for human volunteers to provide blood-meals to mosquitoes not infected with dengue virus (permit CF11/0766-2011000387). One volunteer was involved throughout this study and provided written consent.

Note that full information on the approval of the study protocol must also be provided in the manuscript.