LETTER

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The *w*Mel *Wolbachia* strain blocks dengue and invades caged *Aedes aegypti* populations

T. Walker[1]*, P. H. Johnson[3]*, L. A. Moreira[1]†, I. Iturbe-Ormaetxe[1], F. D. Frentiu[1], C. J. McMeniman[1]†, Y. S. Leong[1], Y. Dong[1], J. Axford[2], P. Kriesner[2], A. L. Lloyd[4,5], S. A. Ritchie[3], S. L. O'Neill[1,6] & A. A. Hoffmann[2]

Dengue fever is the most important mosquito-borne viral disease of humans with more than 50 million cases estimated annually in more than 100 countries[1,2]. Disturbingly, the geographic range of dengue is currently expanding and the severity of outbreaks is increasing[2–4]. Control options for dengue are very limited and currently focus on reducing population abundance of the major mosquito vector, *Aedes aegypti*[5,6]. These strategies are failing to reduce dengue incidence in tropical communities and there is an urgent need for effective alternatives. It has been proposed that endosymbiotic bacterial *Wolbachia* infections of insects might be used in novel strategies for dengue control[7–9]. For example, the *w*MelPop-CLA *Wolbachia* strain reduces the lifespan of adult *A. aegypti* mosquitoes in stably transinfected lines[8]. This life-shortening phenotype was predicted to reduce the potential for dengue transmission. The recent discovery that several *Wolbachia* infections, including *w*MelPop-CLA, can also directly influence the susceptibility of insects to infection with a range of insect and human pathogens[9–11] has markedly changed the potential for *Wolbachia* infections to control human diseases. Here we describe the successful transinfection of *A. aegypti* with the avirulent *w*Mel strain of *Wolbachia*, which induces the reproductive phenotype cytoplasmic incompatibility with minimal apparent fitness costs and high maternal transmission, providing optimal phenotypic effects for invasion. Under semi-field conditions, the *w*Mel strain increased from an initial starting frequency of 0.65 to near fixation within a few generations, invading *A. aegypti* populations at an accelerated rate relative to trials with the *w*MelPop-CLA strain. We also show that *w*Mel and *w*MelPop-CLA strains block transmission of dengue serotype 2 (DENV-2) in *A. aegypti*, forming the basis of a practical approach to dengue suppression[12].

A successful *Wolbachia*-based program for dengue control requires the inherited bacterial infection to efficiently invade wild *A. aegypti* populations. Invasion is based on the induction of a particular reproductive phenotype by *Wolbachia* known as cytoplasmic incompatibility[13,14], which leads to early embryonic death when *Wolbachia*-infected males mate with uninfected females. In contrast, *Wolbachia*-infected females produce viable embryos when mated with either infected or uninfected males, resulting in a reproductive advantage over uninfected females[13]. Although cytoplasmic incompatibility provides a driving force for *Wolbachia* invasion, successful invasion also depends on both the initial frequency of *Wolbachia* as well as any host fitness costs[15]. As fitness costs increase, higher initial *Wolbachia* frequencies are required for invasion. Mathematical predictions indicate that as the fitness cost of infection approaches 0.5 then spatial spreading of *Wolbachia* slows to zero[15]. Therefore, although the *w*MelPop-CLA strain induces complete cytoplasmic incompatibility in *A. aegypti*[8], fitness costs associated with this virulent infection may be sufficient to prevent invasion[15,16].

In *Drosophila*, interference against insect pathogens is induced by several *Wolbachia* strains closely related to *w*MelPop-CLA[10], indicating that strains with more desirable invasion characteristics would also impact human pathogen transmission. The *Wolbachia* strain *w*Mel occurs naturally in *Drosophila melanogaster* flies and has spread globally within the last century[17]. Moreover, it interferes effectively with *Drosophila* RNA viruses in its natural host[10,11]. Considering these properties, we established *A. aegypti* colonies stably infected with this *Wolbachia* strain and determined if *w*Mel might be effective for dengue control programs.

To facilitate transinfection of *A. aegypti*, the *w*Mel strain was first transferred from *Drosophila melanogaster* embryos into the RML-12 mosquito cell line and serially passaged for approximately two years to allow adaptation to the mosquito intracellular environment. A total of 2,541 *A. aegypti* embryos were microinjected with *Wolbachia* purified from this cell line (Supplementary Fig. 1). Three stably infected lines, MGYP1, MGYP2 and MGYP3, were generated independently and PCR screening indicated each line was 100% infected from generations 2–8 (G2–G8) after infection. The MGYP2 line was selected for further characterization and a tetracycline-cured line, MGYP2.tet, was established by G8 after infection. An outbred MGYP2.OUT line was established by backcrossing for three generations to the F1 progeny of wild-caught *A. aegypti* eggs from Cairns, Australia, as outlined elsewhere[16]. The F1 progeny from wild-caught eggs were used as control 'wild-type' mosquitoes for comparison to outbred *w*Mel-infected MGYP2.OUT mosquitoes.

The density of the *w*Mel strain in all three transinfected lines and outcrossed *A. aegypti* female mosquitoes (3 days old) was similar with WSP (*Wolbachia*) to RPS17 (*A. aegypti*) gene ratios of about 10, which was approximately threefold lower than the density of the *w*MelPop-CLA strain as determined by quantitative PCR (qPCR; Supplementary Fig. 2). The tissue distribution of the *w*Mel infection in adult female mosquitoes was also visualized using fluorescence *in situ* hybridization (FISH). The *w*Mel strain has a wide tissue distribution with infection levels in the ovaries and salivary glands (Fig. 1) similar to those of the *w*MelPop-CLA strain[9]. The heavy infection of ovaries by both strains supports the high level of maternal transmission and infection frequency observed in infected lines. However, unlike *w*MelPop-CLA, the *w*Mel strain is not present at high levels in Malphigian tubules and fat bodies (Fig. 1), thoracic ganglia or brain tissues (Supplementary Fig. 3). Therefore, the pathogenic effects of *w*MelPop-CLA infection previously observed in *A. aegypti*[8,9] may be a direct result of *Wolbachia* colonization of specific mosquito tissues.

Mass reciprocal crossing experiments between MGYP2 and MGYP2.tet mosquitoes (Supplementary Table 1) showed that the *w*Mel strain induces strong cytoplasmic incompatibility (no hatching eggs produced). In contrast, *w*Mel-infected females mated to uninfected and

[1]School of Biological Sciences, The University of Queensland, Brisbane, Queensland 4072, Australia. [2]Bio21 Institute, Department of Genetics, The University of Melbourne, Victoria 3010, Australia. [3]School of Public Health and Tropical Medicine and Rehabilitative Sciences, James Cook University, Cairns, Queensland 4870, Australia. [4]Biomathematics Graduate Program and Department of Mathematics, North Carolina State University, Raleigh, North Carolina 27695, USA. [5]Fogarty International Center, National Institutes of Health, Bethesda, Maryland 20892, USA. [6]School of Biological Sciences, Monash University, Victoria 3800, Australia. †Present addresses: Centro de Pesquisas René Rachou-Fiocruz, Belo Horizonte, MG 30190, Brasil (L.A.M.); Laboratory of Neurogenetics and Behavior, The Rockefeller University, 1230 York Avenue, Campus Box 63, New York, New York10065, USA (C.J.M.).
*These authors contributed equally to this work.

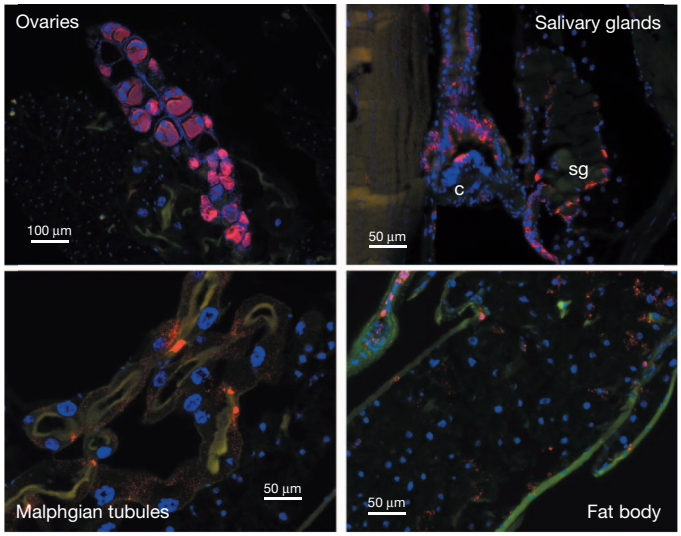

Figure 1 | Tissue distribution of the *w*Mel strain in transinfected *A. aegypti* female mosquitoes. Fluorescence *in situ* hybridization (FISH) of paraffin sections showing the localization of *Wolbachia* (red) in tissues of 7-day-old female *A. aegypti* mosquitoes. Sections were hybridized with two *Wolbachia*-specific 16S rRNA probes labelled with rhodamine. DNA is stained with DAPI (blue). A green GFP filter was used to enhance contrast. sg, salivary gland; c, cardia.

*w*Mel-infected males resulted in embryo hatch rates of approximately 90%. Strong cytoplasmic incompatibility was also observed when *w*Mel was introduced into an outbred *A. aegypti* background using backcrossing[16] to produce an outbred MGYP2.OUT strain (Supplementary Table 1).

We investigated potential fitness effects by examining fecundity and viability of eggs over time, both strongly influenced by the *w*MelPop-CLA strain of *Wolbachia*[16,18]. As shown in Supplementary Figure 4, under semi-field conditions fecundity in outbred MGYP2.OUT mosquitoes was not significantly different to that of uninfected mosquitoes. In contrast to this, a fecundity cost of ~56% was observed for *w*MelPop-CLA-infected females. In addition to fecundity, the viability of eggs is a crucial determinant of *A. aegypti* abundance following a dry season[19] and the *w*MelPop-CLA infection markedly decreased the viability of *A. aegypti* eggs[16,18]. However, the *w*Mel infection had no significant effects on viability of eggs at 5–26 days after oviposition (Supplementary Fig. 5), in contrast to a sharp reduction for *w*MelPop-CLA-infected eggs at 12 days. The *w*Mel strain is therefore much more likely to persist in wild *A. aegypti* populations in areas with a long dry season such as North Queensland, Australia[19,20].

The effect of the *w*Mel strain on immature mosquito stages was measured through larval development time, a potential correlate of mosquito fitness[21]. The *w*Mel strain caused significantly faster larval development for both females and males under high larval nutrition diets (Supplementary Table 2). Under low nutritional levels typically encountered in the field[22], the *w*Mel infection did not affect the development time of either sex. The longevity of adult mosquitoes can influence fitness, and the life-shortening effect of the *w*MelPop-CLA strain was predicted to reduce invasion potential[15]. We tested the effect of the *w*Mel strain on lifespan of outbred MGYP2.OUT females versus wild-type females and found only approximately 10% reduction in mean longevity (Supplementary Fig. 6), compared to more than 40% for the *w*MelPop-CLA strain[16]. Finally, we found that 100% of offspring from 30 MGYP2.OUT females ($n = 30$ offspring per female) were infected by *w*Mel, indicating a high maternal transmission rate of 1.0 (lower 95% confidence interval, 0.89).

We tested the invasion potential of *Wolbachia* in a semi-field facility consisting of two cages (A and B) providing environments that simulate the natural habitat of *A. aegypti* in north Queensland, Australia[23]. We separately tested the potential of the *w*Mel and *w*MelPop-CLA strains to invade uninfected mosquito populations at a starting frequency of 0.65 (likely to be achievable in open releases) and with additional near-weekly supplementary additions of *Wolbachia*-infected mosquitoes[24]. The semi-field cage invasion experiment methodology is summarized in Supplementary Fig. 8. The *w*Mel infection increased rapidly and reached fixation in cage B within 30 days and in cage A within 80 days (Fig. 2a). In contrast, *w*MelPop-CLA increased at a slower rate (Fig. 2b) and reached fixation in cage B after 40 days and approximately 80% after 80 days in cage A. The two cages differed in that there were fewer overall mosquitoes present in cage B, most probably because of predation by two geckos found in this cage. This may have increased adult mortality rates and decreased in later life any fitness costs associated with *Wolbachia* infection. *Wolbachia* therefore seemed to be more invasive when overall mortality rates were high (in cage B), which may be a better reflection of field conditions[25].

To determine if invasion rates could be predicted by model simulations, a deterministic age-structured model was used to describe the population in the cage, tracking daily cohorts of adult mosquitoes, categorized by sex and *Wolbachia* infection status, and their relative fitness (Supplementary Information). Different parameters were used for the two strains of *Wolbachia* because of the lower deleterious fitness effects associated with *w*Mel and lack of substantial longevity effects of this strain. The models provided a good fit to the data (Fig. 2), including

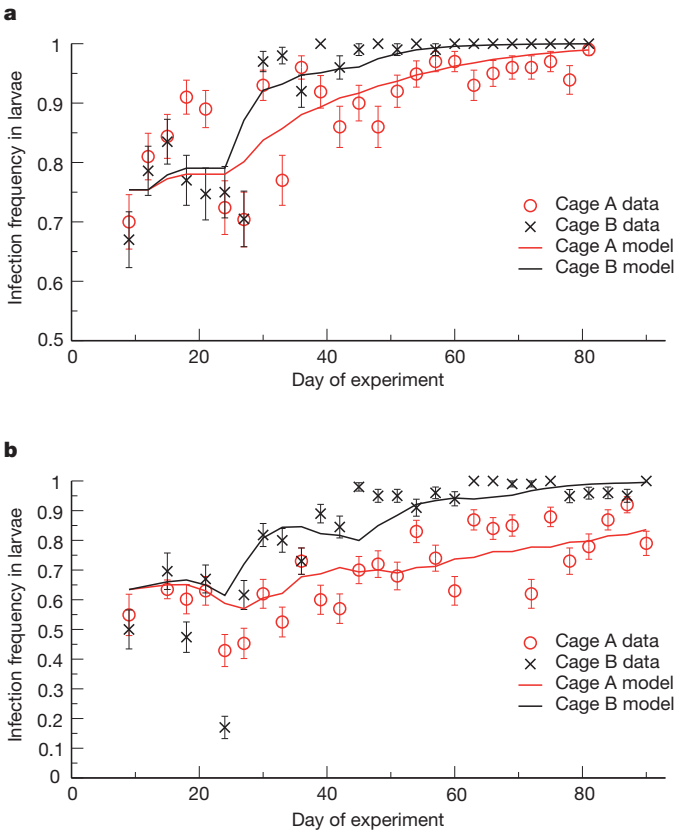

Figure 2 | Predicted and observed invasion dynamics under semi-field conditions. **a**, **b**, *w*Mel strain (**a**) and *w*MelPop-CLA strain (**b**) infection frequencies in larvae in cages A (red curves and symbols) and B (black curves and symbols). Curves indicate model-based predictions, as explained in the supplementary information. Symbols denote frequencies observed in the cages with error bars indicating binomial standard errors. Infected pupae initially comprised 65% of the released population in each cage. Eggs were collected from the cage in ovitraps and pupae developing from these eggs were released back into the cage at 3-day intervals. To simulate field releases involving repeated releases of infected mosquitoes, additional infected pupae were released into the cage, comprising a third of the total pupae released.

the difference between cage A and cage B, when additional mortality levels were introduced.

We tested the vector competence of *w*Mel-infected females by feeding mosquitoes a dengue serotype 2 (DENV-2)-infected blood meal to determine levels of virus in whole bodies, legs for disseminated virus and saliva for transmission. Virus levels were assessed at 14 days after infection, as the extrinsic incubation period (EIP) for dengue before transmission to a new host is typically 7–14 days[26,27]. Vector competence experiments also included outbred *w*Mel-infected MGYP2.OUT mosquitoes to remove any inbreeding effects that might influence dengue vector competence[28,29]. Total DENV-2 levels in *w*Mel-infected MGYP2.OUT females (whole bodies) were markedly reduced by *Wolbachia*, with approximately 1,500-fold (3 logs) fewer copies present compared to *Wolbachia*-uninfected controls (Fig. 3a). Levels of DENV-2 in *w*MelPop-CLA-infected PGYP1.OUT females were even lower with 4 logs less virus present compared to controls. Disseminated virus, measured in the legs of individual mosquitoes, was detected in only 12.5% (3/24) of *w*Mel-infected MGYP2 females compared to 82.6% (19/23) of *Wolbachia*-uninfected MGYP2.tet mosquitoes. Overall disseminated virus levels in the legs of *w*Mel-infected females were approximately 2,600-fold lower than in *Wolbachia*-uninfected females (Fig. 3b), confirming very strong inhibition of dengue virus replication in mosquitoes infected by the *w*Mel strain.

In addition to examining levels of dengue nucleic acid in mosquito extracts, we also examined the presence of infectious virus in mosquito saliva. Pooled saliva samples at 14 days after infection were assayed for infectious dengue virus using plaque assays[30]. Infectious virus was present in 29 out of 36 (80.2%) pooled saliva samples from *Wolbachia*-uninfected MGYP2.tet mosquitoes (Table 1). In contrast, infectious virus was only detected in 2 out of 48 pooled saliva samples from the transinfected MGYP2 line with each DENV-2-positive saliva pool containing 2 plaque-forming units (Supplementary Fig. 7). When the individual mosquitoes in these two positive pools were examined, a single individual in each pool was determined to be uninfected with *Wolbachia*. Moreover, these same individuals tested dengue-positive by PCR. Rare *Wolbachia*-uninfected individuals may be occasionally produced through imperfect maternal transmission[8]. No infectious virus was detected in the 36 pooled saliva samples from MGYP2.OUT females. These data indicate complete blockage of DENV-2 transmission under the experimental conditions used. As shown in Table 1, complete absence of DENV-2 in PGYP1 and PGYP1.OUT saliva extracts indicates that the closely related *w*MelPop-CLA strain also blocks DENV-2 transmission.

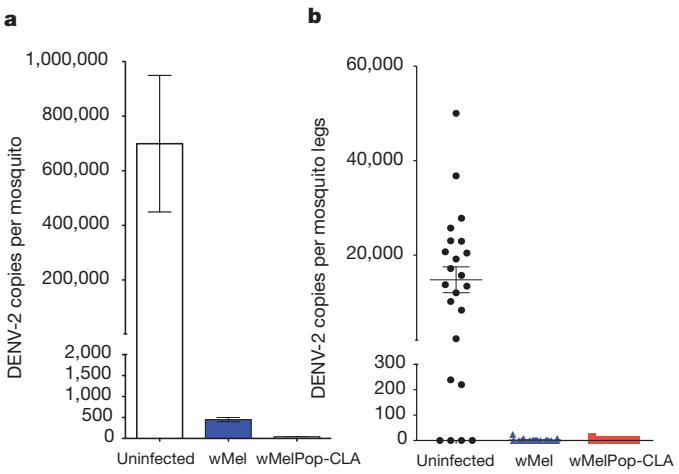

Figure 3 | Dengue infection levels in mosquitoes. Mosquitoes were fed DENV-2-infected blood orally (DENV-2 titre 1.5×10^7 plaque-forming units per ml) and titre determined at 14 days after infection. **a**, qPCR of total dengue virus in whole female mosquitoes ($n = 19–30$). Bars represent overall means ± s.e.m. across three independent replicate experiments. White bar, *Wolbachia*-uninfected wild-type control mosquitoes; blue bar, *w*Mel-infected MGYP2.OUT; red bar, *w*MelPop-CLA-infected PGYP1.OUT. **b**, qPCR of disseminated dengue virus in the legs of individual *Wolbachia*-uninfected MGYP2.tet (black circles), *w*Mel-infected MGYP2 (blue triangles) and *w*MelPop-CLA infected PGYP1 (red squares) mosquitoes ($n = 23–30$).

Table 1 | Prevalence of infectious DENV-2 in pooled saliva extracts.

Mosquito line	*Wolbachia* infection	DENV-2-positive pools/total saliva pools	Saliva pools positive for DENV-2 (%)
MGYP2.tet	Uninfected	29/36	80.2
MGYP2	*w*Mel	2/48*	4.2
MGYP2.OUT	*w*Mel	0/36	0.0
PGYP1	*w*MelPop-CLA	0/14	0.0
PGYP1.OUT	*w*MelPop-CLA	0/22	0.0

Mosquitoes were orally fed DENV-2 in an artificial blood meal (DENV-2 titer 1.5×10^7 plaque-forming units per ml) across three independent replicate experiments. Saliva was collected 14 days after blood feeding by placing the mosquito proboscis into filtered pipette tips and the saliva was pooled into groups of four for plaque assays to determine the presence of infectious virus in the saliva ($n = 56–196$ saliva extracts per strain).
* Both DENV-2-positive saliva pools from the MGYP2 line included saliva contributed from *Wolbachia*-uninfected females.

To our knowledge, this is the first reported case of complete blockage of dengue transmission by *Wolbachia*-infected mosquitoes. No infectious DENV-2 virus was detected in the saliva of any of the 336 *w*Mel-infected *A. aegypti* females used in artificial feeding experiments. The *w*Mel-infected mosquitoes had detectable DENV-2 virus in their bodies (albeit significantly reduced levels compared to control mosquitoes), but dengue virus did not disseminate into mosquito saliva. This may coincide with the heavy *w*Mel infection of salivary gland tissue (Fig. 1). Although the *w*AlbB strain of *Wolbachia* was previously shown to reduce dengue levels in mosquito saliva, infectious virus was still detected in 62.5% of pooled saliva from *w*AlbB-transinfected mosquitoes using similar methodology[30]. The ability of *w*Mel to provide protection against dengue virus in *A. aegypti* is unlikely to be transient; *w*Mel provides protection against insect viruses in its native *Drosophila melanogaster* host[10] despite a long-term evolutionary association. Recently *w*Mel has also been shown to induce strong resistance against West Nile virus in *D. melanogaster*[31], suggesting potential blocking of other human pathogens.

A major advantage of a *Wolbachia*-based biocontrol approach is that cytoplasmic incompatibility can result in invasion from the release of relatively small numbers of individuals. This invasive potential is demonstrated by the successful global invasion of *D. melanogaster* by *w*Mel within the last 80 years[17]. The apparent minimal fitness costs of the *w*Mel infection in *A. aegypti* are critical for ensuring a relatively low unstable point (<40%) from which invasions can occur[15,16], and the semi-field cage experiments demonstrate that rapid invasion is possible.

The direct inhibition of pathogens by *Wolbachia* may also augment cytoplasmic incompatibility as a mechanism for population invasion by providing a positive fitness benefit to insects carrying *Wolbachia*—something that cannot be tested in experimental invasion studies in our semi-field system. This benefit may overlay the traditional 'Bartonian' view of cytoplasmic-incompatibility-based invasion dynamics and provide an additional driving force for *Wolbachia*[14,15]. These results pave the way for an open release of *w*Mel-infected *A. aegypti* mosquitoes in Cairns, Australia[12].

METHODS SUMMARY

The RML-12 cell line was infected with *w*Mel *Wolbachia* from *Drosophila melanogaster* yw^{67c23} embryos using the shell vial technique and maintained by continuous serial passage. Embryos of the JCU strain of *A. aegypti* were microinjected with *Wolbachia* purified from the RML-12 cell line (passages 130–132). Screening for *w*Mel infection was done using PCR primers specific for the IS5 repeat element. Routine mosquito maintenance, tetracycline treatment to remove the *w*Mel infection and lifespan assays were performed as previously described[8]. An outcrossed line (MGYP2.OUT) was established by backcrossing the MGYP2 line for three generations to the F1 progeny of wild-collected *A. aegypti* eggs[16]. Fitness assessments involved a comparison between the outcrossed line and uninfected line established from the wild-collected eggs of similar genetic background. *Wolbachia*

density was estimated using the single-copy *Wolbachia* surface protein (WSP) and *A. aegypti* RPS17 genes. FISH was carried out on 7-day-old MGYP2 and MGYP2.OUT adult females using a *Wolbachia*-specific 16S ribosomal RNA probe[9]. Reciprocal mass crosses were conducted between virgin individuals (3 days old) of each sex to determine cytoplasmic incompatibility levels. Fecundity was assessed using multiple human volunteer feeders with 20–25 mosquitoes per feed. Embryo viability and larval development assays were conducted as previously outlined[16]. Two separate cage invasion experiments were performed by releasing *w*Mel-infected MGYP2.OUT and *w*MelPop-CLA-infected PGYP1.OUT mosquitoes at a starting frequency of 0.65 into two independent semi-field cages (A and B) and competing these with uninfected mosquitoes with a similar genetic background. A total of 1,680 mosquitoes were initially introduced into the cages over 6 days. Mosquitoes were provided access to live human blood almost daily. Eggs were collected from the cages every 3 days (starting at day 9), hatched and reared in the laboratory. A sample of the larvae was tested for *Wolbachia* infection and additional larvae were reared to pupae to be released back into the cages following the 3-day egg collection schedule. In addition, from day 7, supplementary cohorts of *Wolbachia*-infected mosquitoes were released in the cages every 6 days to simulate what would be expected in an open field release program[24]. Dengue vector competence was assessed using oral feeding with DENV-2 (92T strain) at 14 days after infection at a titre of 1.5×10^7 plaque-forming units per ml as determined using immunoplaque assays. Oral feeding, RNA extraction, cDNA synthesis and qPCR analysis for total DENV-2 levels in females was carried out using protocols previously outlined[9]. Disseminated viral infection rates in mosquito legs were measured after RNA extraction using a QIAamp Viral RNA Mini Kit (Qiagen). Mosquito saliva from four mosquitoes was pooled and used in immunoplaque assays.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions T.W. performed transinfection and initial phenotypic characterization of the infection. P.H.J., Y.S.L., Y.D. and S.A.R. performed cage invasion experiments and fecundity assays on outbred mosquito lines. T.W., L.A.M. and F.D.F. carried out vector competence assays. I.I.-O. performed FISH. C.J.M. established cell lines for transinfection. J.A. and P.K. performed cytoplasmic incompatibility and lifespan assays on outbred mosquito lines. A.L.L. undertook modelling studies. T.W. and A.A.H. performed data analysis. T.W., P.H.J., S.L.O. and A.A.H. wrote the paper. S.L.O., A.A.H. and S.A.R. provided oversight of the design and direction of the work.

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METHODS

Transinfection. The naturally uninfected JCU strain of *A. aegypti*, established from field-collected eggs from Cairns, Australia, in 2005, was used as the recipient strain for transinfection. Adults were provided with constant access to 10% sucrose solution and 5-day-old females were blood-fed with human blood (UQ Human Ethics Approval 2007001379). *w*Mel from *Drosophila melanogaster* yw^{67c23} embryos was established in the *Aedes albopictus* cell line RML-12 using the shell vial technique[32] and the infection serially passaged as previously described[33]. Preblastoderm embryos were microinjected in the posterior pole with *w*Mel, purified from RML-12 cells (*w*Mel passage 130–132 in cell lines) as described previously[33]. Microinjection was carried out under ×100 magnification using a FemtoJet microinjector system (Eppendorf) with type II Femtotip microinjection needles (Eppendorf) using a hydrophilic membrane method. After injection, embryos were incubated at 80% relative humidity and 25 °C for approximately 40 min and transferred to wet filter paper. Embryos were then allowed to develop for 4–5 days before being hatched. Adult females (G0) that survived embryo microinjection were isolated as pupae and mated with JCU males. Following blood feeding and oviposition, DNA was extracted from G0 females using the DNeasy tissue kit (Qiagen). Screening for *w*Mel infection was done using PCR primers specific for the IS5 repeat element[17,34]. Females from isofemale lines infected with the *w*Mel strain were backcrossed to JCU males until G4 at which time the lines were closed (restricting matings to mosquitoes from within the line) with the population size maintained at several thousand adults. Routine mosquito maintenance and tetracycline treatment to remove the *w*Mel infection from the MGYP2 line were carried out as previously described[8]. An outcrossed line (MGYP2.OUT) was established by backcrossing the MGYP2 line for three generations to the F1 progeny of wild-caught *A. aegypti* eggs from the Cairns region[16]. The F1 progeny was also used to generate an outbred wild-type population for comparison to the outbred *w*Mel-infected MGYP2.OUT line (that is, with a similar genetic background).

***Wolbachia* density and tissue distribution.** Density of the *w*Mel strain was assessed by quantitative PCR (qPCR) in 3-day-old females (whole bodies) on MGYP2 and outcrossed MGYP2.OUT females ($n = 10$). *Wolbachia* density was estimated by comparing the abundance of the single-copy *Wolbachia* surface protein gene (WSP) to that of the single-copy *A. aegypti* RPS17 gene. For each sample, qPCR amplification of DNA was performed in triplicate using a Rotor-Gene 6000 system (Corbett Research)[34]. Fluorescence *in situ* hybridization (FISH) was carried out on 7-day-old MGYP2 and MGYP2.OUT adult female mosquitoes using a *Wolbachia*-specific 16S rRNA probe[9,34].

Cytoplasmic incompatibility and fitness assays. Reciprocal mass crosses were conducted between 30 virgin individuals (3 days old) of each sex from the MGYP2 and MGYP2.tet lines to determine cytoplasmic incompatibility levels. This was repeated with 10 virgins from the MGYP2.OUT and wild-type lines. Fecundity, the total number of eggs laid by females, was assessed using 3–5 independent human volunteer blood feeders with 15–25 mosquitoes per feed. This was repeated with MGYP2.OUT and wild-type lines using 21–22 mosquitoes and an additional experiment was carried out to determine the total number of eggs laid over a 6-day period after blood feeding. Diapause egg viability was assessed after MGYP2, MGYP2.tet and PGYP1 females were blood-fed on human volunteers and isolated individually for oviposition. Egg batches were transferred to plastic storage boxes and maintained at a temperature of 25 ± 1 °C and 85% relative humidity using saturated KCl solution as previously described[16]. Larval development time was measured as the time between 1st instar hatching and pupation. A low nutritional level, 0.05 mg of food per larva per day, was previously determined to significantly delay larval development time when compared to the standard rearing nutritional level of 1 mg of food per larva per day[16]. Two-sample *t*-tests were used to compare fecundity, embryo viability and larval development times between mosquito lines as well as mean and median longevity per cage. Mosquito survival was also analysed using Cox regression to determine the equality of the survival distributions between treatments after pooling data across replicates.

Semi-field cage invasion experiments. These experiments used the backcrossed lines and controls with a similar genetic background from Cairns. Initial starting frequency of *Wolbachia*-infected mosquitoes for both *w*Mel and *w*MelPop-CLA invasion experiments was 0.65, reflecting a release ratio of around 2 infected mosquitoes to 1 uninfected mosquito; this ratio was considered achievable under field conditions in North Queensland given the relatively low number of adult mosquitoes in houses[35]. The *w*MelPop-CLA strain was tested from November 2009 to March 2010 and *w*Mel from April to July 2010. The same procedure and schedule were used in both experiments. A total of 1,680 mosquitoes were introduced into the cage over a period of 6 days. Each day 120 female pupae (78 infected and 42 non-infected) and 120 male pupae (78 infected and 42 non-infected) were released into to the cage. Mean minimum and maximum temperature and relative humidity conditions in the cages were: cage A, 20.7–30.1 °C and 64.0–98.0%; cage B, 20.5–29.1 °C and 54.1–97.8% respectively. Mosquitoes were provided access to a blood meal almost daily (at least 6 days per week) using 1–3 human volunteers (James Cook University Human Ethics H2250). The volunteers sat in the cages and allowed mosquitoes to feed uninterrupted for 15 min. For each blood-feeding session, the same volunteers were used for both cages. The first-fed cage was alternated between sessions.

Twelve oviposition sites were distributed throughout each cage. An oviposition site consisted of a 4 l white plastic bucket filled with 2 l of a 10% hay infusion solution. A 10 × 15 cm strip of red flannelette cloth (ovistrip) was attached to the inside of each bucket as an oviposition substrate. All ovistrips were retrieved every 3 days (starting from day 9), moved to the laboratory and kept damp for 3 days so that all eggs could embryonate. Ovistrips were partially dried on the third day and hatched in a dilute solution (1 g in 1 l of water) of activated baker's yeast in the afternoon. Thus, the age of the eggs at hatching was between 3 and 6 days.

Two days after hatching, all larvae were pooled and mixed thoroughly in a 10 l bucket, and a sample of at least 100 larvae was collected to assess *Wolbachia* infection frequency. The sampled larvae were placed in clean water for at least 1 h and then killed and stored in 80% ethanol. Samples were stored at 2–5 °C before being processed to determine infection status. The remaining larvae were reared to pupae and a cohort sample of 60 female and 60 male pupae were returned to each cage. Pupae from the midpoint of the pupation period, usually at days 6–8, were returned. Cohorts of pupae were returned to the cages every 3 days, following the egg-collection schedule. By using this procedure, around 500–700 adults were maintained in each cage. Additional cohorts of *Wolbachia*-infected mosquitoes were regularly released in the cages over the course of the experiment, to simulate an ongoing field release program. Starting on day 7, *Wolbachia*-infected pupae were added to the cage so that they constituted a third of the total pupae released over a 6-day period resulting in 120 *Wolbachia*-infected pupae (60 females and 60 males) included for every 240 cage-cohort pupae. For this purpose, *Wolbachia*-infected mosquitoes were reared in the laboratory and added to the cages to coincide with every second cage-cohort release.

Wolbachia infection status of *A. aegypti* was tested with a multiplex PCR assay. Larvae (second instars) hatched from eggs collected in oviposition buckets were preserved in ethanol. Larvae were washed twice individually for at least 10 s in distilled water to remove ethanol residue. Each larva was dried briefly with paper towel and put into a well of a 96-well PCR plate with 20 µl of lysis buffer (10 mM Tris pH 8.4, 1 mM EDTA, 50 mM NaCl, 0.25 µl DNARelease Additive (Finnzymes Oy)). The samples were then incubated at 56 °C for 5 min and then boiled at 95 °C for 5 min. Multiplex PCR was carried out with primers amplifying a fragment of the WSP gene of *Wolbachia* (185 base pairs) and with primers amplifying a fragment of ribosomal protein S17 (RPS17) from *A. aegypti* (305 bp). The RPS17 primers were designed to act as a control for the presence of amplifiable DNA or any PCR inhibition in the reaction. Reactions (20 µl volume) were set up with 1 µl extracted DNA as template, 4 µl 5× Buffer, 2 µl of 1 mM dNTPs, 10.6 µl water, 0.5 µl of 10 µM Seq_rps17F (5′-CTGGAGATTTTCCGTTGTCA-3′), 0.5 µl of 10 µM Seq_rps17R (5′-GACACTTCCGGCACGTAGTT-3′), 0.5 µl of 20 µM wspFQALL (5′-GCATTTGGTTAYAAAATGGACGA-3′), 0.5 µl of 20 µM wspRQALL (5′-GGAGTGATAGGCATATCTTCAAT-3′), and 0.4 µl of PHIRE Hot Start Taq Polymerase (Finnzymes Oy). The temperature profile of the PCR was 98 °C for 3 min, 35 cycles of 98 °C for 5 s, 60 °C for 5 s and 72 °C for 20 s and ended with 72 °C for 1 min. PCR products were analysed by gel electrophoresis in a 2% agarose gel. The larvae were scored as positive for *Wolbachia* infection if *Wolbachia* WSP and RPS17 of the host were positive or if only *Wolbachia* WSP was positive. Larvae were scored as negative for *Wolbachia* infection if *Wolbachia* WSP was negative and RPS17 of the host was positive. If both *Wolbachia* WSP and *RPS17* of the host were negative, the sample was excluded.

Dengue vector competence. Dengue virus serotype 2 (DENV-2), strain 92T, was passaged six times in an *A. albopictus* C6/36 cell line and viral supernatant was collected. Virus titres were determined using immunoplaque assays[36] and kept at −80 °C until use. Oral feeding, RNA extraction, cDNA synthesis and qPCR analysis for total DENV-2 levels in whole female bodies was carried out using protocols previously outlined[37]. Disseminated viral infection rates were measured through quantification of virus copies in mosquito legs after RNA extraction using a QIAamp Viral RNA Mini Kit (Qiagen). Mosquito saliva was collected from orally fed females at 14 days after infection after 24 h of starvation. The legs and wings were removed and the proboscis was inserted into filtered pipette tips containing 20 µl of Opti-MEM medium (Invitrogen) supplemented with 20% heat-inactivated FBS (Invitrogen), antibiotics (gentamicin, penicillin/streptomycin) and antimycotics. Mosquitoes were allowed to salivate for 30 min and then the media was transferred to 130 µl of Opti-MEM media supplemented with 2% FBS.

Mosquito saliva extracts were immediately placed on dry ice and stored at −80 °C until use in plaque assays.

Immunoplaque assays. C6/36 cells were grown to confluence in 24-well plates at 26 °C in RPMI 1640 medium (Invitrogen) supplemented with 1× GlutaMAX (Invitrogen) and 10% FBS and buffered with 25 mM HEPES (pH 8.0). Saliva pools each from four mosquitoes (600 µl total volume) were filtered through a Millex-G 0.22 µm sterile filter and added to the cell monolayers with the growth media removed. Monolayers and saliva samples were incubated with gentle rocking for 10 min, followed by a further incubation for 1.5 h at 26 °C. A further 600 µl of a mixture of RPMI media and carboxymethylcellulose (CMC) containing antibiotics and antimycotics was added to the wells, resulting in a final concentration of 2% FBS and 1.5% CMC. Cell monolayers were incubated at 26 °C for 6 days after infection, followed by fixation with acetone/PBS and plaques were visualized[36]. Briefly, cell monolayers were incubated with a primary antibody against the DENV-2 NS1 protein, washed three times with PBS and incubated with a secondary anti-rabbit IgG horseradish peroxidase. Plaques were developed using SIGMA*Fast* DAB with Metal Enhancer (Sigma-Aldrich). Data was recorded as negative for virus transmission if no plaques were detected or recorded as positive if at least one plaque was present in a particular well.

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