LETTER

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Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission

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Genetic manipulations of insect populations for pest control have been advocated for some time, but there are few cases where manipulated individuals have been released in the field and no cases where they have successfully invaded target populations[1]. Population transformation using the intracellular bacterium *Wolbachia* is particularly attractive because this maternally-inherited agent provides a powerful mechanism to invade natural populations through cytoplasmic incompatibility[2]. When *Wolbachia* are introduced into mosquitoes, they interfere with pathogen transmission and influence key life history traits such as lifespan[3–6]. Here we describe how the *w*Mel *Wolbachia* infection, introduced into the dengue vector *Aedes aegypti* from *Drosophila melanogaster*[7], successfully invaded two natural *A. aegypti* populations in Australia, reaching near-fixation in a few months following releases of *w*Mel-infected *A. aegypti* adults. Models with plausible parameter values indicate that *Wolbachia*-infected mosquitoes suffered relatively small fitness costs, leading to an unstable equilibrium frequency <30% that must be exceeded for invasion. These findings demonstrate that *Wolbachia*-based strategies can be deployed as a practical approach to dengue suppression with potential for area-wide implementation.

Dengue fever is a major health problem throughout the tropics; its geographic distribution and severity of impact are increasing, with more than 50 million people currently estimated to be affected by dengue disease each year[8,9]. Traditional control measures focus on reducing populations of the main transmission vector, the mosquito *A. aegypti*, but these have largely failed to slow the current dengue pandemic. This has led to a search for novel technologies to break dengue transmission cycles.

One promising avenue is provided by the intracellular insect bacterium, *Wolbachia pipientis*, which can spread rapidly into uninfected host populations by inducing cytoplasmic incompatibility. This phenomenon causes embryos from *Wolbachia*-uninfected females to die when they are mated with infected males whereas infected females are not affected in this manner. Because *Wolbachia* are maternally inherited this effect provides a transmission advantage for the symbiont, resulting in rapid invasion of insect host populations[10,11]. Successful invasion depends on the cytoplasmic incompatibility driver overcoming incomplete maternal transmission of the *Wolbachia* infection as well as overcoming a loss of fitness of infected hosts[12,13]. This creates an unstable equilibrium point that must be exceeded for *Wolbachia* to spread rather than be lost from a population[14].

In addition to being able to effectively invade wild insect populations, *Wolbachia* also influence the ability of insects to transmit pathogens. This can be achieved indirectly by reducing insect lifespan[15] or directly by reducing the ability of viruses and other pathogens to proliferate within the insect. The disruption of dengue transmission by *Wolbachia* through saliva has been demonstrated to be nearly complete in laboratory assays, with the strength of the effect dependent on the *Wolbachia* strain[3,6,7]. Strain variability is presumably due to variation in the densities and tissue distributions of *Wolbachia*[16]. Of note is the observation that *Wolbachia* strains that provide greater disruption to dengue transmission also confer greater fitness costs to the mosquito host, and successful invasion for dengue control therefore requires a *Wolbachia* strain that balances these two effects[7].

One such strain has recently been produced; the *w*Mel *Wolbachia* strain from *Drosophila melanogaster* has been stably introduced into *A. aegypti* where it has strong anti-dengue properties and small host fitness costs[7]. Moreover, contained field-cage experiments demonstrated that this strain could successfully invade a small wild-type population and do so more effectively than *w*MelPop-CLA, the strain of *Wolbachia* originally introduced into *A. aegypti* with the goal of indirectly reducing dengue transmission by reducing mosquito lifespan[4,7].

Given these results we have now proceeded to a deliberate *Wolbachia* introduction through open releases of mosquitoes with the *w*Mel *Wolbachia* strain into wild Australian *A. aegypti* populations. Field locations chosen for the study were Yorkeys Knob (614 houses) and Gordonvale (668 houses) near Cairns in north-eastern Australia (Supplementary Fig. 1). These open releases were approved by the Australian Pesticides and Veterinary Medicines Authority (APVMA permit 12311) and were preceded by an extensive period of community engagement and subsequent strong community support.

In the month before release, residential properties within the release area were visited and water was removed from visible breeding containers. In early January 2011 during the wet season, adult female and male mosquitoes were released at 184 (Yorkeys Knob) and 190 (Gordonvale) sites spread uniformly throughout each field location. This was followed by an additional 9 releases at each location over the ensuing 9–10 weeks, with a total of 141,600 (Yorkeys Knob) and 157,300 (Gordonvale) adults released. The number of mosquitoes released in a week varied between 10,000 and 22,000 depending on results obtained from monitoring *Wolbachia* infection frequencies (Fig. 1); extra mosquitoes were released where there were perceived weak spots (low infection frequencies) within the release area.

Following the initial release, *Wolbachia* frequencies were monitored every 2 weeks and monitoring was continued after releases were terminated. For monitoring purposes, each suburb was divided into 40–42 blocks. In the first 10 weeks, 320 ovitraps were placed throughout the release area, but this was decreased to around 100 traps later. From each trap, up to 10 larvae were collected and screened for *Wolbachia* using a multiplex PCR assay to both discriminate *A. aegypti* from other *Aedes* species (*Aedes palmarum*, *Aedes notoscriptus*) present as well as determine the *w*Mel infection frequency. A series of experiments was conducted to provide quality control of the *Wolbachia* screening protocol.

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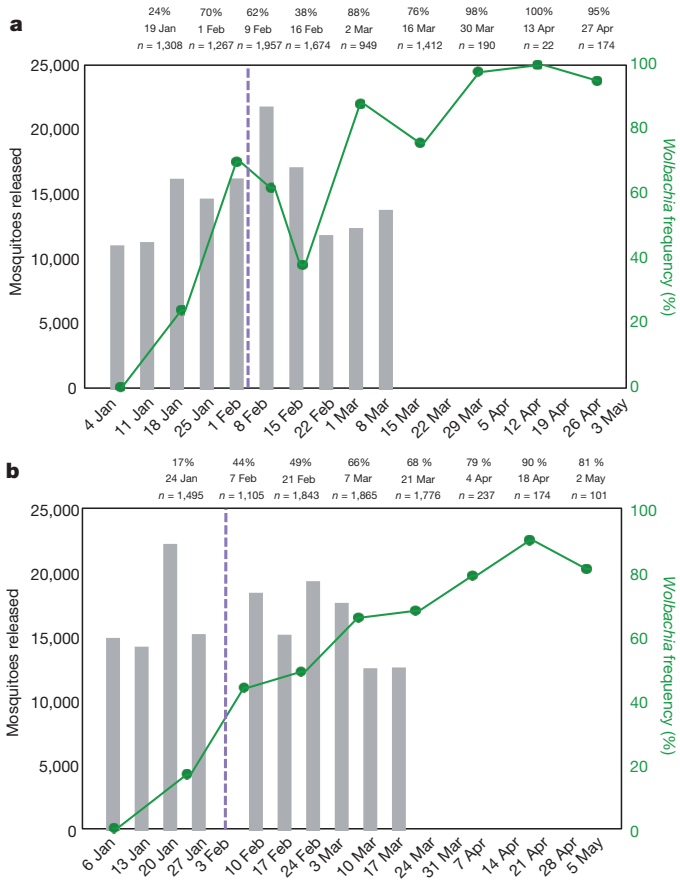

Figure 1 | Number of mosquitoes released, timing of releases, and changes in infection frequencies over time. **a**, **b**, Data based on monitoring with ovitraps at Yorkeys Knob (**a**) and Gordonvale (**b**). Ten releases were carried out at each site. Lower numbers were collected late in the season because of a reduction in trapping intensity and the advent of the dry season. Tropical Cyclone Yasi landed on 3 February (dotted line) and disrupted *Wolbachia* monitoring collections at Yorkeys Knob. A planned release at Gordonvale on 3 February was cancelled.

This included comparisons between samples obtained from the ovitraps with adult samples collected through BioGents Sentinel (BGS) traps and larval samples from natural breeding sites in the same area, as well as experiments to eliminate potential sources of contamination.

The screening procedure indicated that the frequency of *A. aegypti* infected with *Wolbachia* initially increased to more than 15% in both locations 2 weeks after release (Fig. 1). Following further releases, frequencies increased to >60% at Yorkeys Knob. At this stage Cairns was exposed to a Category 5 Tropical Cyclone (Yasi) although the city suffered only minor damage. It is not clear what effects the cyclone had on the resident mosquito population although winds were predominantly offshore during the event. The *Wolbachia* frequency decreased at Yorkeys Knob 6 weeks after the first release, then after the 7th release increased and reached near fixation 5 weeks after releases were terminated. At Gordonvale the *Wolbachia* frequency increased more slowly but steadily to reach 90% at 5 weeks after releases stopped (Fig. 1). In both areas there was a small decrease in *Wolbachia* frequency at the end of the monitoring period as the onset of the dry season caused a sharp decrease in the size of the mosquito population and most probably increased movement of mosquitoes into release areas as reflected by previously detected changes in population genetic structure[17].

Detailed monitoring of *Wolbachia* frequencies in blocks across the release area pointed to significant spatial heterogeneity in the incidence of *Wolbachia* (Fig. 2 and Supplementary Fig. 2 for block numbering). At Yorkeys Knob in the first monitoring period there were lower frequencies of *Wolbachia* in some blocks (for example, blocks 15 and 24). These low areas tended to persist in the ensuing 16 February monitoring period; however, following adaptive management involving the release of additional mosquitoes in such blocks, the incidence of *Wolbachia* increased across the entire area. At Gordonvale block-level monitoring also indicated significant spatial structure with some areas having low infection frequencies (for example, blocks 26 and 28) which were identified for adaptive management through additional releases into blocks. Releases in one area of Yorkeys Knob (blocks 17, 19, 20 and 23, Supplementary Fig. 2) ceased on 21 February because of the notification of a single dengue case most likely acquired in Innisfail outside the release area, which led to insecticide intervention by health authorities in a 150-m radius from the case house. This did not adversely influence *Wolbachia* frequencies, which continued to increase despite the absence of further releases within the affected area (Fig. 2 and Supplementary Fig. 2). No local transmission of dengue occurred from the identified case.

Adult surveys in the release area with BioGents Sentinel (BGS) traps (Supplementary Fig. 3) pointed to an increase in numbers at the start of the release period in both locations, followed by a decrease at the end of the release period. By using the average number of adults in the preceding month in each location, we estimated that during the release period the number of adults doubled in Yorkeys Knob and increased 1.5-fold in Gordonvale (there was no equivalent increase in adult numbers in BGS traps placed at Parramatta Park, a suburb in Cairns several kilometres from the release area). Adult numbers in all locations declined in April at the onset of the dry season.

The observed increases in *Wolbachia* frequency, the number of adults released, and estimates of resident population sizes were incorporated into a model of *Wolbachia* population dynamics[12] to infer plausible fitness costs and approximate the resulting unstable equilibrium frequency. This heuristic analysis (Supplementary Information) was consistent with fitness costs on the order of 20% (Supplementary Fig. 4). Estimates are uncertain because population sizes and daily survival rates of *A. aegypti* are poorly known. Fitness costs may be overestimated because our data conflate transient fitness effects associated with laboratory rearing of released *A. aegypti* with inherent costs associated with *Wolbachia* infection, and the model ignores movement into the population of uninfected individuals (for example, from newly submerged eggs). If intrinsic fitness costs are lower than 30%, a spreading wave of infection would be relatively easy to initiate from localized introductions[18]. The data from Gordonvale and Yorkeys Knob were consistent with the same estimated fitness costs; the slower increase of *Wolbachia* frequency in Gordonvale most probably reflects a larger resident *A. aegypti* population (and lower ratio of released to resident mosquitoes).

Monitoring of the *Wolbachia* infection outside the release area during and directly after the release pointed to some spread of infected mosquitoes (Fig. 3). *Wolbachia*-infected larvae were found in Pyramid Estate (Fig. 3b), a part of Gordonvale that is separated by a major highway from the release zone. Highways are often viewed as a physical barrier blocking mosquito movement and gene flow in *A. aegypti*[19]. Around Yorkeys Knob there was movement into a nearby suburb, with *Wolbachia* being detected on two occasions 4 weeks apart within the same region of Holloways Beach (Fig. 3), 1.86 km from the nearest release site. The infection was also detected on one occasion at Trinity Park, 1.03 km from the nearest release site. These results point to occasional long distance movement of *A. aegypti* either through vehicles or adult dispersal, which is inconsistent with the view that *A. aegypti* is a weak disperser but consistent with some experiments and observations[20–22]. Unless fitness costs are essentially zero or unless there are unexpected fitness benefits, we do not expect the infection to spread further in these areas but instead to be swamped by local wild types[18].

Releases of *Wolbachia*-infected males have previously been used to suppress natural populations of *Culex* mosquitoes through cytoplasmic

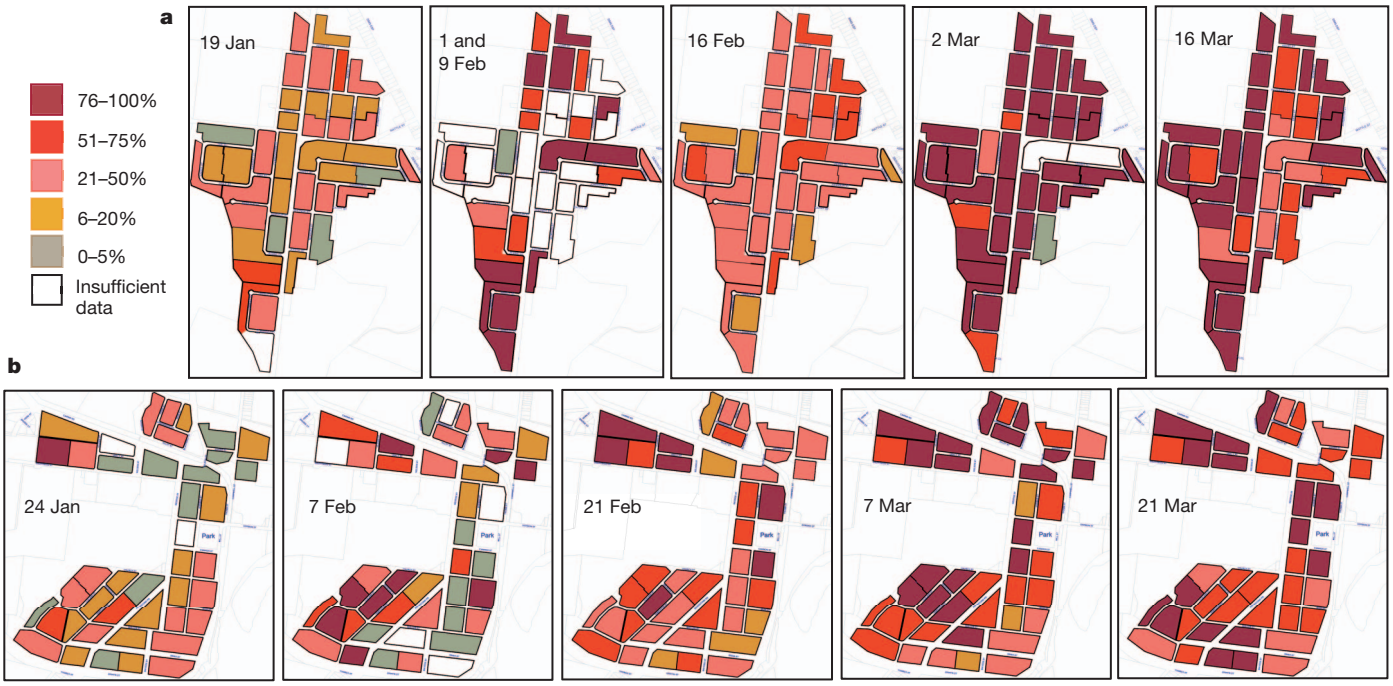

Figure 2 | Changes in frequencies of *Wolbachia*-infected mosquitoes. **a**, **b**, Data collected in individual blocks within Yorkeys Knob (**a**) and Gordonvale (**b**). Block frequencies are based on samples of 10 or more (usually >30) larvae from 1–8 (usually >3) ovitraps. If fewer than 10 *A. aegypti* were scored from any given block then the block was scored as having insufficient data. Block data are provided in Supplementary Figure 2. Adaptive management involving additional releases was applied in areas where *Wolbachia* frequencies were relatively low.

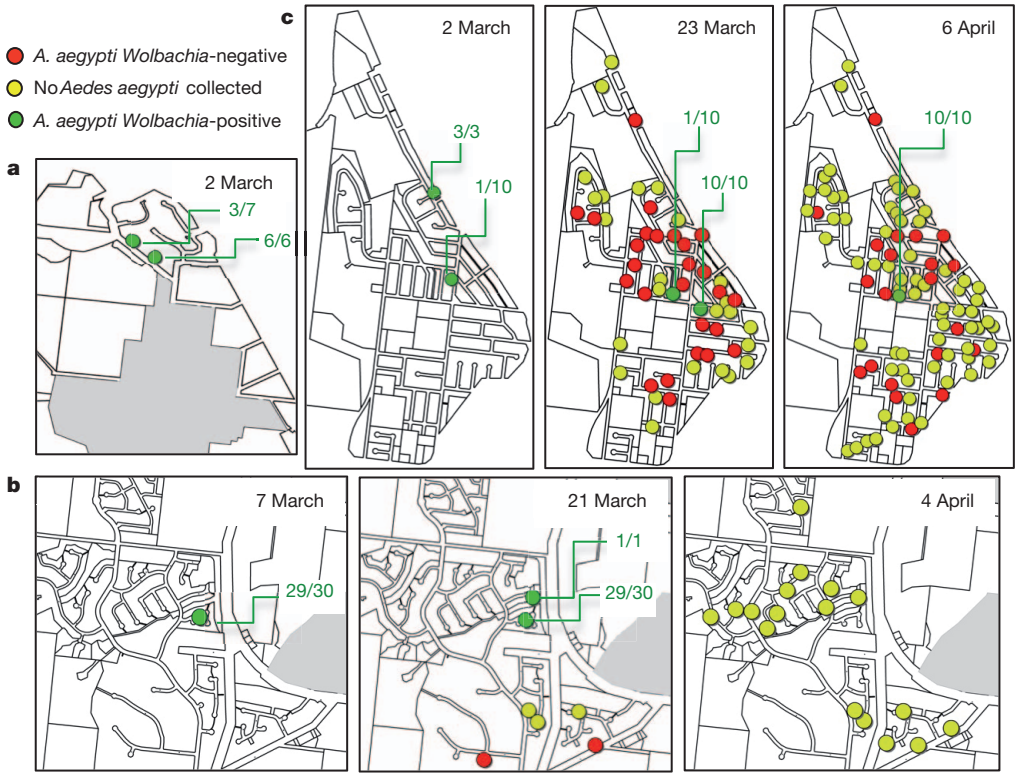

Figure 3 | Occurrence of *Aedes aegypti* and *Wolbachia* infection in ovitraps outside release areas. **a**–**c**, The release areas were adjacent to the Yorkeys Knob release area (**a**), Pyramid Estate, adjacent to the Gordonvale release zone (**b**) and Holloways Beach (**c**; see Supplementary Fig. 1 for locations). Green symbols indicate the presence of *A. aegypti* infected by *Wolbachia*, red symbols indicate only uninfected *A. aegypti*, whereas yellow symbols indicate no *A. aegypti*. Shaded areas indicate release zones. Numbers on graphs show the number of infected larvae (from total collected). Collections occurred at the onset of the dry season when *A. aegypti* abundance was declining.

incompatibility[23]. However, the present results provide the first case where wild insect populations have been transformed to reduce their ability to act as vectors of human disease agents. This success with the deliberate release of *Wolbachia*-infected insects follows early unsuccessful attempts to manipulate insect populations through other genetic control strategies, including chromosomal manipulations and lethal genes[24,25]. Because *w*Mel and other *Wolbachia* strains inhibit dengue virus transmission in the laboratory[3,7], there is potential to use this technology for area-wide suppression of dengue transmission. We have demonstrated that it is possible to produce *Wolbachia*-infected mosquito populations that can act as 'nursery' areas for future human-assisted collection and further dispersal of *Wolbachia*-infected mosquitoes, without the need to rear additional mosquitoes in an insectary. This should provide a strategy for sustainable dengue control at low cost, with a relatively simple deployment system suitable for implementation in developing countries. The next step is a disease endpoint trial to test efficacy of the method for dengue and dengue haemorrhagic fever control, ongoing monitoring in and around the release area to test for persistence, and releases to test the spatial spread of the infection across a populated area.

METHODS SUMMARY

The outcrossed *A. aegypti w*Mel-infected line described elsewhere[7] was backcrossed to the offspring of Cairns field-collected mosquitoes. Adult mosquitoes from the backcrossed line were maintained in a semi-natural field cage. Offspring were reared to the adult stage in a separate field cage to ensure ambient conditions during development, and then released on 10 occasions at weekly intervals throughout release zones at Yorkeys Knob and Gordonvale starting in early January 2011. Releases and monitoring were interrupted by up to a week when tropical cyclone Yasi made landfall south of Cairns. To monitor *Wolbachia* frequencies, a grid of up to 320 ovitraps was placed at each release area every two weeks. Eggs were hatched and larvae rinsed before being tested for infection status through a multiplexed PCR assay. *Wolbachia* frequencies in release areas were pooled into 40–42 blocks (up to 8 ovitraps per block) and blocks with particularly low infection frequencies were identified for adaptive management consisting of doubling release rates. Releases were terminated after the overall *Wolbachia* frequency exceeded 70%. Traps were also deployed in suburbs adjacent to each release zone to monitor the wider spread of *Wolbachia*. A series of quality control procedures was followed to ensure that frequencies assessed through individual larvae were reliable. This included checking frequencies in independent laboratories and from different trapping methods, and excluding the possibility of cross contamination due to re-use of containers. Community support for the releases was very strong following an extensive period of consultation before commencement of the releases and ongoing communication throughout the release and monitoring periods.

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

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1. Gould, F. & Schliekelman, P. Population genetics of autocidal control and strain replacement. *Annu. Rev. Entomol.* **49,** 193–217 (2004).
2. Turelli, M. & Hoffmann, A. A. Cytoplasmic incompatibility in *Drosophila simulans*: dynamics and parameter estimates from natural populations. *Genetics* **140,** 1319–1338 (1995).
3. Moreira, L. A. *et al.* A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, chikungunya, and *Plasmodium*. *Cell* 139**,** 1268–1278 (2009).
4. McMeniman, C. J. *et al.* Stable introduction of a life-shortening *Wolbachia* infection into the mosquito *Aedes aegypti*. *Science* **323,** 141–144 (2009).
5. Kambris, Z., Cook, P. E., Phuc, H. K. & Sinkins, S. P. Immune activation by life-shortening *Wolbachia* and reduced filarial competence in mosquitoes. *Science* **326,** 134–136 (2009).
6. Bian, G. W., Xu, Y., Lu, P., Xie, Y. & Xi, Z. Y. The endosymbiotic bacterium Wolbachia induces resistance to dengue virus in *Aedes aegypti*. *PLoS Pathog.* **6,** e1000833 (2010).
7. Walker, T. *et al.* A non-virulent *Wolbachia* infection blocks dengue transmission and rapidly invades *Aedes aegypti* populations. *Nature* doi:10.1038/nature10355 (this issue).
8. Kyle, J. L. & Harris, E. Global spread and persistence of dengue. *Annu. Rev. Microbiol.* **62,** 71–92 (2008).
9. Guzman, M. G. *et al.* Dengue: a continuing global threat. *Nature Rev. Microbiol.* 8, S7–S16 (2010).
10. Turelli, M. & Hoffmann, A. A. Rapid spread of an inherited incompatibility factor in California *Drosophila*. *Nature* **353,** 440–442 (1991).
11. Riegler, M., Sidhu, M., Miller, W. J. & O'Neill, S. L. Evidence for a global *Wolbachia* replacement in *Drosophila melanogaster*. *Curr. Biol.* **15,** 1428–1433 (2005).
12. Turelli, M. Cytoplasmic incompatibility in population with overlapping generations. *Evolution* **64,** 232–241 (2010).
13. Yeap, H. L. *et al.* Dynamics of the 'popcorn' *Wolbachia* infection in *Aedes aegypti* in an outbred background. *Genetics* **187,** 583–595 (2011).
14. Caspari, E. & Watson, G. S. On the evolutionary importance of cytoplasmic sterility in mosquitos. *Evolution* **13,** 568–570 (1959).
15. McMeniman, C. J. & O'Neill, S. L. A virulent *Wolbachia* infection decreases the viability of the dengue vector *Aedes aegypti* during periods of embryonic quiescence. *PloS Neglect. Trop. Dis.* **4,** e748 (2010).
16. Osborne, S. E., Leong, Y. S., O'Neill, S. L. & Johnson, K. N. Variation in antiviral protection mediated by different *Wolbachia* strains in *Drosophila simulans*. *PLoS Pathog.* **5,** e1000656 (2009).
17. Endersby, N. M. *et al.* Changes in the genetic structure of *Aedes aegypti* (Diptera: Culicidae) populations in Queensland, Australia, across two seasons: implications for potential mosquito releases. *J. Med. Entomol.* (in the press).
18. Barton, N. H. & Turelli, M. Spatial waves of advance with bistable dynamics: cytoplasmic and genetic analogs of Allee effects. *Am. Nat.*. doi:10.1086/661246 (2011).
19. Hemme, R., Thomas, C., Chadee, D. & Severson, D. Influence of urban landscapes on population dynamics in a short-distance migrant mosquito: evidence for the dengue vector *Aedes aegypti*. *PLoS Negl. Trop. Dis.* **4,** e634 (2010).
20. Reiter, P., Amador, M. A., Anderson, R. A. & Clark, G. G. Dispersal of *Aedes aegypti* in an urban area after blood feeding as desmonstrated by rubidium-marked eggs. *Am. J. Trop. Med. Hyg.* **52,** 177–179 (1995).
21. Harrington, L. C. *et al.* Dispersal of the dengue vector *Aedes aegypti* within and between rural communities. *Am. J. Trop. Med. Hyg.* **72,** 209–220 (2005).
22. David, M. R., Lourenco-de-Oliveira, R. & de Freitas, R. M. Container productivity, daily survival rates and dispersal of *Aedes aegypti* mosquitoes in a high income dengue epidemic neighbourhood of Rio de Janeiro: presumed influence of differential urban structure on mosquito biology. *Mem. Inst. Oswaldo Cruz* **104,** 927–932 (2009).
23. Laven, H. Eradication of *Culex pipiens fatigans* through cytoplasmic incompatibility. *Nature* **216,** 383–384 (1967).
24. Curtis, C. F. Genetic control of insect pests: growth industry or lead balloon? *Biol. J. Linn. Soc.* **26,** 359–374 (1985).
25. Foster, G. G. Genetic control of sheep blowfly (*Lucilia cuprina*) and the logistics of the CSIRO control program. *Wool Technol. Sheep Breed.* **28,** 5–10 (1980).

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

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Author Contributions A.A.H. and S.L.O. provided oversight of the releases and drafted most of the paper. S.A.R. and B.L.M. provided knowledge of local mosquito populations and liaison with authorities. J.P., I.I.-O., Y.D. and Y.S.L. carried out the *Wolbachia* screening. P.H.J., C.O., J.A., N.K., E.A.M. and A.G.C. were responsible for mosquito culture and backcrossing. Field releases and monitoring collections were undertaken by F.M., M.G., M.D. and B.L.M. and coordinated by B.L.M. and P.A.R. M.T. developed models to interpret the results and A.A.H. interpreted data during the release. H.C., S.L.O., S.A.R., M.D. and P.A.R. were involved in community engagement and mapping. S.L.O. and I.I.-O. were responsible for gaining regulatory approvals for the releases.

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METHODS

Regulatory approval. Approval for the release of *Aedes aegypti* containing *Wolbachia* was provided by the Australian Pesticides and Veterinary Medicines Authority. Considering the novelty of the proposed experiment it was not initially clear how the open release of *Wolbachia* infected mosquitoes should be regulated in Australia[26]. Finally after considerable consultation the Australian Government chose to regulate the release under existing legislation as a Veterinary Chemical product[26]. This is the approach taken to regulate microbial pesticides within Australia.

Community engagement. An extensive community engagement program ran for 2 years before the open release experiments reported here[27,28], with consultation taking place through public meetings, one-on-one meetings, and through engagement with existing community groups. Resident concerns were canvassed and addressed through communication materials and in some cases additional experimental studies[27]. In general community support was extremely high (>85%) as measured through random telephone surveys on two occasions ($n = 300$). Prior to the field trials residents were asked to provide written permission for three separate activities to take place on their properties. These included pre-release suppression of mosquitoes by removing water from potential breeding sites, release of mosquitoes containing *Wolbachia* and the placement of monitoring traps in resident yards. It is notable that recruitment of resident participation at one location, Yorkeys Knob, was led by a local community group, the State Emergency Service, indicating considerable local ownership of the research program. Approximately 66% of 599 households were able to be contacted at Yorkeys Knob and 54% of 652 households at Gordonvale. Of these households 97% agreed and 3% elected not to participate at both locations ($n = 17$ and 11 declining households for Yorkeys Knob and Gordonvale, respectively, on 4 January). Mosquitoes were only released at properties where residents had given permission. No mosquitoes were released at properties of households that chose not to participate (or at properties adjacent to these households). In addition, mosquito control at the household level was offered to a concerned household. During the release period a small number of households became concerned about mosquito releases and their properties were excluded from subsequent releases and monitoring. We also encountered residents that wished to take part during the study and these households were recruited.

During the release period we continued to undertake close engagement with the local community to ensure that residents were informed about the study and were comfortable with continuation of the program. This included monthly random household surveys and monthly meetings with a reference group of residents and community leaders, and ongoing promotion of a free phone number and accessible city project office. Finally, periodic result updates were provided to the community through letterbox leaflet drops, paid advertisements in a local newspaper and engagement with local newspaper and radio media outlets.

Mass rearing and backcrossing. We initiated crosses with a *w*Mel infected population that had already been backcrossed to a population originating from collections around the Cairns area for three generations[7]. This population was backcrossed again to F1 offspring of females collected from Cairns field females for another three generations (that is, nuclear background was expected to be 99.9% from the Cairns region, which forms a genetically homogeneous group[29]). For the backcrosses, eggs from field females were collected and stored at approximately 80% relative humidity in diapause and then hatched as required to be used in successive backcross generations[13]. Populations were then expanded for a generation and used to establish a release population at James Cook University as well as backup colonies at The University of Queensland and The University of Melbourne.

Mosquitoes for release were reared at James Cook University, Cairns campus, in the Mosquito Research Facility which comprises a laboratory and two large ($8.0 \times 9.0 \times 4.1$ m) semi-field cages[30]. The adult colony was allowed to fly freely in one cage, and larvae and adults prepared for release were maintained in the other cage under ambient conditions. Mean daily minimum and maximum temperature and relative humidity conditions in the adult cage were 23.5–29.4 °C and 78.1–95.5%, whereas in the rearing cage it was 23.9–30.1 °C and 76.6–95.7%.

A population of 800–1,600 female *A. aegypti* (and a similar number of males) was maintained in the adult cage. This cage was initially populated with 900 mosquitoes from the backcrossed colony replenished weekly by 240–720 additional female and male pupae from the larval rearing cage. To minimise laboratory adaptation, male *A. aegypti* pupae from field-collected *A. aegypti* (F1-F2 eggs) were introduced to the cage each week so that they constituted around 20% of the new male population. Mosquitoes were provided access to human volunteer bloodfeeders almost daily in this colony (JCU Human Ethics Approval H2250). Volunteer blood feeders were initially bled and tested for dengue infection by IgM ELISA and PCR, then aural temperature was taken before each feeding to exclude individuals with fever (temperature > 38 °C). Eggs were collected on red flannel cloth in twelve 4.2 l plastic ovibuckets spread throughout the cage, incubated for 3 days and stored in the laboratory for further use.

Three cohorts of approximately 10,000 adults (15,000 larvae) were reared each week. Eggs were hatched in a fresh baker's yeast solution (0.8–1.0 g in 2 l tap water). The following day, larvae were transferred to buckets (4 l) in the rearing cage at a density of approximately 150–200 larvae per 2 l water. Larvae were fed TetraMin Tropical Tablets (total 0.90–1.20 g per rearing bucket over 6 days plus 0.08 g at hatching). Seven days after hatching, when approximately 75% of larvae had pupated, larvae and pupae were concentrated by decanting excess water from the buckets. Pupae/larvae were transferred with a large pipette to adult release cups. These consisted of 750 ml clear plastic cups with the inside surface scratched and roughened with sandpaper, covered with mesh cloth and secured with plastic lids with holes (Supplementary Fig. 5). Approximately 50–70 pupae/larvae were normally added to each release cup with 300 ml clean water and no food. Adults were allowed to emerge and mature in the release cups over 6 days and were provided with a 50% honey solution on a sponge resting on top of each cup. On the day before a release, water was poured off and the honey-soaked sponge was replaced. Adults were 2–6 days old at the time of release, and the sex ratio in the cups was close to 1:1 (51% females). Mortality was minimal (<5%) in the release cups.

Two backup colonies each capable of producing around 10,000 adults per week were maintained in insectaries at The University of Queensland (UQ) and The University of Melbourne (UM). These colonies were maintained with human blood feeding (UQ Human Ethics Approval 2007001379, UM Human Ethics Approval 0723847.2) of adults in cages (around 200 adults per cage, eight cages per colony) and rearing larvae in trays (around 50 trays per colony). These colonies were maintained as a contingency in the event that the main rearing was compromised, but they were not needed for the releases.

Releases. Field teams visited all residential properties within both study locations (Yorkeys Knob, 614 properties; Gordonvale, 668 properties) 1–4 weeks before the commencement of the adult release schedule and were provided access to around half of them. Water bearing containers were emptied, and larger containers were siphoned out whenever possible. Chemicals were not applied, to minimize disruption to potential mosquito breeding sites following adult releases. Where possible, *Aedes* eggs were removed by scrubbing the inside of these containers with a brush.

Weekly releases of adults were undertaken for 10 consecutive weeks, starting 4 January 2011. Adult release cups were transported from the Mosquito Research Facility in vehicles with dampened black cloth covering crates to maximise humidity. A minimum number of 184 (Yorkeys Knob) or 190 (Gordonvale) release points were evenly distributed throughout the release area. Each week release points were rotated between adjacent properties to minimize any discomfort to residents due to mosquito biting activity. Adults were released at the property fence line or front yard by removing the container lids and gently shaking out any mosquitoes that did not immediately fly out. Releases commenced at 08:00 a.m. and were usually completed within 2 h. We removed mosquitoes in the delivery vehicles at the conclusion of each release event with a sweep net. Five unopened cups were immediately returned to James Cook University to assess the impact of transportation on mortality.

Release management and monitoring. Adult *A. aegypti* populations were monitored in the release area by BioGents Sentinel mosquito traps (BGS) which are an effective surveillance tool for adult *A. aegypti*[31]. A network of these traps was established in residential properties ($n = 16$ and 13 for Yorkeys Knob and Gordonvale, respectively) and serviced each week since October 2009. In Yorkeys Knob, 16 BGS traps collected a mean of 0.48 (confidence interval = 0.25–0.70) female *A. aegypti* over a 4-week period (2–30 December 2010), while in Gordonvale the 14 BGS traps collected a mean of 0.92 (confidence interval = 0.38–1.46) females. Mark-release-recapture trials conducted in houses in Cairns indicated that BGS traps captured 25% (confidence interval 21–29%) of released female *A. aegypti* within 24 h (P. H. Johnson, unpublished data). Thus the BGS mean daily trap collection needs to be multiplied by at least four to estimate the population of female *A. aegypti* per premise. However, these are underestimates because both teneral (recently emerged adults) and engorged, blood-fed female *A. aegypti* are poor fliers and undersampled by BGS traps[32]. Furthermore, *A. aegypti* in upper level rooms, other buildings and cryptic sites are not included in the mark-release study; the number of female *A. aegypti* per premise is therefore likely to have been substantially higher, most likely five or more per premise for Yorkeys Knob and double this figure for Gordonvale.

A confirmed case of dengue fever was reported by Queensland Health at a Yorkeys Knob address on 21 February 2011. The case originated in Innisfail where dengue transmission occurred in 2011. All properties within the dengue response zone (approximately 150 m radius from case house) were subjected to the emergency response protocols activated by Queensland Health. Control activities included yard inspections to treat all water-bearing containers with *s*-methoprene (an insect growth regulator) and the application of residual insecticide (deltamethrin) to mosquito harbourage areas (for example, underside of furniture) within houses

with permission. No further releases were conducted in the affected area after 15 February 2011 (week 7) following this health advice. No cases of dengue were subsequently reported.

Tropical Cyclone Yasi made landfall south of Cairns on 3 February 2011 and resulted in the suspension of a scheduled release at Gordonvale for a week. Releases at Yorkeys Knob were not affected but only half the ovitraps used for monitoring (below) could be collected before the cyclone, and the other half were collected a week later.

Monitoring in release areas. *Wolbachia* infections were monitored fortnightly for 9–10 collection events at each area (January to May, Fig. 1). The ovitraps consisted of 1.2 l black plastic golf-divot buckets (Clayton Plastics) fitted with a red flannel (Spotlight) strip (15 × 12 cm)[33]. The cloth strip provided a vertical oviposition substrate within the bucket and was secured with a clip (Supplementary Fig. 5). Ovitraps were filled with tap water on site and an infusion created by adding half an alfalfa pellet (approximately 0.5 mg).

Ovitraps were deployed at fixed addresses after release zones were subdivided into blocks (40 and 42 in Yorkeys Knob and Gordonvale, respectively, area 0.0085 to 0.0328 km^2). Initially two ovitraps were deployed per property with four properties per block to provide an array of 320 ovitraps per zone, but as *Wolbachia* infection rates increased a single ovitrap was placed at each property and this was further reduced to 100 ovitraps per release zone. This reduction was due to a redeployment of the trapping grid outside of the release area into adjacent suburbs when *Wolbachia*-infected mosquitoes were first detected there (see below). Each ovitrap was bar-coded to enable each trap to be re-deployed to the same address.

Ovitraps were collected 7 days after deployment, and strips were detached from the ovitrap wall before buckets were flooded with water and a 5% yeast extract to induce hatching. Approximately 40 h later each ovitrap was inspected for larvae. The larvae were transferred through a sieve and placed into a 50 ml container holding approximately 200 ml of water and left for 1 h to purge their gut content and minimize contamination due to ingestion of *Wolbachia*-infected exuvia. All larvae were transferred into 80% ethanol vials (5 ml) with barcodes before shipping overnight to University of Queensland for PCR analyses.

We used Spatial Analysis by Distance IndicEs (SADIE) (http://www.rothamsted.bbsrc.ac.uk/pie/sadie) to test for non-randomness of *Wolbachia* frequencies at the property level across the release areas in the January (both locations) and 7 February (Gordonvale) monitoring events. Significant structure (randomization test, $P < 0.01$) was detected in both release zones on these occasions, pointing to areas with consistently low or high *Wolbachia* frequencies, and providing impetus for adaptive management.

Adaptive management. In the first two monitoring periods, blocks with a low frequency of *Wolbachia* were identified and release rates doubled within these blocks in the ensuing release. Individual properties with particularly low frequencies of *Wolbachia* were subject to property inspections (when permitted by the resident) to identify and remove any containers where mosquitoes were breeding.

Because *Wolbachia* frequencies increased rapidly in Yorkeys Knob, additional traps were deployed in adjacent communities (Supplementary Fig. 1) at Holloway's Beach (92 traps), Trinity Park (96 traps), and Pyramid Estate (25 traps).

Wolbachia screening. *Wolbachia* infection status of *A. aegypti* larvae collected in ovitraps was tested with a multiplex PCR assay. DNA was extracted from larvae as described previously[7]. Multiplex PCR was carried out with primers amplifying a fragment of the IS5 repeat element of *Wolbachia* (820 bp) and with primers amplifying the ribosomal DNA internal transcribed spacer 2 (ITS2) from *A. aegypti* (approximately 320 bp). The ITS2 primers were designed to act as a control for the presence of amplifiable DNA or any PCR inhibition in the reaction and to discriminate between the different *Aedes* species present in North Queensland: *Aedes aegypti* (approximately 320 bp), *Aedes palmarum* (approximately 380 bp) and *Aedes notoscriptus* (approximately 400 bp). Volume reactions (10 µl) were set up with 1 µl of extracted DNA as template, 2 µl of 5× buffer, 2 µl of 1 mM dNTPs, 3.55 µl water, 0.125 µl of 20 µM ITS2A (5′-TGTGAACTGCAGGACACAT-3′), 0.125 µl of 20 µM ITS2B (5′-TATGCTTAAATTCAGGGGGT-3′)[34], 0.5 µl of 20 µM IS5F (5′-GTATCCAACAGATCTAAGC-3′), 0.5 µl of 20 µM IS5R (5′-ATAACCCTACTCATAGCTAG-3′), and 0.2 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, 55 °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* IS5 and ITS2 of *A. aegypti* were present. Larvae were scored as negative for *Wolbachia* infection if *Wolbachia* IS5 was absent and ITS2 of *A. aegypti* was present. If both *Wolbachia* IS5 and ITS2 of the host were absent, the sample was excluded. If the ITS2 product had a size ≥ approximately 380 bp, the larvae were designated as 'other species' and not *A. aegypti*.

Quality control. We validated the PCR method across laboratories by transferring larval samples from the same eight containers to screening laboratories in both The University of Queensland and The University of Melbourne. Screening indicated that four of the containers were completely infected based on samples of 10 or 20 individuals ($n = 60$ in total). The same containers also produced only infected individuals in the assay run at The University of Melbourne using samples of 12–20 individuals ($n = 70$ in total). The other containers were polymorphic in the The University of Queensland assay, with a total of 57 infected larvae and 18 uninfected larvae. At The University of Melbourne there were 39 infected larvae and 21 uninfected larvae. The proportion of uninfected and infected larvae did not differ between laboratories by a contingency test ($G = 1.96$, df = 1, $P = 0.161$).

To confirm that *Wolbachia*-infected larvae were breeding in natural resources and not just in ovitraps, larvae were collected from pot plant bases, buckets and other containers, and tested for infection status by PCR. For Yorkeys Knob, larvae were collected from 14 containers between 7 March and 1 April. There were 237 *A. aegypti* larvae, of which 208 (87.8%) were infected. For Gordonvale, larvae were retrieved from 8 containers in late February/early March, and yielded 126 infected larvae out of a total of 146 (*Wolbachia* frequency of 86.3%).

Ovitraps were cleaned in different ways before being used in separate collections. This raises the possibility of cross contamination due to residues from a high abundance of infected larvae in the previous trapping period. To test this, 12 ovitraps from Yorkeys Knob where infection frequencies were high (>90%) were retested. The ovitraps were left dry for one week from the last time they were collected, subjected to different treatments and placed inside cages with uninfected adult *A. aegypti* mosquitoes. In all cases, there was no contamination from previously infected larvae which had developed in the same container: all 470 uninfected larvae were scored as negative in the PCR assays.

26. De Barro, P., Murphy, B., Jansen, C. & Murray, J. The proposed release of the yellow fever mosquito, *Aedes aegypti* containing a naturally occurring strain of *Wolbachia pipientis*, a question of regulatory responsibility. *J. Cons. Protect. Food Safety* **6** (suppl. 1), 33–40 (2011).
27. Popovici, J. *et al.* Assessing key safety concerns of a *Wolbachia*-based strategy to control dengue transmission by *Aedes* mosquitoes. *Mem. Inst. Oswaldo Cruz* **105,** 957–964 (2010).
28. McNaughton, D., Clough, A., Johnson, P. H., Ritchie, S. A. & O'Neill, S. L. Beyond the 'back yard': lay knowledge about *Aedes aegypti* in northern Australia and its implications for policy and practice. *Acta Trop.* **116,** 74–80 (2010).
29. Endersby, N. M. *et al.* Genetic structure of *Aedes aegypti* in Australia and Vietnam revealed by microsatellite and exon primed intron crossing markers suggests feasibility of local control options. *J. Med. Entomol.* **46,** 1074–1083 (2009).
30. Ritchie, S. A. *et al.* A secure semi-field cage for the study of *Aedes aegypti*. *PLoS Negl. Trop. Dis.* **5,** e988 (2011).
31. Williams, C. R. *et al.* Field efficacy of the BG-Sentinel compared with CDC backpack aspirators and CO_2-baited EVS traps for collection of adult *Aedes aegypti* in Cairns, Queensland, Australia. *J. Am. Mosq. Control Assoc.* **22,** 296–300 (2006).
32. Ball, T. S. & Ritchie, S. A. Sampling biases of the BG-sentinel trap with respect to physiology, age, and body size of adult *Aedes aegypti* (Diptera: Culicidae). *J. Med. Entomol.* **47,** 649–656 (2010).
33. Ritchie, S. A. Effect of some animal feeds and oviposition substrates on *Aedes* oviposition in ovitraps in Cairns, Australia. *J. Am. Mosq. Control Assoc.* **17,** 206–208 (2001).
34. Beebe, N. W. & Saul, A. Discrimination of all members of the *Anopheles punctulatus* complex by polymerase chain reaction restriction-fragment-polymorphism analysis. *Am. J. Trop. Med. Hyg.* **53,** 478–481 (1995).