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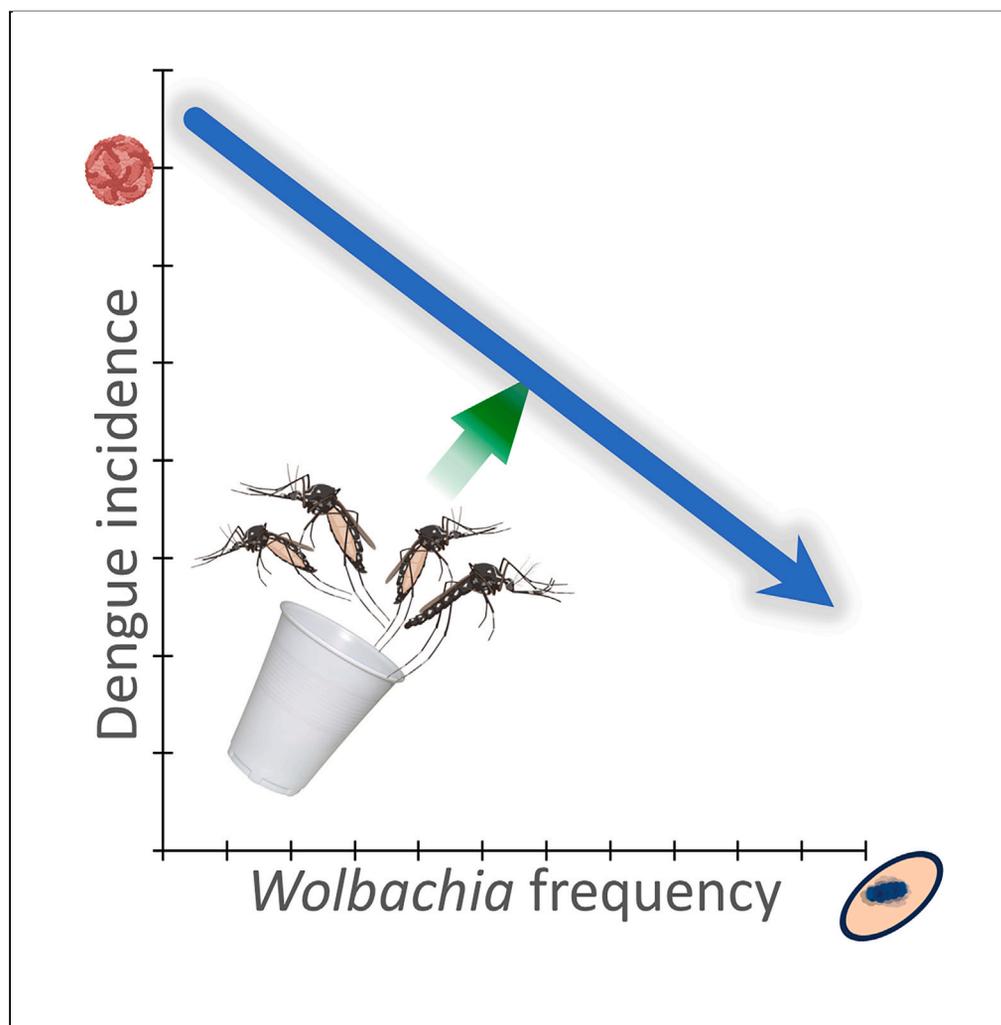
Introduction of *Aedes aegypti* mosquitoes carrying *w*AlbB *Wolbachia* sharply decreases dengue incidence in disease hotspots

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Highlights

Dengue data collated from 20 high-rise residential areas with *Wolbachia* releases

Comparisons with controls indicate a reduction of 62.4% in dengue fever incidence

Dengue reduction increased with *Wolbachia* frequency to a predicted 75.8% at 100%

Article

Introduction of *Aedes aegypti* mosquitoes carrying *w*AlbB *Wolbachia* sharply decreases dengue incidence in disease hotspots

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SUMMARY

Partial replacement of resident *Aedes aegypti* mosquitoes with introduced mosquitoes carrying certain strains of inherited *Wolbachia* symbionts can result in transmission blocking of dengue and other viruses of public health importance. *Wolbachia* strain *w*AlbB is an effective transmission blocker and stable at high temperatures, making it particularly suitable for hot tropical climates. Following trial field releases in Malaysia, releases using *w*AlbB *Ae. aegypti* have become operationalized by the Malaysian health authorities. We report here on an average reduction in dengue fever of 62.4% (confidence intervals 50–71%) in 20 releases sites when compared to 76 control sites in high-rise residential areas. Importantly the level of dengue reduction increased with *Wolbachia* frequency, with 75.8% reduction (61–87%) estimated at 100% *Wolbachia* frequency. These findings indicate large impacts of *w*AlbB *Wolbachia* invasions on dengue fever incidence in an operational setting, with incidence expected to further decrease as wider areas are invaded.

INTRODUCTION

Wolbachia are common symbiotic, inherited intracellular bacteria that can affect the reproduction of their arthropod hosts, allowing them to invade populations. The mosquito *Aedes aegypti*, the primary vector of dengue virus (DENV) and several other arboviruses, does not naturally carry the symbiont[1]; following laboratory introduction into this species, however, some *Wolbachia* strains are able to block the transmission of DENV. Releases of *Wolbachia*-carrying *Ae. aegypti* were first initiated in northeastern Australia in 2010[2] with the aim of replacing *Wolbachia*-free populations with populations carrying a high frequency of this bacterium, and thus decrease the transmission of DENV (e.g., [3–5]). The initial releases were undertaken with a *Wolbachia* strain (*w*Mel) that originated in *Drosophila melanogaster*.[2] Since that time releases have also been initiated with other *Wolbachia* including the *w*AlbB strain originating in *Aedes albopictus*, which causes similar levels of virus transmission blockage in multiple population backgrounds.[3,4,6,7] Virus blockage with a different *w*AlbB variant has been found to vary between genetic backgrounds.[8] Both invasions of *w*Mel and *w*AlbB have had a substantial impact on the local incidence of dengue fever.[6,7,9–11] However, in some areas there have been issues in achieving and maintaining the high *Wolbachia* population frequencies in *Ae. aegypti* that are necessary for effective disease control.[7,9,12]

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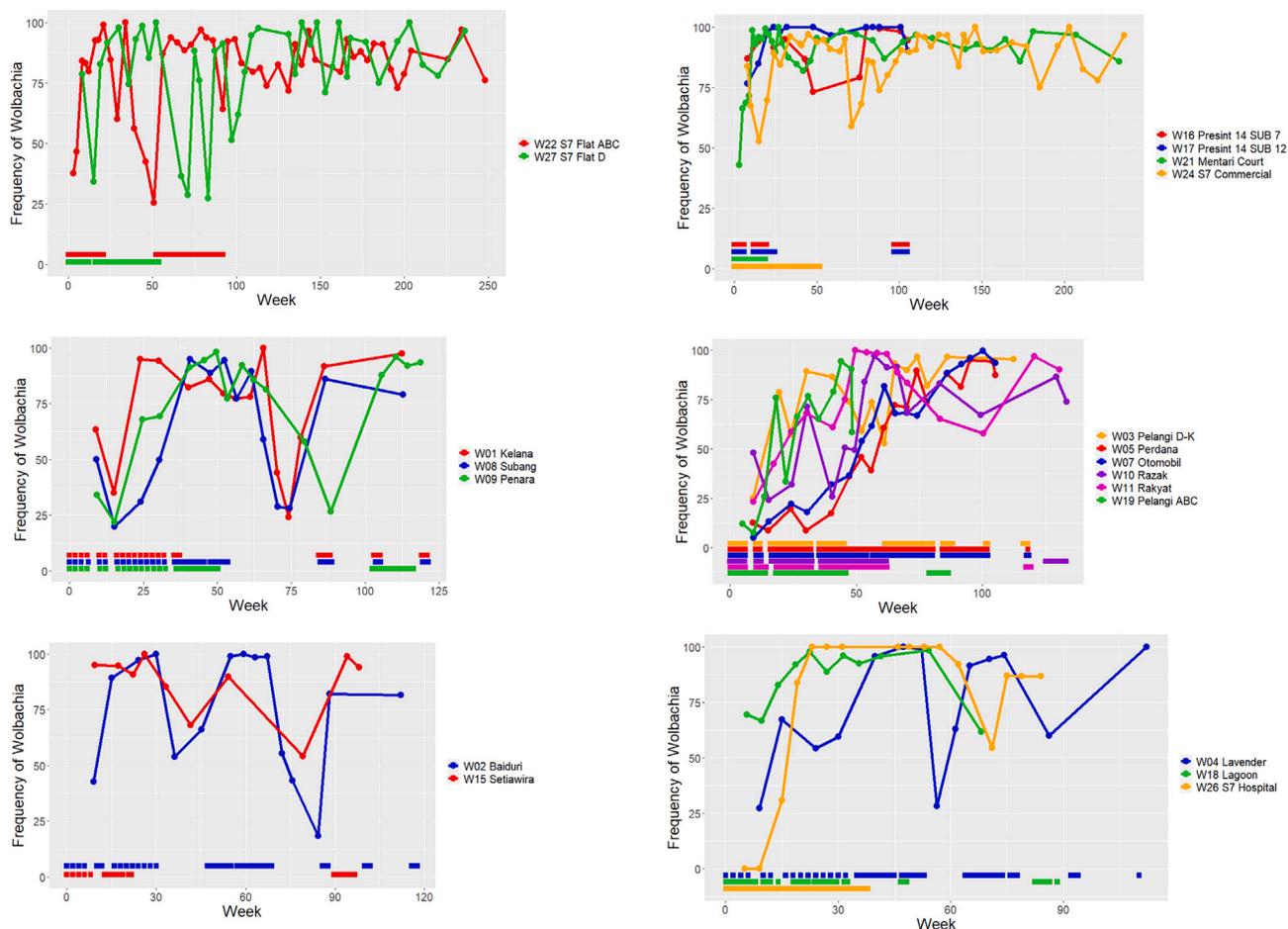[11]MRC-University of Glasgow Centre for Virus Research, Glasgow, UK
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Figure 1. Map of Selangor region showing operational release sites used in evaluating *Wolbachia* impact and control sites
Districts within the region are labeled. Release sites are marked (see Table S1 for code).

For *w*Mel, local transmission cycles of seasonally imported DENV have been virtually eliminated in *Wolbachia*-invaded areas in Australia.[10] In Yogyakarta in Indonesia[11] where dengue is endemic there has been an estimated 77% reduction in dengue fever incidence following releases. Releases of *w*AlbB in dengue hotspots that acted as initial release sites resulted in an estimated decrease in dengue of 40.3% compared to control sites around the Kuala Lumpur region.[9] Reductions have been obtained using *w*Mel in sites around Rio de Janeiro where invasion has been challenging, with *Wolbachia* frequencies often failing to reach 50% in *Ae. aegypti* populations and averaging 38%.[6]

The relative success of these interventions at research sites despite some challenges has increased interest around the world from health authorities wanting to use *Wolbachia* releases routinely to suppress dengue fever. A range of *w*Mel and *w*AlbB *Wolbachia* strains as well as others are starting to be developed for in country use (e.g.,[13,14]). Releases have been initiated in multiple locations around the world with local resources, and cost-effectiveness analyses have been completed to support the widescale use of this technology.[15]

In Malaysia, releases have focused on a *Wolbachia* *w*AlbB strain introduced into a local genetic background.[4] The *Wolbachia* Malaysia project was launched in 2017, releasing *w*AlbB-carrying *Ae. aegypti* in 11 dengue hotspots in the Klang Valley around Kuala Lumpur.[9] Since then, *Wolbachia* density and frequency as well as dengue blocking ability have remained stable in many of the original release areas.[16,17]

Because of the promising effect of the initial releases in reducing dengue incidence, the Disease Control Department of the Malaysian Ministry of Health, in collaboration with the Institute for Medical Research Malaysia, deployed *Wolbachia*-carrying *Ae. aegypti* to various additional dengue hotspot localities around Kuala Lumpur in a move to make *Wolbachia* releases part of the normal operations the government takes against dengue fever. These activities coupled with ongoing monitoring at some of the original release sites (Figure 1) and the availability of long-term data on dengue fever incidence provide a unique opportunity to reassess impact on dengue fever incidence associated with *w*AlbB invasion. They also provide an opportunity to assess the impact of different *Wolbachia* frequencies attained at sites on dengue fever incidence.

RESULTS

Wolbachia invasion and persistence

Because releases were started in different areas at different times, changes in *Wolbachia* frequency are presented as weeks since the releases were initiated (Figure 2). The weeks when releases were undertaken are also presented on these graphs. *Wolbachia* frequencies increased rapidly at some sites and stayed high (W16, W17, W21, and W24) which included two research sites. At others (W03, W05, W07, W10, W11, and W19) they increased more slowly to reach a high frequency. At still others (W22, W27) there seemed to be a period of instability before frequencies settled at a high level. The remaining sites such as W02 and W15 showed instability which contributed to subsequent releases being undertaken (as marked on the graphs). Some of this instability may have reflected small sample sizes although points are based on a minimum of 10 individuals. Moreover, for some sites showing variable *Wolbachia* frequencies like W22 and W15, the average mosquito numbers scored per monitoring point was relatively high (280.2 and 167 respectively) whereas the site (W26) with the lowest average number of mosquitoes per monitoring point (40.2) showed a consistent pattern of change in *Wolbachia* frequency.

Figure 2. Changes in the frequency of *Wolbachia* across time in the release sites
The sites with similar patterns of *Wolbachia* changes are grouped together. Frequency estimates are based on a minimum of 10 adults being screened. Periods of mosquito release are also indicated on the graphs along the bottom color coded for each release site.

Dengue impact

Across all 20 release sites, the mean *Wolbachia* frequency in the release + post-release monitoring period averaged 82.3%, ranging from 56.2% to 96%. A Bayesian model provided the posterior mean estimate of the reduction in dengue fever incidence at this average *Wolbachia* frequency of 62.4% (95% credible interval: 50–71% reduction), ranging from a 42.6% (95% CI: 34–49%) decrease in the lowest-frequency site to a 72.8% (95% credible interval: 59–83%) decrease in the highest frequency site. At a hypothetical 100% *Wolbachia* frequency, the model estimated a posterior mean reduction in dengue incidence of 75.8%, with a 95% credible interval from 61 to 87%. Table 1 summarizes the posterior distributions over the model parameters. The posterior probability of *Wolbachia* releases resulting in a reduction in dengue incidence was greater than 0.999.

DISCUSSION

With the additional release sites and extended period of dengue fever monitoring, we find stronger evidence for dengue reduction following release of *w*AlbB-carrying mosquitoes than recorded in our earlier work,[9] particularly at high *Wolbachia* frequencies in release sites. At some research sites like Mentari Court and S7 Commercial, dengue fever cases have now been reduced for a number of years and the *Wolbachia* has remained stably high without further releases; in high-rise blocks at Mentari Court, the *Wolbachia* also remains well spread throughout the apartment blocks where interventions took place.[9,17] Dengue reduction has persisted despite no further community engagement in these sites. Clearly as *Wolbachia* frequencies stabilize in new areas there is the potential for further increasing the impact of the infection on dengue fever cases.

The level of dengue fever reduction is similar to that achieved in Yogyakarta with *w*Mel where the *Wolbachia* frequency in invaded areas was 95.8%[11] although that project employed releases over larger areas in a randomized design rather than through an operationalized approach aimed at specific areas where dengue was common. *Wolbachia* frequencies in large, invaded areas are expected to be more stable than small areas since there is a lower likelihood of movement of *Wolbachia*-free mosquitoes into an area,[18] although this will also depend on

Table 1. Parameter estimates for variables in the model estimating *Wolbachia* release effects

Parameter	Posterior mean	95% credible interval
ρ (autoregression parameter in the random timeseries model for weekly dengue cases)	0.937	0.93–0.94
σ (noise parameter in the random timeseries model for weekly dengue cases)	0.482	0.47–0.5
β (proportional change in dengue cases if Wolbachia is at 100%	−0.758	−0.87––0.61

the structure of the urban environment including the presence of wide roads.[19] DENV will be acquired outside release areas and contribute to the local incidence of dengue fever in an area, making it less likely to detect large reductions in small areas. While the incidence of dengue fever reduction in some areas have been more moderate than achieved with *w*Mel in Yogyakarta, such as a 38% reduction in Rio de Janeiro,[6] the *w*Mel *Wolbachia* incidence was also lower in this area at 33.8% overall.

There was a substantial impact of *Wolbachia* frequency on dengue reduction with the Bayesian model indicating a strong overall negative association between *Wolbachia* frequency and dengue fever incidence. This pattern was clear at sites such as W05, W08, and W10, where the *Wolbachia* frequency was relatively low reflecting a slow invasion or instability in *Wolbachia* frequencies (Figure 2), while the incidence of dengue fever remained relatively high after releases started (Figure 3). The reverse pattern was evident at other sites like W16, W17, and W24, although W15 was an exception. Overall, these findings highlight the importance of continued *Wolbachia* monitoring and taking the necessary steps when there is a decrease in *Wolbachia* frequencies. Booster releases were successful in re-establishing or maintaining high frequencies (such as locations Kelana and Subang in Figure 2) but this does entail ongoing monitoring of release sites.

The reasons for the observed variation in *Wolbachia* stability remain unclear, with no obvious connection to any of the variables defining the release sites (Table 1). However, it is consistent with data from release programs in other countries that highlight variable success in obtaining introductions which include slow increases in some populations.[6,20] Identifying the factors involved remains an intriguing area for future research, with a number of factors that could be important such as environmental conditions, local mosquito movement patterns (including immigration from neighboring areas such as construction sites with high mosquito density), and the nature of breeding sites (predominance of permanent versus temporary, periodically inundated breeding sites, which will affect *Wolbachia* fitness costs).[12,21,22] Ongoing fogging in neighbourhoods adjacent to release sites is another possibility. Theory also emphasizes the importance of density dependent interactions.[23] A combination of local *Wolbachia* and entomological monitoring as well as molecular approaches such as tracking mtDNA variation[24] and new ways of tracking breeding sites[25] could provide useful tools in developing this understanding.

The outcome of this research program followed by an expanded operational program serves as the basis for future expansion of releases in additional dengue-prone areas. To date, a total of 40 localities inclusive of research sites have been used for releases with *Ae. aegypti* carrying *w*AlbB *Wolbachia* in 8 states in Malaysia. The Malaysian Ministry of Health has established plans for future release of *Wolbachia* mosquitoes in dengue hotspots as a national rollout program. The results support the continued use of *w*AlbB in the Kuala Lumpur area, where temperatures in breeding sites can be high, so the proven strong stability of *w*AlbB *Wolbachia* under hot conditions[26,27] remains important.

Limitations of the study

Because of the operationalized nature of the releases, we were unable to successfully randomize control and release sites prior to the start of the study. When health authorities are tackling dengue fever outbreaks with limited resources, the focus is on responding rapidly to existing outbreaks as well as local community pressure. This prevented the adoption of a "gold standard" intervention design. Because of funding restrictions, we also relied on local health officers with different levels of experience in undertaking mosquito releases and establishing a trapping network, rather than being able to use the same team for releases and monitoring in all areas. Finally, we were required to change the nature of the releases in several areas due to restrictions imposed by the covid outbreak (see STAR Methods). Nevertheless, these limitations are in some ways also the strength of the study which provides an indication of the level of dengue fever suppression one might expect to be achieved by *w*AlbB invasion in a realistic setting.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- METHOD DETAILS
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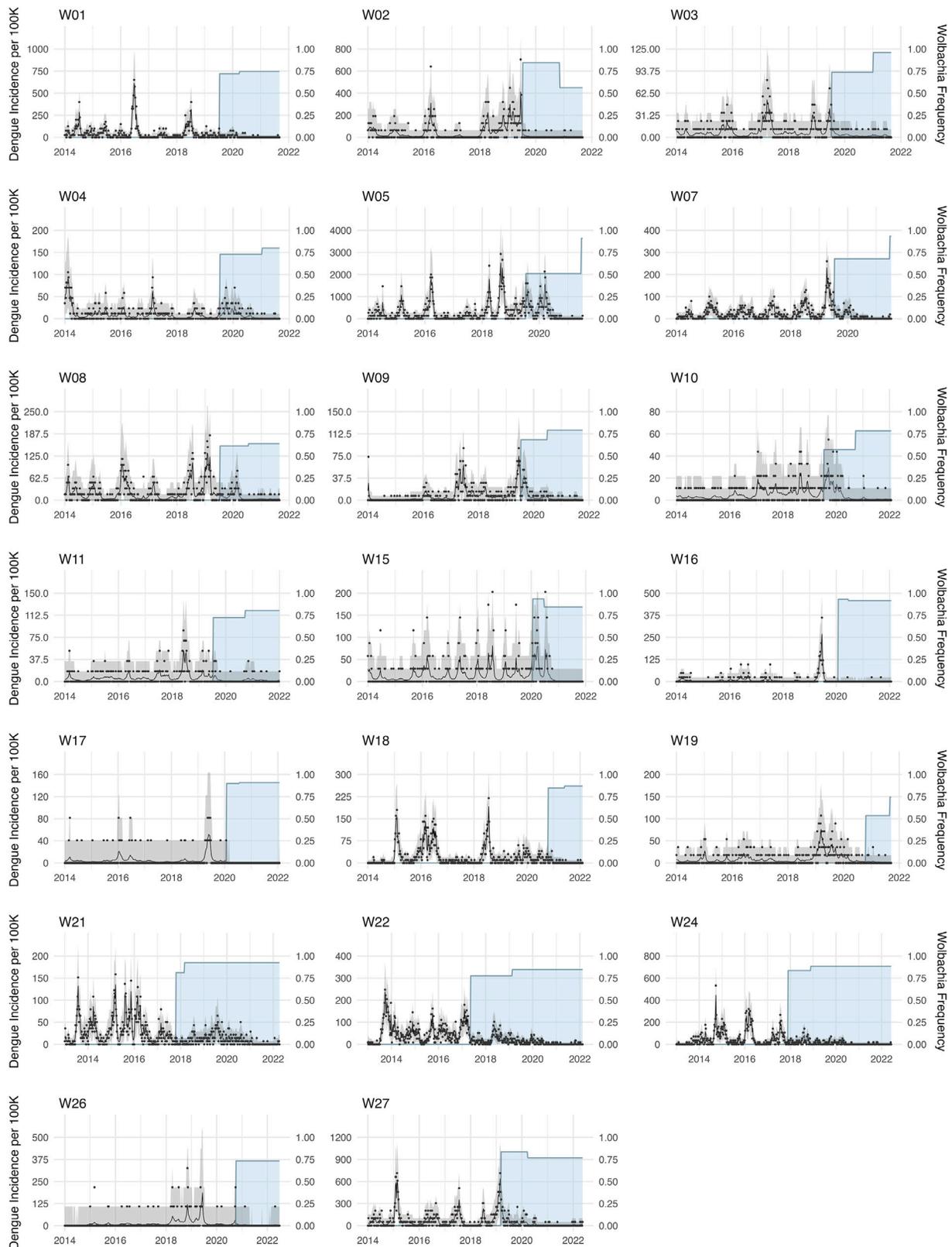
Figure 3. Incidence of dengue in sites the 20 release sites

Data are black dots, the posterior mean predicted incidence is represented by the black lines, with the 95% predictive interval in gray. The period of *Wolbachia* release and mean frequency of *Wolbachia* are also provided (blue region; first level is the release period, second is after releases finished).

- ○ Operational program
- ○ Community engagement
- ○ Operational releases
- ○ Research and control sites
- ○ Wolbachia monitoring
- ○ Dengue data
- ● QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.108942.

AUTHOR CONTRIBUTIONS

Conceptualization, A.A.H., N.W.A., S.P.S., T.A., and W.M.K.; Methodology, N.W.A., A.A.H., S.P.S., and C.Y.L.; Investigation, N.W.A., N.A.A., J.J., P.W.T., N.M., S.S.S., L.S.M., K.K., K.D., N.M.R., H.S., T.O., M.K.R.G., N.Z., M.A.A.K., M.I.S., and M.N.M.N.; Formal Analysis, N.G., N.T., C.Y.L., and A.A.H.; Visualization: N.G., N.T., and C.Y.L.; Writing – Original Draft, A.A.H., S.P.S., N.W.A., and N.G.; Writing – Review and Editing, A.A.H., S.P.S., and N,W,A; Funding Acquisition, S.P.S., T.A., and W.M.K.; Resources, S.P.S., T.A., and W.M.K.; Supervision, N.W.A., M.K.R.G., and N.A.A.; Management, N.W.A.,T.A., W.M.K., S.P.S., and A.A.H.

DECLARATION OF INTERESTS

The authors declare that they do not have conflicting interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
wAlbB carrying *Aedes aegypti*	Microinjected line from Ant et al.[4] and field sourced infected mosquitoes from areas where line established[9]	N/A
Deposited data		
Data generated during the study	This study	https://doi.org/10.26188/24314689.v1
Software and algorithms		
Analysis code	Similar to Nazni et al.[9]	https://github.com/goldingn/wolbachia_kl
Vegan package	N/A	https://cran.r-project.org/web/packages/vegan/vegan.pdf
greta	N/A	https://github.com/greta-dev/greta
coda	N/A	https://rdrr.io/cran/coda/

RESOURCE AVAILABILITY

Lead contact

- Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Ary Hoffmann (ary@unimelb.edu.au).
- For enquiries about the *Ae. aegypti-w*AlbB line, contact Steven Sinkins (steven.sinkins@glasgow.ac.uk).

Materials availability

- This study did not generate new unique reagents.
- There are MTA restrictions to the availability of the *Ae. aegypti-w*AlbB line, due to collaborative agreements put in place to ensure all further releases of this line are conducted in a carefully controlled manner.

Data and code availability

- Dengue and *Wolbachia* data are available as MS Excel sheets in a public repository at https://doi.org/10.26188/24314689.v1.
- R code for Bayesian analysis is freely available at https://github.com/goldingn/wolbachia_kl.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

The wAlbB *Wolbachia* line that formed the basis for the releases had previously been produced by microinjection[4] into an uninfected *Aedes aegypti* line originating from Kuala Lumpur. The line was transferred from Glasgow University to the Institute for Medical Research, Kuala Lumpur, where backcrosses were undertaken prior to mass rearing to maximize the fitness and competitiveness of the mosquitoes to be released.

All aspects of rearing the mosquitoes and quality control follow the description in Nazni et al.[9] with minor modifications. Briefly, weighed eggs from colonies were submerged in seasoned tap water (after desiccation) and exposure to an air vacuum to stimulate hatching. The seasoned water consisted of tap water stored overnight to dechlorinate the water. Eggs were hatched in beakers with the seasoned water. These eggs were used for mass rearing and for release where egg containers were used. In other releases, adults rather than eggs were released and eggs were shipped to local field workers to produce the adults. Quality control measures during culture included wing measurements.

Releases were undertaken as part of a program (the "operational program") run by the Ministry of Health targeting multi-storey buildings where dengue incidence was historically high. This program followed the successful introduction of *Wolbachia* at research sites which was described in Nazni et al..[9] The *Wolbachia* operational program started on 7 July 2019 and has been undertaken in several phases as resources and other factors such as COVID lockdown have allowed. The operational program has so far targeted 40 sites but here we focus on 15 sites as well as five sites from the initial research program where continued monitoring has taken place and where there is an adequate elapsed time for dengue monitoring since releases have started. Changes in dengue incidence at these sites was compared to those recorded at 76 control sites consisting of high dengue areas close to the release sites which also consisted of multi-storey developments (Figure 1).

A fundamental requirement for successful implementation of a mosquito release program is community engagement involving comprehensive education of the local community. This ensures that the community appreciates the underlying objectives and strategies associated with the release initiative. The engagement efforts were undertaken by the Institute of Behavioural Research (IPTK) in collaboration with the Institute of Medical Research (IMR).

Following releases, *Wolbachia* frequencies were assessed using mosquito larvae collected with 100 ovitraps per release site. Mosquitoes were identified to species and *Wolbachia* status was assessed through qPCR. Dengue data for each site was obtained from the Disease Control Division, Ministry of Health, with each recorded dengue case confirmed by clinical and laboratory criteria. A Bayesian time series analysis was used to assess the impact of *Wolbachia* releases on dengue fever incidence following Nazni et al.[9] but with a consideration of *Wolbachia* frequency averaged across the release and post-release period.

METHOD DETAILS

Mosquito culture

For mass rearing adults, mosquito eggs were laid on paper and around 15000 eggs estimated by weight were soaked in 1 l of seasoned water in a 36 x 26 x 5.5 cm container. Two days after the hatching process, the larvae were filtered and put into a beaker containing 500 ml of seasoned water and 10 aliquots of larvae (1 ml per aliquot) were taken from the beaker using a 10 ml plastic pipette (approximately 50 larvae per 1 ml aliquot). 10 of these aliquots were placed into a 36 x 26 x 5.5 cm plastic container with 1 l of seasoned water. Sera Vipan powder (Heinsberg, Germany) was given daily to ensure even pupation.

To separate sexes, 12,000 larvae and pupae were introduced into a pupal separation system (Orinno Technologies, Singapore). These immature stages were separated over a period of around 13 min, producing groups of separated (mostly) male pupae, (mostly) female pupae and larvae.

For producing the next generation of the stock population of mosquitoes, we used 3750 female and 1250 male pupae which were transferred to a plastic container filled with seasoned water and placed in a mosquito cage (30.5 x 30.5 x 30.5 cm) to emerge into adults. Emerged mosquitoes were fed on laboratory-reared mice before oviposition on paper to restart the process. Wing measurements were carried out periodically for quality control and wild type males collected from field stock were introduced every 7 generations to ensure fresh material entered the culture regularly.

Operational program

Phase 1 of the operational program involved releasing eggs in containers provided to the Ministry of Health field workers. Two other phases have since been added, resulting in 40 sites now where releases have been conducted. The focus here is on the first 15 operational sites representing the first two operational phases and the 5 sites from the original program[9] where sufficient *Wolbachia* and dengue data is available across 5 years to test for intervention effects.

Wolbachia release areas in the Selangor region (Figure 1) were based on selection criteria important to local health staff and managers which focused on the incidence of dengue, presence of mosquitoes, and perceived barriers (mostly roads) around the release sites given that most movement of mosquitoes tends to be within buildings c. f..[28] The majority of the research sites, and thirteen operational release sites were situated in Selangor, while five sites were in Kuala Lumpur and two sites were in Putrajaya.

Release sites (Table S1) consisted of areas that varied in size (19,321 to 276,261 m^2) where *Ae. aegypti* numbers were relatively high (index value derived from the proportion of positive ovitraps placed for 7 days in an area > 40%). Sites with a high *Aedes aegypti* index and low *Ae. albopictus* index were preferred. For each defined area constituting a site, data on the incidence of dengue cases across the previous 5 years were collected, along with information on the relative abundance of *Ae. aegypti* and *Ae. albopictus* mosquitoes (based on prior ovitrap surveillance).

For each individual site nominated by authorities, a site profile was generated in terms of the number of residential housing blocks, number of stories, whether the area was treated with outdoor residual sprays and whether it was surrounded by any potential barriers to mosquito movement, particularly multi-lane highways or vegetated areas.

Community engagement

Community members were regularly invited to IMR and provided with transparent updates of release progress. Community members were encouraged to be actively engaged in discussions to encourage shared responsibility and ownership. The local community were positive about the release of both male and female *Wolbachia* infected mosquitoes as a way of suppressing dengue and in many cases the mosquitoes encountered after releases were viewed as "good" mosquitoes with a reluctance to kill them even though continued local steps to control were encouraged by engagement staff. The significant reduction in dengue cases within release areas instilled a sense of protective custodianship which led to communities becoming strong advocates for the continuation of the *Wolbachia* program.

A novel communication tool involved the creation of a WhatsApp group to facilitate exchange of ideas, insights, and concerns within the community. Members sought clarifications, shared observations and engaged in dialogues that helped foster collective commitment to disease prevention. This contributed to vigilance in the community about pesticide fogging activities near to release areas communicated to local health departments which often led to the cessation of fogging operations. This represents a good example of community advocacy

for the program. Community champions emerged which shared firsthand experience of release related activities and the insights of these community members extending to other release sites. In control sites community engagement consisted of larval source reduction activities.

Three days prior to the first release, a program of fogging, larval control, breeding site search and destroy activities, and health promotion activities were undertaken as outlined for previous releases.[9] These are normal procedures carried out in dengue hotspot areas as represented by the release and control sites assessed here. For the promotion activities, brochures on the benefits of releasing *Aedes Wolbachia* were generated and distributed to households.

Operational releases

Eggs were initially used in the operational releases but there was a switch to adults later during the COVID -19 period (below). We aimed to release a total of 10 mosquitoes per house unit per week, with equal numbers of males and females being released. The number of eggs released was always double this number, with the expectation that 50% of the eggs would not hatch due for example to predation of eggs and adults emerging from egg release containers (see below).

For egg releases, 200 eggs were introduced into a release container as described in Nazni et al..[9] Egg containers were placed in sites within buildings such as passageways and staircases to ensure minimum disturbance by the community, and in shaded spots to avoid direct exposure to sunlight, which can result in high larval mortality. Egg release containers were visited 3 times; on day 2 after placement to introduced food for larvae, on day 5 to open the stoppers or lids for adults to escape into the open, and on day 7 to collect the egg release containers and to replace these with another set of egg release containers.

The switch from eggs to adults related to the fact that the early release period coincided with COVID-19 pandemic, whereby the several phases of control on the human population were present. The Movement Control Order (MCO) was implemented, then the Conditional Movement Control Order (CMCO) and Recovery Movement Control Order (RMCO) until the end of May 2021. During the MCO, only key government and private services were allowed to operate, while the CMCO and RMCO aimed to revive the economy while implementing social distancing measures to effectively curb the progression of the pandemic. Standard operating procedures (SOPs) and guidelines consisting of health and safety measures was issued by the Malaysian National Security Council via mass media using radio, television, newspapers, government websites, social media, and text messages. During the MCO, the 3Cs (Crowded places, Confined spaces, Close conversation) and applying the 3Ws (Wash hands, Wear masks, Watch your distance, were strictly followed. Due to the introduction of MCO, egg releases were no longer possible and were changed to adult releases to reduce issues around communication and social distancing by MOH staff releasing the *Wolbachia* mosquitoes.

For adult releases, eggs were provided by IMR to the Botanic Laboratory Selangor (Ministry of Health) where eggs were hatched and reared to the adult stage, with releases involving 3-day-old adults (sexes mixed) held in small containers as described in Nazni et al..[9]

Research and control sites

Monitoring continued at 5 of the release sites involving multistorey buildings described in Nazni et al.[9] This included *Wolbachia* monitoring yearly in Mentari Court, 6 monthly Flat A, B and C in Shah Alam, and 3-monthly in Section 7 Commercial Centre (see results).

The control sites were selected to match operational and research sites in terms of involving multi-story residential buildings with a similar area size and population size (Table S1) as well as dengue burden. The control sites were selected based on the highest dengue burden districts with prolonged outbreaks in Malaysia, the districts Hulu Langat, Gombak, Petaling and Klang in Selangor. The ratio of intervention sites to the control sites was ideally set as at least 1:3. We collated the 353,683 reported dengue cases of all four districts from 2014 till 2021. The data was grouped by locality and sorted from highest to lowest total dengue cases according to three time periods, i. e. year 2014 till 2018, 2015 till 2019 and 2016 till 2020, as the release of *Wolbachia* mosquitoes in the intervention sites were started in different phases. The intervention sites were then matched based on high dengue burden, high rise building type and pre-release time period. Seventy-six control sites were identified by the Vector Control Department (Figure 1). However matched groupings had no impact on dengue reduction effects when included in the Bayesian models (below) so only analyses based on pooled control site data are presented.

Wolbachia monitoring

Each ovitrap for collecting mosquitoes consisted of a plastic container (96 mm height, 67 mm diameter) with 150 mL water and a wooden paddle (2 cm x 7 cm). In all *Wolbachia* release operational localities and research localities, 100 ovitraps were set up in the apartment buildings. The ovitraps were spread across every 2-3 floors. If there were carparks for the respective buildings, ovitrap were placed on alternate floors. Ovitraps were collected after a week and the paddle + water was transferred to a plastic container (12 x 3 x 12 cm). All emerging mosquitoes were identified to species and a maximum of 5 *Ae. aegypti* per trap were used for *Wolbachia* screening. *Wolbachia* frequency was only computed in releases (with three exceptions, 2 from W26 (with n=5) and 1 from W27 (with n=7) if at least 10 adults were scored from the ovitraps (with a maximum of 500 possible given that 100 traps were placed at each locality).

In all operational release sites, monitoring was done after 4 weeks from the first release. To ensure that the release procedures and subsequent activities were consistent across operational locations, a Standard Operation Procedure (SOP) for adult releases was developed, involving six stages and implemented as required: (i) an initial release phase; (ii) an ongoing monitoring phase; (iii) booster releases as required; (iv) a second monitoring phase; (v) further booster releases as required; and (vi) a maintenance phase. Adult releases and egg releases were stopped when frequency of *Wolbachia* in field population reached 80% and remained at 80% or more for 3 consecutive monitoring periods across 3 months. Monitoring was undertaken irregularly after that period while research sites were also irregularly monitored.

Adults from ovitraps were stored in absolute ethanol at - 80°C. DNA was extracted from individual mosquitoes using a glass bead and Chelex solution in an extraction tube. Mosquitoes were homogenized in 175 μL of 5% Chelex solution using TissueLyser II machine (QIAGEN) and with 5 μL of proteinase K (20 mg/mL) (Bioron Life Science). The extraction plate was spun down at 4000 rpm for 5 mins and the mosquito aliquot was transferred into 96 plates and spun down again for another 5 minutes. The extraction was incubated in a thermocycler at 65°C for 1 hour, followed by incubation for 10 min at 90°C. The plate was removed and spun down for another 5 mins. *Wolbachia* was detected by high-resolution melting polymerase chain reaction (qPCR-HRM)[27] with 1:10 diluted DNA using the following wAlbB1-specifc primers: wAlbB1-F (50-CCTTACCTCCTGCACAACAA) and wAlbB1-R (50 – GGATTGTCCAGTGGCCTTA), as well as universal mosquito primers: mRpS6_F (50-AGTTGAACGTATCGTTT CCCGCTAC) and mRpS6_R (5 0 -GAAGTGACGCAGCTTGTGGTCGTCC), which target the conserved region of the RpS6 gene, and *Ae. aegypti* primers aRpS6-F (5 0 -ATCAAGAAGCGCCGTGTCG) and aRpS6-R (5 0 -CAGGTGCAGGATCTTCATGTAT TCG), which target the *Ae. aegypti*-specific polymorphisms within RpS6 and do not amplify *Ae. albopictus*.

Reactions were run as 384-well plates in a LightCycler 480 II (Roche). qPCR-HRM was performed in 10μL reactions containing 2 μL of DNA, 0.08 μL of 50 mM forward + reverse primer, 2.92 μL Milli-Q water and 5 μL Ronald's Real-Time Buffer (3.28 μL Milli-Q water, 0.4 μL MgCl2 (50 mM), 1.0 μL ThermoPol Reaction Buffer with 20 mM Magnesium (10x), 0.25 μL HRM Master (Roche), 0.064 μL dNTPs (25 mM) and 0.01 μL Immolase (20 U/μL). qPCR was run following cycling conditions: 95°C for 10 min, followed by 50 cycles of 95°C for 10 s, 58°C for 15 s, 72°C for 15 s. High resolution melting was performed by heating the PCR product to 95°C, and then cooling to 40°C. Then the temperature was increased to 65°C. Samples were considered positive for *Wolbachia* when the Tm for the amplicon produced by the *Ae. aegypti* primers was at least 84°C and the Tm for the *Wolbachia*-primer amplicon was around 80°C.

Dengue data

The dengue incidence for both intervention sites (Figure 3) and control sites (Figures S1, S2, S3, and S4) were obtained starting from 2013. Based on guidelines published by Disease Control Division, namely the Case Definition for Infectious Diseases in Malaysia (2017), a confirmed dengue case was defined as fulfilling both the clinical and laboratory criteria. Clinical criteria include acute onset of high-grade fever of 2–5 days associated with 2 or more clinical features such as headache, retro-orbital pain myalgia, arthralgia, rash and mild haemorrhagic manifestation. All patients presented with relevant symptoms and be diagnosed with suspected dengue will be notified by attending doctor and confirmatory laboratory test will be conducted. The laboratory criteria for dengue included detection of dengue non-structural protein 1 (NS1) or dengue antibody (IgM/IgG) or dengue virus genome by PCR or dengue virus isolation from serum or dengue virus antigen in tissue biopsy. Patients that tested positive for dengue with any of these tests are registered in national dengue registry, also known as eDengue. Dengue combo rapid test kits containing NS1 and IgM/IgG are used as point of care testing in most government health clinics for early diagnosis and treatment.

QUANTIFICATION AND STATISTICAL ANALYSIS

A Bayesian time series model was used to estimate reduction in dengue cases as a result of the increased frequency of *Wolbachia* following releases. The model follows that in Nazni et al.,[9] but the reduction in dengue cases is modelled as proportional to the frequency of *Wolbachia*. The model structure was as follows:

$$y_{i,t} \sim Poisson(\lambda_{i,t} N_i)$$
$$ln(\lambda_{i,t}) = \alpha_i + \gamma_{i,t} + \log(1 + \beta x_i)$$

$$\gamma_{i,t} = \rho\,\gamma_{i,t-1} + \epsilon_{i,t}; \quad \gamma_{i,0} = 0$$

$$\epsilon_{i,t} \sim N(0, \sigma^2); \quad \alpha_i \sim N(0, 10^2);$$

with global parameter prior distributions:

$$\rho \sim U(-1, 1); \quad \sigma \sim N^+(0, 10^2); \quad \log(1 + \beta) \sim N(0, 1^2)$$

where the number of cases y at each site i and week t were assumed to follow a Poisson distribution, with the expected count given by the product of population at that site (N), and the per-capita incidence (λ) which varied varying through time t and between sites. Each site i had a separate time series of log-incidences $\alpha_i + \gamma_{i,t}$ with temporal correlation driven by an autoregressive model of order one, with global parameters ρ and σ^2 describing the prior distribution shared by all sites. Each observation therefore had a separate temporally-correlated random effect on the log scale, to account for extra-Poisson dispersion and temporal correlation in case counts. Both release sites (including pre- and post-release periods) and non-release sites were included, enabling us to estimate expected levels of volatility in case count time series in this part of the model.

The intervention effect was represented by a parameter β and a covariate $x_{i,t}$ giving the mean frequency of *Wolbachia* in the mosquito population in each site and time. The values of $x_{i,t}$ were assumed to be constant over the release and post-release monitoring periods (set to the mean of observations) and set to 0 in pre-release times and in non-release sites. Model parameters were assigned vague priors with normal; positive-truncated normal; or uniform distributions. The $\log(1 + x)$ transformations applied to the intervention effect term ensures that the parameter β can be interpreted as the proportional change in dengue cases if *Wolbachia* frequency was at 100%, which is scaled

linearly according to the achieved *Wolbachia* frequency; i.e. at a *Wolbachia* frequency of 100%, dengue cases are changed by percentage $100 * \beta$, whilst at a frequency of 25%, dengue cases are changed by percentage of $25 * \beta$. This choice of prior implies a median value of 0% change in dengue cases and a 50% interval ranging from a 49% reduction to a 96% increase in dengue cases. The model therefore assumes *a priori* that there is a 50% probability of *Wolbachia* presence reducing dengue cases, and a 50% probability of it increasing cases. The model was fitted to the dengue incidence data to estimate the impact of the releases, and to assess the evidence from the dengue case data that releases lead to a reduction in incidence - quantified as the posterior probability that β is negative.

Posterior samples of model parameters were simulated by Hamiltonian Monte Carlo in greta (https://github.com/greta-dev/greta) with 4 chains, each yielding 4000 posterior samples of model parameters after a warmup period of 1000 iterations during which period the leapfrog step size and diagonal mass matrix parameters were tuned. The number of leapfrog steps was sampled uniformly from between 30 and 40 throughout. Convergence was assessed by the Gelman-Rubin $\widehat{R}$ diagnostic, using the coda R package[29] ($\widehat{R} \leq 1.01$ for all parameters) and visual assessment of trace plots. Model fit was assessed by posterior predictive simulation: a random dataset of $y_{i,t}$ values was generated according to each posterior sample of $p_{i,t}$ and r, and the distributions of the simulated $y_{i,t}$ values were compared with the observed $y_{i,t}$. The analysis code is freely available online at https://github.com/goldingn/wolbachia_kl.