RESEARCH ARTICLE

UPDATE Scaled deployment of *Wolbachia* to protect the community from dengue and other *Aedes* transmitted arboviruses [version 3; peer review: 2 approved]

Scott L. O'Neill [1], Peter A. Ryan[1], Andrew P. Turley[1], Geoff Wilson[1], Kate Retzki[1], Inaki Iturbe-Ormaetxe[1], Yi Dong[1], Nichola Kenny[1], Christopher J. Paton[2], Scott A. Ritchie[2], Jack Brown-Kenyon[1], Darren Stanford[1], Natalie Wittmeier[1], Nicholas P. Jewell[3-5], Stephanie K. Tanamas[1], Katherine L. Anders [1], Cameron P. Simmons[1]

[1]Institute of Vector-Borne Disease, Monash University, Clayton, VIC, 3800, Australia
[2]College of Public Health, Medical and Veterinary Sciences, James Cook University, Cairns, QLD, 4878, Australia
[3]Division of Epidemiology and Biostatistics, School of Public Health, University of California, Berkeley, USA
[4]Centre for Statistical Methodology, London School of Hygiene and Tropical Medicine, London, UK
[5]Department of Medical Statistics, London School of Hygiene & Tropical Medicine, London, WC1E 7HT, UK

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Abstract

Background: A number of new technologies are under development for the control of mosquito transmitted viruses, such as dengue, chikungunya and Zika that all require the release of modified mosquitoes into the environment. None of these technologies has been able to demonstrate evidence that they can be implemented at a scale beyond small pilots. Here we report the first successful citywide scaled deployment of *Wolbachia* in the northern Australian city of Townsville.

Methods: The *w*Mel strain of *Wolbachia* was backcrossed into a local *Aedes aegypti* genotype and mass reared mosquitoes were deployed as eggs using mosquito release containers (MRCs). In initial stages these releases were undertaken by program staff but in later stages this was replaced by direct community release including the development of a school program that saw children undertake releases. Mosquito monitoring was undertaken with Biogents Sentinel (BGS) traps and individual mosquitoes were screened for the presence of *Wolbachia* with a Taqman qPCR or LAMP diagnostic assay. Dengue case notifications from Queensland Health Communicable Disease Branch were used to track dengue cases in the city before and after release.

Results: *Wolbachia* was successfully established into local *Ae. aegypti* mosquitoes across 66 km^2 in four stages over 28 months with full community support. A feature of the program was the development of a

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1 **Gregor J. Devine**, QIMR Berghofer Medical Research Institute, Herston, Australia

scaled approach to community engagement. *Wolbachia* frequencies have remained stable since deployment and to date no local dengue transmission has been confirmed in any area of Townsville after *Wolbachia* has established, despite local transmission events every year for the prior 13 years and an epidemiological context of increasing imported cases. **Conclusion**: Deployment of *Wolbachia* into *Ae. aegypti* populations can be readily scaled to areas of ~60km^2 quickly and cost effectively and appears in this context to be effective at stopping local dengue transmission

Keywords
Dengue, World Mosquito Program, Eliminate Dengue, Aedes aegypti, mosquito release, community engagement

2 **Horacio M. Frydman**, Boston University, Boston, USA

Heverton Leandro Carneiro Dutra, Boston University, Boston, USA

Any reports and responses or comments on the article can be found at the end of the article.

Corresponding author: Scott L. O'Neill (scott.oneill@monash.edu)

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UPDATE **Updates from Version 2**

This new version adds new data on both *Wolbachia* monitoring in the local mosquito population as well as dengue case notification and extends the data to cover an additional dengue transmission season in Townsville (new Figure 3 and Figure 4). In addition, a new Interrupted Time Series analysis of the data has been added. Nicholas Jewel and Stephanie Tanamas contributed to the new Interrupted Time Series analysis, and so they have been added to the author list of this version.

See referee reports

Introduction

A growing body of evidence shows that the *w*Mel strain of *Wolbachia*, when introduced into *Aedes aegypti*, reduces the mosquito's ability to transmit key human viruses such as dengue[1], Zika[2,3] and chikungunya[4,5], and this reduction is estimated to have the potential to significantly reduce disease transmission in affected communities[6]. The World Mosquito Program (formerly known as the Eliminate Dengue Program), a not-for-profit consortium, has demonstrated previously that, after small-scale releases, the *w*Mel strain of *Wolbachia* can be established and maintain itself within isolated *Ae. aegypti* populations around the city of Cairns in Australia[7,8]. Subsequent pilot releases have also shown that *Wolbachia* can be established in contiguous urban habitats[9]. In this report, we present the results of the first large-scale deployment of *Wolbachia* across Townsville, a medium-sized city in northern Australia with a population of ~187,000 residents.

Our goals for this work were to demonstrate that large scale deployment of *Wolbachia* was possible[10], that it could be done quickly and efficiently at low cost, and that it was acceptable to communities. In addition, while not designed as a clinical trial, it also provided an opportunity to examine a time series of observational data on dengue transmission, for 13 years before deployment and four consecutive dengue transmission seasons since deployment began.

Methods

Community engagement

One of the key objectives of the Townsville project was establishing a community engagement framework that could be suitably scaled for a citywide deployment and could be used cross-culturally for future deployments. Previous deployments in Cairns had relied on obtaining individual consent from community members for the release activities, an approach that was unsuitable for the required scaling. Instead we developed a Public Acceptance Model (PAM) for our engagement that formed the basis for obtaining community support for the research activities. The PAM was based on a set of Public Participation Principles described in Table 1.

The PAM consisted of four key components

1. **Raising awareness** by providing information to residents and key stakeholders about the program. These activities included face to face meetings, media events, stalls at community markets, community presentations utilising existing community networks such as community associations, information kiosks in public spaces, traditional and electronic mail outs of information letters and deployment coverage updates, a public billboard and newspaper advertising, a school outreach program and social media incentive program.

Table 1. Public participation principles of the World Mosquito community engagement approach.

Principle	Measure of Success
Respectful Caring for and heeding the interests and concerns of others	1. Issues raised by people are treated as valid and properly considered
Inclusive Making an effort to include everyone within its scope	2. Efforts are made to include all people with a potential interest in the project in project communications 3. People are able to nominate their interest in being included in the project communications
Transparent Being clear, open, and not hiding anything	4. Project information relevant to community understanding and interest is readily available and kept up to date
Responsive Showing that requests or concerns have been heard and trying hard to accommodate them	5. Commitments made by project personnel are met 6. Public requests for information are responded to promptly 7. Concerns raised are listened to and efforts taken to resolve them
Honest Telling the truth, not trying to deceive or allowing untruths to prevail	8. All communications about the project are factual and cover the information of potential interest to people 9. Information is presented in appropriate forms and languages so that all interested people can understand

2. **Quantitative surveys** that measured community awareness and acceptance conducted by an external market research company, Compass Research. Each telephone survey was undertaken at roughly six monthly intervals, the first survey being undertaken in March 2014 prior to our community engagement activities starting in the city and each involved 200–600 participants (Table 2).

3. **An issues management system** that allowed community members to easily contact the program with questions or concerns and have them addressed by program staff typically within 24 hours of receipt. This also allowed residents to opt out of direct participation if they had concerns.

4. **A community reference group** that consisted of respected community members from key stakeholder groups and included representation from Townsville City Council, Queensland Health, the local indigenous community, the Defence Force, local business, community development and environmental groups, the tourism sector and the education sector. The reference group's primary function was to independently review our activities to ensure that we had carried out our engagement in accordance with our commitments and stated Public Participation Principles (Table 1). The reference group was tasked to evaluate our activities and make a recommendation to the program management that community engagement had been sufficient for releases of mosquitoes to commence. Before releases began this group met monthly; after releases started they continued to meet every 6–8 weeks. The secondary functions of this group were to test and comment on the suitability of engagement materials and approaches, and to provide the program with feedback on community sentiment towards the program and identify potential issues that might require a proactive response. The reference group was also kept regularly updated on the latest results of the program.

Rearing

In order to establish the colony for release, wild mosquito eggs were collected from ovitraps set at 49 sites across Townsville and used to produce a wildtype colony. Material from this colony was stored as dried eggs and amplified only as required. Amplification of material from this colony was limited to F3 for use in outcrossing during colony maintenance. For stage 1 of the Townsville releases, eggs were produced from insectaries at Monash University, Melbourne or James Cook University, Cairns and shipped to the Townsville field office. For stages 2–4 all mosquito material was produced at Monash University.

The wildtype colony was backcrossed for three generations to a laboratory line infected with the *w*Mel strain of *Wolbachia*[11]. This new colony, TSV *w*Mel.f was continuously maintained in order to produce ~800,000 eggs per week. To maintain the material during mass production, the TSV *w*Mel.f line was divided into two distinct colonies: 'broodstock' and 'release material'. The 'broodstock' colony was reared under the more relaxed conditions described in 12 but kept at 26°C. Its purpose was to produce eggs for amplification and production of the 'release material colony'. In order to prevent inbreeding, 10% wildtype males (from the same wildtype material as was used for backcrossing) were added to each generation of the 'broodstock'. The purpose of the 'release material' colony was to produce eggs for release; it did not provide any material for the next generation in the laboratory. In order to facilitate mass production, the 'release material' colony was maintained as described for the broodstock with the following modifications. No wild material was added to the 'release material' colony. Once eggs were hatched, first instar larvae were aliquoted into 500 ml plastic cups

Table 2. Results of telephone surveys seeking to understand community awareness and support for the program.

	Jul 2013 (stage 1 area) n=300	Jan 2014 (stage 1 area) n=300	Sept 2014 (stage 1 area) n=600	Dec 2014 (stage 1 area) n=300	Oct 2015 (stages 2, 3, 4 area) n=600
Awareness (unprompted)	17%	29%	49%	51%	62%
Awareness (prompted)	52%	59%	69%	80%	62%
Awareness via media (TV, radio, paper)	N/A	69%	66%	65%	78%
Very Comfortable or Comfortable with the research	91%	85%	89%	95%	92%
Very Comfortable or Comfortable with community mosquito releases	N/A	N/A	N/A	95%	87%

at a ratio of 150–180 larvae/400 ml of water. The larvae were fed once with half a fish food tablet (Tetramin Tropical Tablet, Tetra Holding (US) Inc., Germany) until pupation. Larval rearing cups were transferred to adult cages for emergence once 60% of larvae had pupated. Cages were stocked at a rate of ~600 adults per (30 X 30 X 30cm) cage.

For both colonies, females (5–7 days old) were fed with human blood (Monash University Human Ethics approval CF11/0766 – 2011000387). They were provided the bloodmeal by introducing the arm of a volunteer into the selected cage. Females were fed until repletion (usually 10–15 minutes). Females were fed once per week, for one or two weeks depending on requirements. For safety, only one bloodfeeder was used per cage and bloodfeeders who showed any signs of fever or who were taking antibiotics were excluded.

Three 22 cm oviposition strips of red cotton duck cloth were placed in each cage three to five days after bloodfeeding. Oviposition strips were removed from cages four days later, and sandwiched between two double layers of 3mm thick kitchen sponge that had been covered with a single layer of paper towel, covered with a 3mm thick Perspex sheet and placed on a rack. Eggs were allowed to dry this way in an 80%RH controlled-temperature room for up to 24 hours before being placed in humidified containers. The humidity in these containers was maintained at ~80%RH by providing a saturated KCl solution inside the containers.

After the oviposition strips had been dried, the density of eggs/cm on each strip was estimated to determine the length of egg strip to be cut for subsequent use in Mosquito Release Containers (MRCs). Eggs were then shipped to the Townsville field lab.

Hatch rate was tested for every batch of eggs produced. Matched sets of eggs were taken from a number of strips and photographed to assess desiccation and overall quality of the eggs. One portion of each matched set was shipped to the release site, and one set kept at the rearing facility. Once the eggs reached the release site, both sets of eggs were counted, hatched, and hatch rate determined by counting larvae. Hatch rate of 70% or above was considered acceptable. If hatch rate fell below 70%, the cause of this drop was investigated. In most cases, the cause was determined to be due to fluctuating environmental conditions or to slight changes made to the drying procedure, which was altered slightly throughout releases.

Wolbachia infection frequency was also tested each week of production. 80 females and 80 males were screened from each broodstock cohort using diagnostic qPCR as described below. If *Wolbachia* frequency fell below 97% in any broodstock cohort, the eggs from their resultant 'release material colony' would not be used for release, however this issue never arose.

The James Cook University rearing strategy differed slightly from the Monash rearing strategy. A single colony of ~10,000 Townsville *w*Mel-infected *Ae. aegypti* sourced from Monash was created in a semi-field flight cages[13] in the Tropical Medicine Mosquito Research Facility located at James Cook University in Cairns. Based upon experience with earlier releases, we assumed that there is a loss of ~50% of the colony per week. The colony was therefore refreshed with 2500 males and 2500 females each week. We also conducted backcrossing to maintain genetic diversity by adding males (10% of cage male population) sourced from an uninfected wildtype Townsville colony (< F4). To prevent introduction of wild females and potential loss of *Wolbachia* infection into the colony, we only added males. This was achieved by placing suspected male pupae based on size into cups of 10; any cups containing emerged females were discarded.

Females (5–7 days old) were fed with human blood on volunteers (JCU Human Ethics H4907). They were provided the bloodmeal by introducing 5 volunteer blood feeders into the field cage 3–5 times/week who let mosquitoes feed for 10 minutes. For safety bloodfeeders were screened at every feed for possible exposure to dengue infected mosquitoes using a questionnaire to access travel history, and their temperature was taken to detect fever. Any volunteers with fever, a possible exposure to dengue infected mosquitoes or who were taking antibiotics were excluded for a minimum of 2 weeks.

Eggs were harvested from partially flooded 10 L buckets containing 26 × 30 cm strips of red felt cloth placed in the semi-field cage. A perspex template 31cm in length with 12 1-cm holes drilled into it was placed over the cloth to limit oviposition to the exposed 1 cm area of the ovistrip. The ovistrips were collected 3 times/week, embryonated and dried three days later. Once removed from the cages, oviposition strips were placed on moist paper towel in a sealed plastic container, after 3 days the lid of the sealed container was removed and the eggs were allowed to dry this way in an 80%RH controlled temperature room for up to 24 hours before being placed in humidified containers. The humidity in these containers was maintained at ~80%RH by providing a saturated KCl solution inside the containers. The cloth was then cut into individual eggstrips containing a single egg clump that could be deployed into egg release containers in the field. The number of eggs on each eggstrip was estimated by using reference photographs of eggstrips with known egg numbers as visual guides for fast estimation.

Mosquito releases

The municipal area of Townsville is ~190km^2. However, within this area there were many areas where releases did not take place due to the lack of suitable *Ae. aegypti* habitat. Releases were restricted to residential and business areas within the city where *Ae. aegypti* breeding was likely to occur. This resulted in the actual area for release being reduced to approximately 66km^2 to effectively cover the city. The release program was divided into four stages (Figure 1).

Stage 1 covered a release area of 20km^2 and included the suburbs with known highest dengue transmission risk: South Townsville, Railway Estate, North Ward, Townsville City, Belgian Gardens, Castle Hill, West End, Garbutt, Currajong, Vincent,

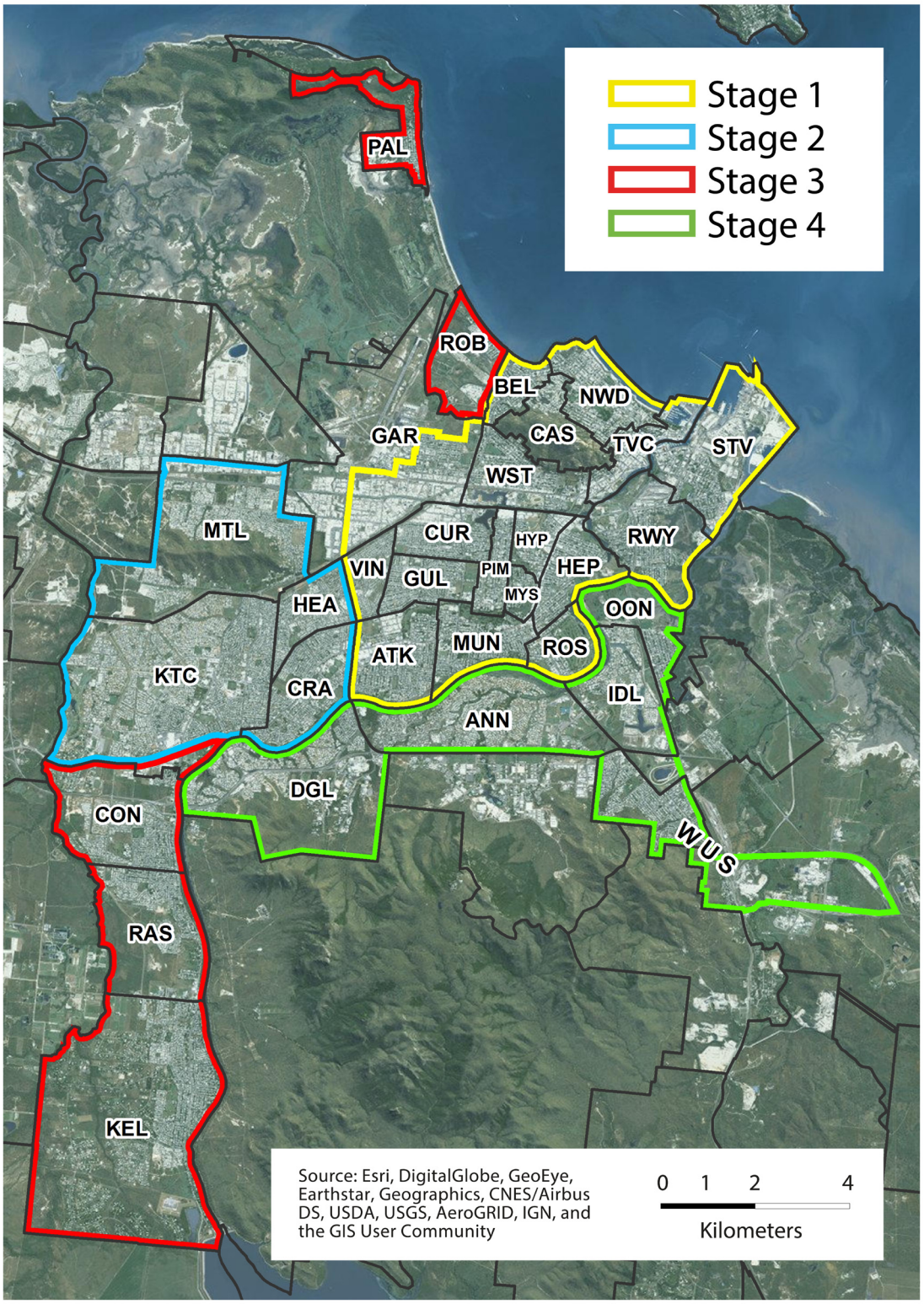


Figure 1. Release site. Map of Townsville city showing the boundaries of the four release stages.

Gulliver, Aitkenvale, Mundingburra, Rosslea, Hyde Park, Pimlico, Mysterton and Hermit Park. In this stage, all releases were undertaken using bucket mosquito release containers (MRCs). These were 2.3L white polypropylene pails with lid (Peopleinplastic, Australia), with top 164mm diameter, base 145mm diameter, and height 147mm. Each bucket had four 6mm holes drilled 20mm apart in a square pattern in the side (Figure 2A). The inside of each bucket was roughened with sandpaper to allow mosquitoes to rest upon emergence. Into each bucket MRC was placed an egg strip containing approximately 100 viable eggs (estimated from hatch rate QA), 5 (summer) or 6 (winter) wafers of Aqua One vege wafer fish food (Aqua Pacific, UK) and 1L water. More food was provided in winter and the servicing cycle for these buckets was extended from 2 to 3 weeks to allow for longer emergence times.

Bucket MRCs for stage 1 were placed by program staff in outdoor shaded areas at approximately 20% of all residential properties in a roughly evenly spaced arrangement in each suburb. They were serviced every two weeks by tipping out the water, cleaning the bucket and adding new food, water and eggs. An average of 88 adult mosquitoes were released from each bucket MRC in stage 1. Releases continued in each suburb until the frequency of *Wolbachia* in samples of field-caught mosquitoes from that suburb was above 50% for two consecutive weeks. For stage 1, it required between 7 and 19 weeks of releases for each suburb to reach that target

Stages 2, 3 & 4 covered release areas of 18, 18 and 10 km^2 respectively, and included the following suburbs. Stage 2: Cranbrook, Heatley, Kirwan/Thuringowa Central and Mount Louisa; stage 3: Condon, Pallarenda, Rowes Bay, Rasmussen and Kelso; stage 4: Idalia, Oonoonba, Wulguru/Stuart, Annandale and Douglas. Releases for these later stages did not rely on program team members to place all release containers. Instead, they utilised strategies that directly involved the community, such as the use of school students, direct community release, or through collaboration with local businesses. Releases for these stages also used Mozzie Box MRCs (Figure 2C) which consisted of a 775ml Food Pail (Detpak, Australia) without handle, and with measurements top 104×92mm, base 79×61mm, height 104mm. Four 5mm holes were punched into each MRC – one hole approximately 1cm from the top right and top left corners of each long-side face of the box. Each Mozzie Box MRC received 100 viable eggs (estimated from hatch rate QA), 4 (summer) or 5 (winter) wafers of Aqua One vege waters, and 400ml tap water. Mozzie Box MRCs were not re-used.

In stages 2–4 the goal was again to place MRCs at 20% of residences in the release area. This was done by using community

Figure 2. Release containers. Photos illustrating different mosquito release containers used in the deployment. (**A**) Bucket mosquito release containers (MRCs) used in stage 1 releases (**B**) Clear bucket MRC used in Wolbachia Warriors school program in stage 1 (**C**) Mozzie Box MRC that was used in stages 2–4 (**D**) Material given to school children as part of the Wolbachia Warriors program.

engagement activities to identify participants who would agree to host an MRC. In areas where there were large spatial gaps in participation, the program team would then supplement coverage by visiting additional houses in these areas and obtaining consent to leave MRCs with residents at these locations. Finally, in the last two suburbs of stage 3 (Kelso & Rasmussen) and across stage 4, releases of adult mosquitoes[7] were used to fill in gaps in MRC coverage.

During the 28 months of the release phase (stages 1–4), a total of approximately 4 million mosquitoes were released. Releases were undertaken with regulatory approval from the Australian Pesticides and Veterinary Medicines Authority (APVMA permit numbers PER14797 and PER82947).

School releases

The Wolbachia Warriors Program was developed both as a tool to engage children and their parents and make them aware of the program, and as an alternative channel to release mosquitoes. Five different primary schools were selected to run the program over the duration of the Townsville project. One school participated in each stage except for stage 2 where two schools participated. In total 943 students aged 6–12 participated in these programs.

School children were provided with a bucket MRC in stage 1 as used in operational releases in stage 1 but made of clear plastic to encourage student observation (Figure 2B) and Mozzie Box MRCs in stages 2–4 (Figure 2C), complete with mosquito eggs, food, instructions, a calendar to track progress, a magnifying glass, a badge for participation, and an educational booklet tailored for either lower (grade P-2) or upper primary (grade 3–6) students (Figure 2D). Each student was expected to undertake three consecutive releases with their MRC over a six-week period.

Materials were distributed at the schools by program communication and engagement staff, who gave presentations encouraging participation prior to each of the three mosquito release cycles. Students were asked to use their calendar to record the progress of the mosquito life cycle in their MRC, and to return it to program staff at the end of the release.

Direct community release

In these releases, a Mozzie Box MRC was provided directly to residents who set it up and reared the mosquitoes themselves at their place of residence. In stages 2-4, more than 6,000 households directly participated in establishing *Wolbachia* by managing their own release container. Almost half of these participants contacted the program team to receive an MRC, which was subsequently delivered to their house. The remaining participants were recruited through doorknocking, or through other recruitment methods such as community groups. Additional Mozzie Box MRCs were distributed through large local employers including the City Council, Telstra, The Townsville Hospital, James Cook University and Queensland Nickel. More than 200 people participated in these programs.

Quality assurance procedures

In stage 1, program staff checked 5–10% of all bucket MRCs to determine whether the bucket had failed or not, and if not to count pupal skins to obtain an estimate of adult emergence from which they could estimate release rates. In stage 2 – 4, a random selection of 5–15% of all MRCs were checked to determine if they were set correctly. Larvae, pupae and pupal skins were counted to estimate emergence rates in these stages (accounting for potential delayed development of mosquitoes at time of QA due to community members setting up MRCs later than day of delivery). This approach was supplemented in stage 3 with additional sentinel buckets that were set and checked by staff to determine average emergence rates. These data were then used to adjust numbers of eggs placed in MRCs.

Monitoring

Up to 172 Biogents Sentinal (BGS) traps were progressively rolled out across stage 1 during releases at a density of approximately 8 BGS traps per km^2. For stages 2–4 the BGS trap density was reduced to 4 per km^2, resulting in 74 – 115 traps being deployed per stage. Exact trap numbers fluctuated due to operational considerations (i.e. trap location no longer suitable, trap broken or missing, community request for trap to be removed or resident moved etc.).

Samples from BGS traps were collected weekly and returned to the field office for morphological identification. *Ae. aegypti* samples were stored in 70% ethanol and shipped to Monash University for diagnostic determination of *Wolbachia* infection status. After Feb 2016, samples were collected fortnightly instead of weekly as occurred in stage 1 until traps were finally removed from each suburb (Figure 3). Sites then moved to long-term annual monitoring.

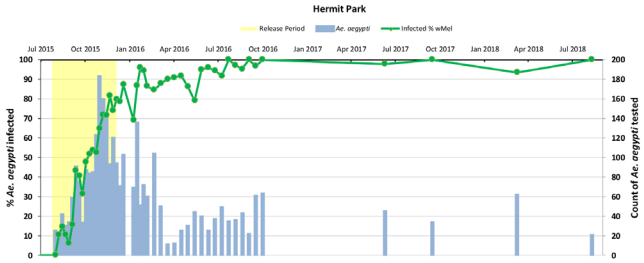
Diagnostics

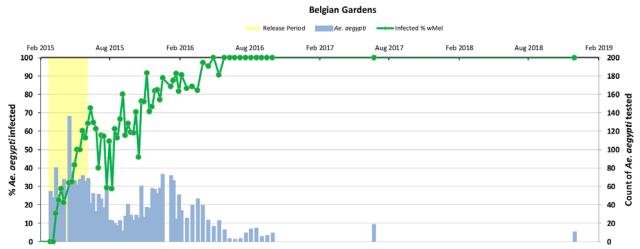
Adult *Ae. aegypti* samples collected from BGS traps in the field were screened for *Wolbachia* using Taqman qPCR on a Roche LightCycler 480 using a qualitative assay for presence or absence of *Wolbachia* as previously described[14] but with the replacement of the Cy5-BHQ3 fluorophore-quencher pair in the *w*Mel probe with the fluorophore-quencher LC640-IowaBlack (Integrated DNA technologies) to remove some of the Cy5 probe instability observed under varying light and ozone levels[15].

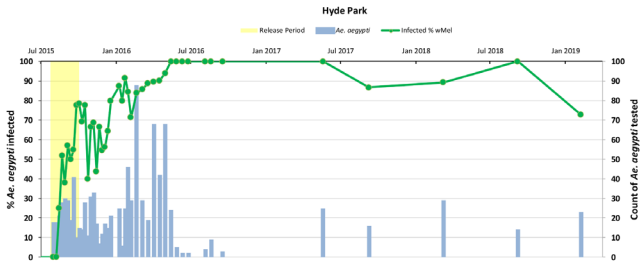
From Septemeber 2018 diagnostics was done by LAMP. LAMP primers (Integrated DNA Technologies, Singapore) were designed to detect the *wsp* gene from *w*Mel and *w*MelPop-CLA strains using the software LAMP Designer 1.02 (PREMIER Biosoft International). Individual reactions consisted of 2X WarmStart® Colorimetric LAMP Master Mix (New England BioLabs, Cat# M1800S), primers according to the manufacturer recommendation (Table 1), and 1 µL of target DNA in a total reaction volume of 17 µL. Reactions for individual samples were performed in 96-well PCR plates (LabAdvantage 96-well PCR plates, full skirt, clear). Plates were incubated in a thermocycler (BioRad C1000) at 65°C for 30 minutes then held at 12°C until scoring. Within one hour of incubation, colour changes of

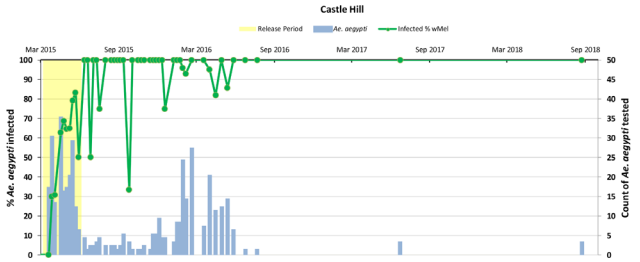
A

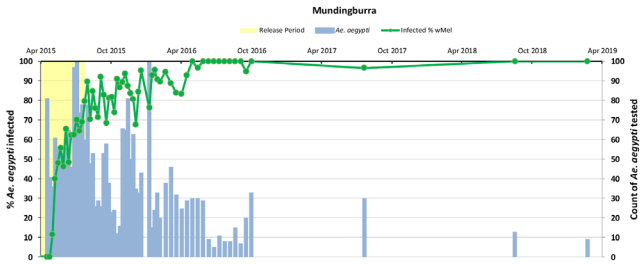

Aitkenvale
Release Period
Ae. aegypti
Infected % wMel
% Ae. aegypti infected
Count of Ae. aegypti tested

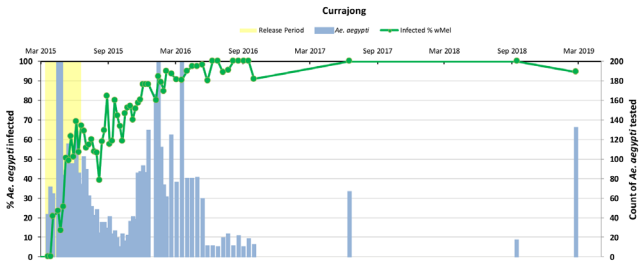

Hermit Park
Release Period
Ae. aegypti
Infected % wMel
% Ae. aegypti infected
Count of Ae. aegypti tested

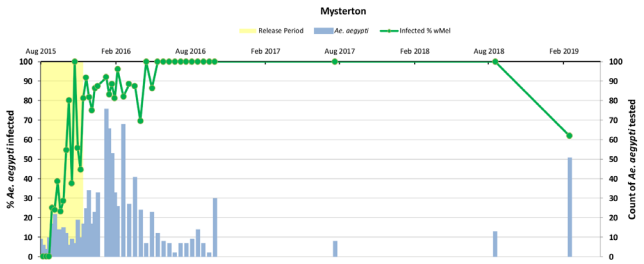

Belgian Gardens
Release Period
Ae. aegypti
Infected % wMel
% Ae. aegypti infected
Count of Ae. aegypti tested


Hyde Park
Release Period
Ae. aegypti
Infected % wMel
% Ae. aegypti infected
Count of Ae. aegypti tested


Castle Hill
Release Period
Ae. aegypti
Infected % wMel
% Ae. aegypti infected
Count of Ae. aegypti tested


Mundingburra
Release Period
Ae. aegypti
Infected % wMel
% Ae. aegypti infected
Count of Ae. aegypti tested


Currajong
Release Period
Ae. aegypti
Infected % wMel
% Ae. aegypti infected
Count of Ae. aegypti tested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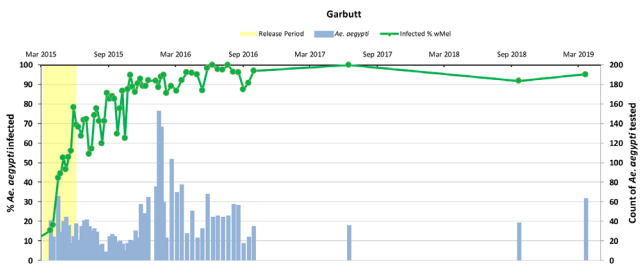
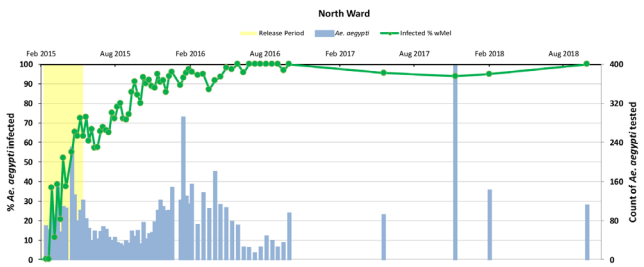
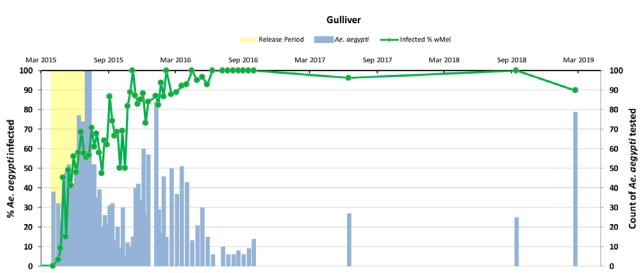
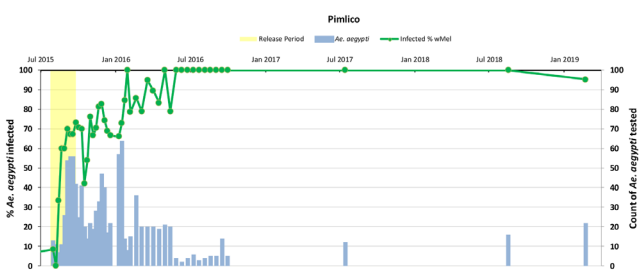
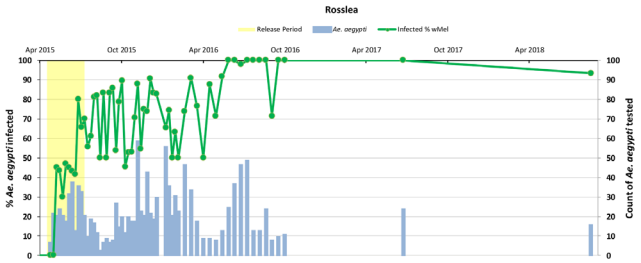
Release Period
Ae. aegypti
Infected % wMel
% Ae. aegypti infected
Count of Ae. aegypti tested

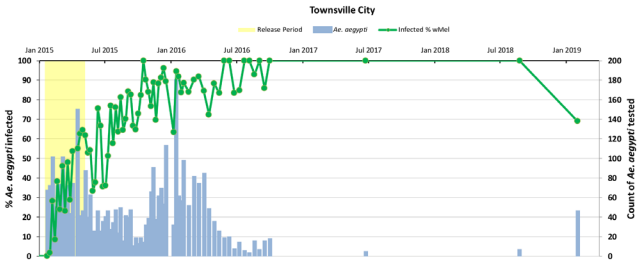

Garbutt
Release Period
Ae. aegypti
Infected % wMel
% Ae. aegypti infected
Count of Ae. aegypti tested

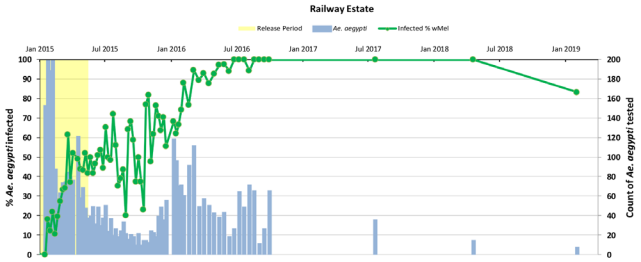

North Ward
Release Period
Ae. aegypti
Infected % wMel
% Ae. aegypti infected
Count of Ae. aegypti tested

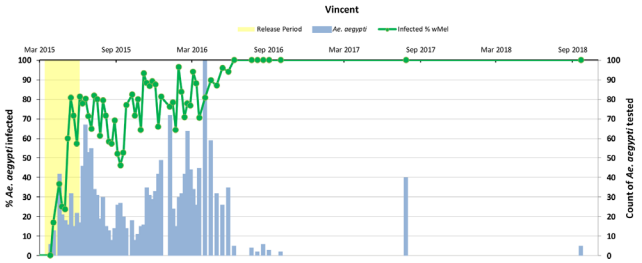

Gulliver
Release Period
Ae. aegypti
Infected % wMel
% Ae. aegypti infected
Count of Ae. aegypti tested

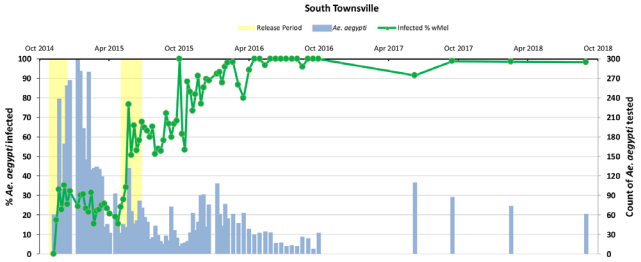

Pimlico
Release Period
Ae. aegypti
Infected % wMel
% Ae. aegypti infected
Count of Ae. aegypti tested

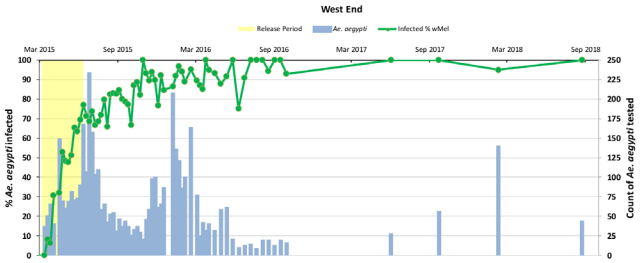

Rosslea
Release Period
Ae. aegypti
Infected % wMel
% Ae. aegypti infected
Count of Ae. aegypti tested


Townsville City
Release Period
Ae. aegypti
Infected % wMel
% Ae. aegypti infected
Count of Ae. aegypti tested

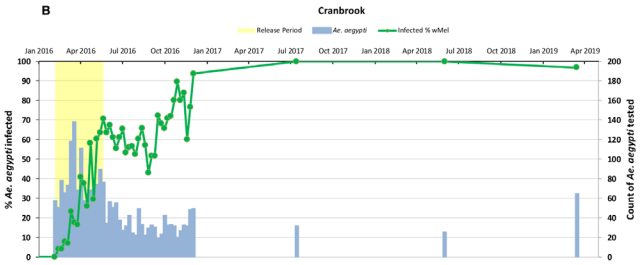

Railway Estate
Release Period
Ae. aegypti
Infected % wMel
% Ae. aegypti infected
Count of Ae. aegypti tested

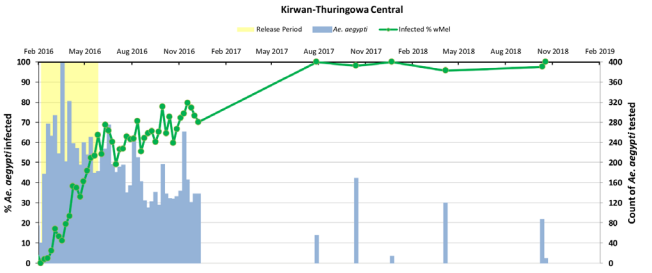

Vincent
Release Period
Ae. aegypti
Infected % wMel
% Ae. aegypti infected
Count of Ae. aegypti tested

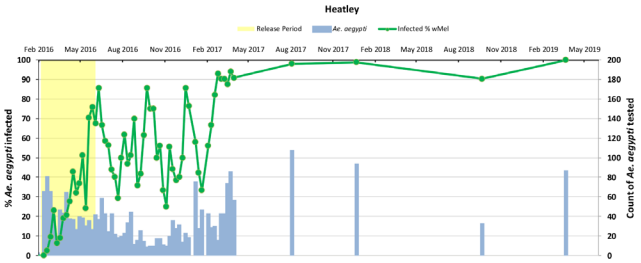

South Townsville
Release Period
Ae. aegypti
Infected % wMel
% Ae. aegypti infected
Count of Ae. aegypti tested

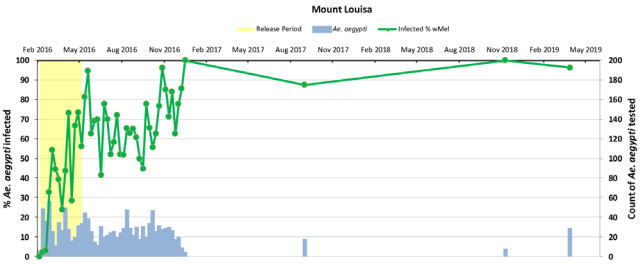

West End
Release Period
Ae. aegypti
Infected % wMel
% Ae. aegypti infected
Count of Ae. aegypti tested

B


Cranbrook
Release Period
Ae. aegypti
Infected % wMel
% Ae. aegypti infected
Count of Ae. aegypti tested


Kirwan-Thuringowa Central
Release Period
Ae. aegypti
Infected % wMel
% Ae. aegypti infected
Count of Ae. aegypti tested


Heatley
Release Period
Ae. aegypti
Infected % wMel
% Ae. aegypti infected
Count of Ae. aegypti tested


Mount Louisa
Release Period
Ae. aegypti
Infected % wMel
% Ae. aegypti infected
Count of Ae. aegypti tested

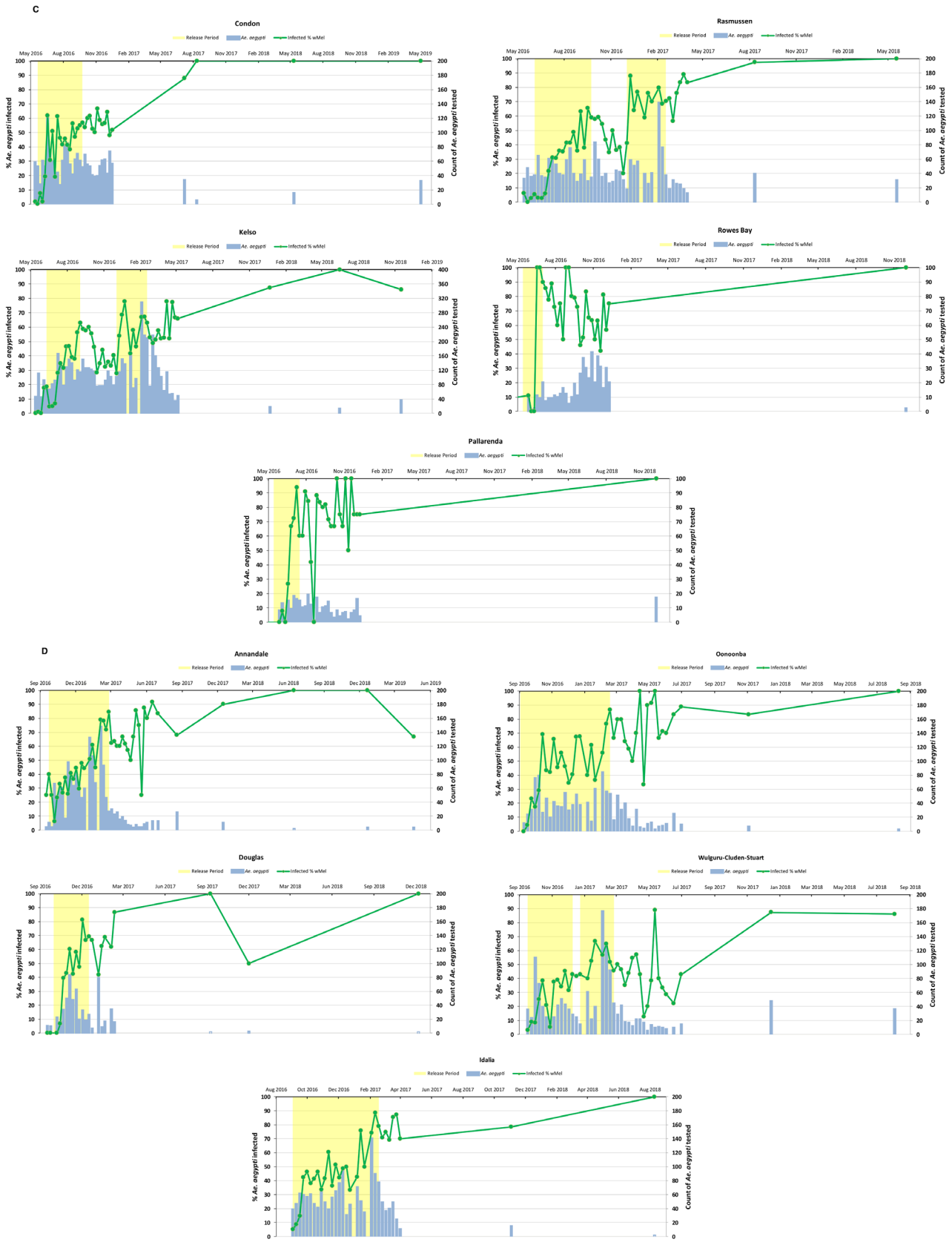


Figure 3. Wolbachia establishment by suburb. For each stage (**A**–**D**) and suburb *Wolbachia* frequency is plotted against time. Yellow shading indicates periods when releases were undertaken. Bars show the number of mosquitos captured in Biogents Sentinel (BGS) traps and tested for *Wolbachia*.

individual samples were recorded. Primers were as follows FIP 5' TGTATGCGCCTGCATCAGCTTCGGTTCTTATGGTGCTAA, BIP 5' GCAGAAGCTGGAGTAGCGTTGTGTCATGCCACTTAGATGG, F3 5' TGATGTAACTCCAGAAGTCA, B3 5' CTTATTGGACCAACAGGATCG, LpF 5' AGCCTGTCCGGTTGAATT, LpB 5' CAGTCTTGTTATCCCAGTGAGT.

Dengue case notification data

Dengue is a notifiable disease in Australia, which mandates clinicians and laboratories to report confirmed and suspected cases to local health authorities (See Queensland Dengue Management plan). Non-identifiable data was provided by Queensland Health Communicable Diseases Branch for all laboratory-confirmed and clinically diagnosed (probable) dengue cases with illness onset between 1 January 2000 and 31 March 2019, extracted from the Notifiable Conditions System (NOCS) on 3 July 2018. Case notifications within the Townsville local government authority were tabulated by month of illness onset and history of recent overseas travel during the 3 – 12 days prior to illness onset; a variable that is routinely captured in case notifications based on interview by public health teams (see 16 for interview protocol). The suburb of residence of four locally-acquired dengue cases notified in Townsville since *Wolbachia* deployments commenced in October 2014 was determined from situation reports published by the local public health unit.

Interrupted time series analysis

Negative binomial regression was used to model monthly counts of locally-acquired dengue cases (January 2001 – March 2019) in aggregate *Wolbachia*-treated and untreated areas of Townsville. The regression model was fitted in Stata (SE version 14.2, StataCorp, TX) using generalised estimating equations, with epidemic year (September – August) as a cluster variable to account for temporal autocorrelation in the monthly case counts, adjusting for monthly imported dengue cases (any vs none) and season (wet: June – November vs dry: December – May), with a population size offset. A binary intervention variable was included in the regression model to distinguish the pre- or post-intervention status of each area in any given month, the coefficient of which provided the estimate of intervention effect (incidence rate ratio). Robust standard errors were used.

Populations were derived from mesh blocks (Australian Bureau of Statistics, 2016) aggregated to the boundaries of each operational release area. Aggregate treated and untreated areas (and their resident populations) were dynamic over time, with the treated area in any given month defined as the total area where *Wolbachia* deployments had been completed to date. Cases' location, for the purpose of classifying *Wolbachia* exposure status in this analysis, was determined using address information from the Townsville Public Health Unit (PHU) operational database. The address classified in the PHU dataset as the probable location of dengue acquisition was used where available (95/468 cases, 20%); if unavailable then the primary residential address was used (248/468, 53%). For 108/468 cases (22%) the address in the operational database was not designated as 'acquired' or 'residential', and for the remaining 17 cases (6%) no address was available in the PHU database, and the suburb of residence from the NOCS dataset was used to define the case's location.

Ethical considerations and consent

Ethics approval for human blood feeding mosquito colonies in Melbourne was issued from Monash University CF11/0766 a 2011000387 (Rearing mosquitoes using blood from human volunteers). All volunteers (no children involved) provided written consent.

In Cairns, Human Ethics approval for bloodfeeding (H6286) was provided by Human Research Ethics Committee, James Cook University. All adult subjects provided informed oral consent (no children were involved). Names of subjects providing oral consent were recorded in writing.

Townsville community mosquito releases were covered under Monash University ethics: MUHREC Approval CF16/763 - 2016000370 - Eliminate Dengue - Community based field releases of Wolbachia infected mosquitoes in Townsville, Queensland.

Surveys were undertaken under Monash ethics: MUHREC Approval CF13/2805 - 2013001515 - Eliminate Dengue - Community knowledge of dengue and Wolbachia based dengue control in Townsville, Queensland

Verbal and/or written consent from participants was obtained by phone, online or face-to-face to set BG traps, set MRCs (phase 1), or participate in Community Mosquito Releases.

Ethical approval was not required to access non-identifiable dengue case notification data collected as part of routine disease surveillance.

Results and discussion

Prior deployments of *Wolbachia* in Australia by the World Mosquito Program utilised a traditional individual informed-consent approach to obtaining community authorisation for the releases[7]. While this approach was adequate for small deployments, it was not considered scalable for an entire city. We therefore developed a Public Acceptance Model (PAM) that proved highly effective in ensuring community awareness and acceptance of the mosquito deployment program in Townsville. We believe this model will be suitable for other settings with appropriate local adaptation, and provides a framework for scaled deployment of this type of intervention globally.

Releases of mosquitoes in Townsville began in Oct 2014 with strong community support (Table 2) and lasted for 28 months. The release program was divided into 4 sequential stages. The approach used in Townsville relied on the use of Mosquito Release Containers (MRC) as the preferred method of deployment (Figure 2). In each suburb of the city MRCs were set at approximately 20% of residences and then refreshed with new food, water and eggs every 2–3 weeks. MRC release cycles continued until 2 consecutive samples of adult mosquitoes taken from the suburb showed a *Wolbachia* frequency above 50%; *Wolbachia* frequency in these areas was then monitored without additional releases. While the city occupies a municipal area of 190km^2, releases were undertaken over a reduced area of ~66km^2 as not all areas of the greater municipal area were inhabited or provided suitable *Ae. aegypti* habitat (Figure 1). The targeted release areas covered all of the suburbs where local dengue transmission had occurred during the prior 10 years and known

high-risk suburbs for dengue transmission were targeted in stage 1. *Wolbachia* monitoring was conducted and infection frequency reported aggregate to suburb boundaries, encompassing an area greater than the 66km^2 of actual release areas. The total area considered 'covered' by *Wolbachia* in Townsville is 128km^2, with a residential population in 2016 of 140,000.

Wolbachia establishment across the different suburbs of Townsville for the four stages is shown in Figure 3. In general, establishment of *Wolbachia* occurred reliably after releases stopped once the 50% threshold was met. In some suburbs, *Wolbachia* frequencies fluctuated for a number of months before eventually rising to above 80%. In five suburbs, a small number of supplementary releases were undertaken to ensure establishment. In all suburbs, the infection frequency has remained stable without any signs of *Wolbachia* being lost from the mosquito population (Figure 3).

Laboratory experiments have suggested that maternal transmission of *w*Mel can become unstable in *Ae. aegypti* at high temperatures and plausibly might limit the field usefulness of the *w*Mel strain[17]. The temperatures used in these incubator experiments were meant to mimic larval rearing temperatures in north Queensland. However, our field data shows long-term stability of *w*Mel, presumably because temperatures used in this study were not truly representative of those experienced by mosquitoes in the field. We assume that mosquitoes predictably seek out non-stressful microhabitat when it exists[18] and larval rearing temperatures do not mirror measured ambient temperatures. Empirical data from this study and other sites[9] suggests that *w*Mel is much more robust to deployment than predicted by 17.

A key feature of using MRCs for mosquito releases is the possibility of mobilising the community to undertake the deployment instead of employed program staff. In stage 1 of the release program staff undertook the deployment by setting and maintaining MRC buckets themselves. In stages 2–4 we used a blended approach of community members setting their own MRCs and then program staff members supplementing these deployments by distributing additional MRCs to meet the target of 20% of residences, to ensure adequate coverage without major spatial gaps. Community-based releases were undertaken in three ways; school programs where students were given MRC kits to take home, direct community releases where MRC kits were given to householders who had signed up to participate through community engagement activities, and finally by having large employers within the city distribute MRCs to staff who were willing to participate. Of the three methods, providing MRCs directly to the community was the most cost effective. It also allowed for more targeted deployment and better coordination with field staff, ensuring adequate coverage across a suburb. This blended approach of community-based deployment supplemented with programmatic targeted deployment is considered the most appropriate for future large-scale operations. The schools program – while being less efficient and costlier – proved to be an excellent community engagement vehicle, with the release outcome of secondary importance. Its success was highly dependent on working with an actively engaged teacher who could serve as a champion for the program.

Episodic outbreaks of locally transmitted dengue have occurred annually in Townsville since 2001. Outbreaks occur against a background of regular importations of dengue into Townsville by international travellers (Figure 4). In the period since

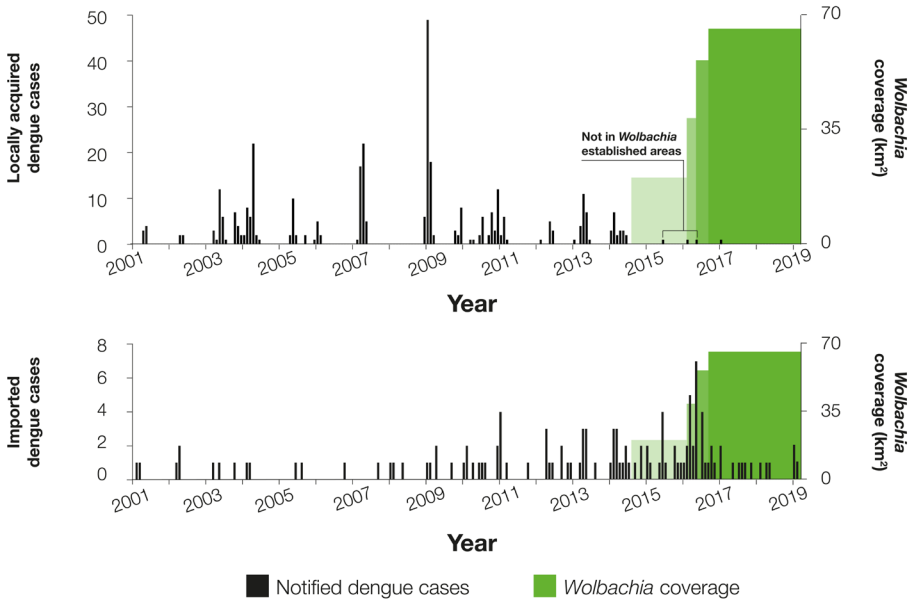


Figure 4. Dengue case notifications. Dengue case notifications per month in Townsville, Australia, January 2001 – March 2019, before and after *Wolbachia* mosquito deployments. Notifications include laboratory-confirmed and probable dengue cases, classified as locally-acquired (**A**) or imported (**B**) based on a history of overseas travel to a dengue-affected country during the period 3 – 12 days prior to illness onset. Data was extracted from the Queensland Health Notifiable Conditions System (NOCS) on 11 April 2019. Green shading shows the four stages of *Wolbachia* deployment conducted in Townsville since October 2014.

Wolbachia deployments began in Townsville in 2014, dengue case importations have continued to occur, with 54 imported cases in the 53 months from November 2014 – March 2019 compared to 41 in the preceding 53-month period. Notably, only four locally-acquired dengue cases have been identified in the post-release period, compared to 94 in the equivalent preceding period and a median of 131 (IQR 101-143) in all 53-month moving windows since 2001. In none of the previous 53-month moving windows since 2001 were there fewer than 69 locally-acquired cases notified. Importantly, only one of the four local cases since November 2014 was resident in an area where *Wolbachia* had been established. However, public health investigation found that this case was highly mobile and therefore the likely place of acquisition was uncertain. The model-based estimate of intervention effect from the interrupted time series analysis suggests a 95% reduction in dengue incidence in *Wolbachia* treated populations (95% confidence interval: 84–98%), adjusted for season, imported cases, and allowing for temporal autocorrelation of cases (Table 5). These findings, coupled with continuous validation of the impaired vector competence of *w*Mel-infected *Ae. aegypti* in release areas[19], represent empirical epidemiological evidence consistent with modelling projections of *w*Mel-mediated elimination of dengue transmission in most settings[6].

The cost of undertaking the program per person, and per km^2, varied between stages, and when time to complete each stage was also considered stage 2 was most efficient (Table 3). Considering the low population density of this city we expect the cost per person, for the same deployment methodology, would be dramatically reduced in many tropical cities with much higher population densities. Furthermore, the costs for the deployment in Townsville were inflated as the work was undertaken as a research activity, with much more monitoring than would be expected in an operational public health intervention. The breakdown of costs by major activity are shown in Table 4. Community engagement activities accounted for a significant

Table 3. Cost per person and cost per km² for each of the four release stages in Townsville.

Stage	Release area km²	Months required to deploy	Average FTE[1]	Cost per person AUD$	Cost per km² AUD$
Stage 1	20.3	14	10	29	69,762
Stage 2	18.2	6	12	16	37,268
Stage 3	17.6	4	11	19	23,231
Stage 4	9.7	5	8	13	37,313

[1]Average number of full-time equivalent (FTE) staff used to undertake deployment. It excludes staff required to produce mosquitoes for release or undertake diagnostics.

Table 4. Costs by major activity class for entire deployment.

Expense category	% of total costs	Major cost components
Community Engagement	23	Staff, surveys, advertising & media, events, catering, overheads
Field Deployment	41	Staff, transport, equipment, MRCs, overheads
Monitoring	24	Staff, transport, BGS traps, GIS, supplies, overheads
Diagnostics	9	Staff, reagents
Production	2	Staff, consumables

MRC - mosquito release containers, BGS- Biogents Sentinel, GIS- Geographic Information Systems

Table 5. Model estimates from negative binomial regression of monthly locally-acquired dengue case counts in *Wolbachia*-treated vs untreated populations, adjusted for temporal autocorrelation within each transmission season.

Variable	IRR	95% CI	Robust SE	p value
Wolbachia intervention (treated vs untreated)	0.05	0.02 – 0.16	0.03	<0.001
Season (dry vs wet)	0.27	0.10 – 0.50	0.09	<0.001
Monthly imported dengue cases (≥1 vs 0)	2.21	1.08 – 4.54	0.81	0.031

IRR: incidence rate ratio; CI: confidence interval; SE: standard error.

part of the cost of deployment, which shows the prioritization of and importance given to these activities by the World Mosquito Program. This, together with the cost of deployment (staff, vehicles etc.), accounted for more than half the cost of the implementation, and represents the areas where significant cost reductions might occur in future operational deployments. Given the costs for this study, and considering that future deployments should utilize less monitoring and occur in settings of higher population density, we estimate that deployment cost should be able to be reduced to less than US$1 per person. Additionally, in contrast to most other interventions, this cost should not be ongoing since once *Wolbachia* is introduced it is expected to maintain itself in populations. This suggests that the use of *Wolbachia* for arbovirus control as described in this study has the potential to be an extremely cost effective intervention compared with existing methods and many other proposed interventions that feature the release of modified mosquitoes[10].

This study demonstrates that: the *w*Mel strain of *Wolbachia* can be deployed effectively across large geographic areas at low cost; that once the intervention is deployed it is stable and self-sustaining; and that communities are accepting of the release of mosquitoes and are willing to participate in deployments when effectively engaged. From this study, we were able to identify a number of key learnings to take into future studies. These include: the understanding that community engagement approaches can be successfully scaled without compromising their quality, that shipping eggs from a remote production facility is possible but that care is needed with the shipping method to avoid excessive mortality, that managing egg strips for quality and to estimate quantity was laborious and a key step to improve in future scale-up. Finally, a time series analysis of notified dengue cases within the city over a 18-year period is consistent with modelling predictions of a large impact on dengue transmission[6] – and indeed in this city the observational data is consistent with elimination of local transmission.

Data availability

The data underlying Figure 3 is available from Figshare.

O'Neill, Scott (2019): Graph Data Version 3. figshare. Dataset. https://doi.org/10.6084/m9.figshare.8282306.v1[20].

This dataset is available under a CCO license.

Human dengue case notification data was provided to us by Queensland Health. The conditions of release of the raw dengue case notifications data to us by the Communicable Disease Branch of Queensland Health do not permit further sharing to a third party. This data (local and acquired dengue case notifications from Townsville local government area, Jan 2001 - June 2018) can be acquired by application to Queensland Health:

https://www.health.qld.gov.au/clinical-practice/guidelines-procedures/diseases-infection/surveillance/reports/notifiable/data-request

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References

1. Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, *et al.*: **A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, Chikungunya, and Plasmodium.** *Cell.* 2009; **139**(7): 1268–1278.
PubMed Abstract | Publisher Full Text

2. Dutra HL, Rocha MN, Dias FB, *et al.*: ***Wolbachia* Blocks Currently Circulating Zika Virus Isolates in Brazilian *Aedes aegypti* Mosquitoes.** *Cell Host Microbe.* 2016; **19**(6): 771–4.
PubMed Abstract | Publisher Full Text | Free Full Text

3. Aliota MT, Peinado SA, Velez ID, *et al.*: **The *w*Mel strain of *Wolbachia* reduces transmission of Zika virus by *Aedes aegypti*.** *Sci Rep.* 2016; **6**: 28792.
Publisher Full Text

4. van den Hurk AF, Hall-Mendelin S, Pyke AT, *et al.*: **Impact of *Wolbachia* on infection with chikungunya and yellow fever viruses in the mosquito vector *Aedes aegypti*.** *PLoS Negl Trop Dis.* 2012; **6**(11): e1892.
PubMed Abstract | Publisher Full Text | Free Full Text

5. Aliota MT, Walker EC, Uribe Yepes A, *et al.*: **The *w*Mel Strain of *Wolbachia* Reduces Transmission of Chikungunya Virus in *Aedes aegypti*.** *PLoS Negl Trop Dis.* 2016; **10**(4): e0004677.
PubMed Abstract | Publisher Full Text | Free Full Text

6. Ferguson NM, Kien DT, Clapham H, *et al.*: **Modeling the impact on virus transmission of *Wolbachia*-mediated blocking of dengue virus infection of *Aedes aegypti*.** *Sci Transl Med.* 2015; **7**(279): 279ra237.
PubMed Abstract | Publisher Full Text | Free Full Text

7. Hoffmann AA, Montgomery BL, Popovici J, *et al.*: **Successful establishment of**

Wolbachia **in** ***Aedes*** **populations to suppress dengue transmission.** *Nature.* 2011; **476**(7361): 454–457.
PubMed Abstract | Publisher Full Text

8. Hoffmann AA, Iturbe-Ormaetxe I, Callahan AG, *et al.*: **Stability of the *w*Mel *Wolbachia* Infection following invasion into *Aedes aegypti* populations.** *PLoS Negl Trop Dis.* 2014; **8**(9): e3115.
PubMed Abstract | Publisher Full Text | Free Full Text

9. Schmidt TL, Barton NH, Rašić G, *et al.*: **Local introduction and heterogeneous spatial spread of dengue-suppressing *Wolbachia* through an urban population of *Aedes aegypti*.** *PLoS Biol.* 2017; **15**(5): e2001894.
PubMed Abstract | Publisher Full Text | Free Full Text

10. Flores HA, O'Neill SL: **Controlling vector-borne diseases by releasing modified mosquitoes.** *Nat Rev Microbiol.* 2018; **16**(8): 508–518.
PubMed Abstract | Publisher Full Text

11. Walker T, Johnson PH, Moreira LA, *et al.*: **The *w*Mel *Wolbachia* strain blocks dengue and invades caged *Aedes aegypti* populations.** *Nature.* 2011; **476**(7361): 450–453.
PubMed Abstract | Publisher Full Text

12. McMeniman CJ, O'Neill SL: **A virulent *Wolbachia* infection decreases the viability of the dengue vector *Aedes aegypti* during periods of embryonic quiescence.** *PLoS Negl Trop Dis.* 2010; **4**(7): e748.
PubMed Abstract | Publisher Full Text | Free Full Text

13. Ritchie SA, Johnson PH, Freeman AJ, *et al.*: **A secure semi-field system for the study of *Aedes aegypti*.** *PLoS Negl Trop Dis.* 2011; **5**(3): e988.
PubMed Abstract | Publisher Full Text | Free Full Text

14. Yeap HL, Axford JK, Popovici J, *et al.*: **Assessing quality of life-shortening *Wolbachia*-infected *Aedes aegypti* mosquitoes in the field based on capture rates and morphometric assessments.** *Parasit Vectors.* 2014; **7**: 58.
PubMed Abstract | Publisher Full Text | Free Full Text

15. Dar M, Giesler T, Richardson R, *et al.*: **Development of a novel ozone- and photo-stable HyPer5 red fluorescent dye for array CGH and microarray gene expression analysis with consistent performance irrespective of environmental conditions.** *BMC Biotechnol.* 2008; **8**: 86.
PubMed Abstract | Publisher Full Text | Free Full Text

16. Vazquez-Prokopec GM, Montgomery BL, Horne P, *et al.*: **Combining contact tracing with targeted indoor residual spraying significantly reduces dengue transmission.** *Sci Adv.* 2017; **3**(2): e1602024.
PubMed Abstract | Publisher Full Text | Free Full Text

17. Ross PA, Wiwatanaratanabutr I, Axford JK, *et al.*: ***Wolbachia* Infections in *Aedes aegypti* Differ Markedly in Their Response to Cyclical Heat Stress.** *PLoS Pathog.* 2017; **13**(1): e1006006.
PubMed Abstract | Publisher Full Text | Free Full Text

18. Ritchie SA: **Dengue vector bionomics: why *Aedes aegypti* is such a good vector.** In *Dengue and Dengue Hemorrhagic Fever: CAB International.* (eds D. Gubler, J. Farrar, E. E. Ooi, & S. Vasudeban) Ch. 24, (CAb International) 2014; 455–480.
Publisher Full Text

19. Frentiu FD, Zakir T, Walker T, *et al.*: **Limited dengue virus replication in field-collected *Aedes aegypti* mosquitoes infected with *Wolbachia*.** *PLoS Negl Trop Dis.* 2014; **8**(2): e2688.
PubMed Abstract | Publisher Full Text | Free Full Text

20. O'Neill S, Ryan P, Turley A, *et al.*: **GraphData_Figure 3.docx.** 2018.
Reference Source

Open Peer Review

Current Peer Review Status: ✔ ✔

Version 2

Reviewer Report 02 November 2018

https://doi.org/10.21956/gatesopenres.13975.r26733

✔ **Horacio M. Frydman**
Department of Biology, National Emerging Infectious Diseases Laboratories (NEIDL), Boston University, Boston, MA, USA
Heverton Leandro Carneiro Dutra
Department of Biology, Boston University, Boston, MA, USA

We consider that the authors have addressed our questions.

Competing Interests: No competing interests were disclosed.

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 28 August 2018

https://doi.org/10.21956/gatesopenres.13925.r26600

? **Horacio M. Frydman**
Department of Biology, National Emerging Infectious Diseases Laboratories (NEIDL), Boston University, Boston, MA, USA
Heverton Leandro Carneiro Dutra
Department of Biology, Boston University, Boston, MA, USA

Overall comments:
The manuscript “Scaled deployment of *Wolbachia* to protect the community from *Aedes* transmitted arboviruses” reports a successful strategy for the long-term establishment of *Wolbachia* on a citywide scale. Previous pioneer work from this group have shown that *Aedes w*Mel *Wolbachia* strain can establish itself in the field after surpassing a certain unstable equilibrium threshold (Hoffmann et al., Nature 2011). However, in the current study, the establishment of *Wolbachia* is done at a larger scale, across 66 km^2 of Townsville, a city in northern Australia with a population of approximately 187,000 residents. Interestingly enough, the authors also report details on a community engagement approach used to achieve *Wolbachia* establishment in a cost-effective manner.

Remarkably, the results presented here provide the first field-based evidence that the self-sustainable *Wolbachia* deployment strategy works against dengue. That being said, this study was not designed as a clinical trial experiment, as pointed out by the authors. Although extremely promising and exciting, the results presented here should not be taken, at least at this point, as a definitive evidence that *Wolbachia* will block dengue transmission in endemic areas. Townsville, has a relatively low number of dengue cases a year, compared to a great number of cities in tropical and sub-tropical areas of the globe. On that regard, this non-profitable consortium has ongoing field-trials in places like South America and Asia, serious contenders to the *Wolbachia*-based strategy.

Major comments:

1. The reader is left wondering if non-*Wolbachia* factors in Townsville (e.g. climate) are contributing to the remarkable decrease of reported locally acquired dengue cases after *Wolbachia* release (2016-2018). This work would greatly benefit from providing additional epidemiological data. Showing the dengue cases for other cities in Northern Australia with no *Wolbachia* release over the same period as shown in Figure 4 (2002-2018). This data could be incorporated in Figure 4 as an additional histogram. Ideally this histogram should show an average of locally acquired dengue case notifications from 2002 to 2018 from several cities with epidemiology similar to Townsville from 2002 to 20014 (prior to *Wolbachia* release). Although not a rigorous control, this data would at least give some indication if non-*Wolbachia* factors in that region (may be climate?) could be playing a role for the dengue decrease.

   (note added in proof: the other reviewer also had a somewhat similar comment).

2. A small discussion of the challenges faced by their approach as well as lessons learned from Townsville that should be considered in areas were *Wolbachia* deployment is imminent would be valuable to the field.

Minor comments and suggestions by page

Main Text: Original text in *italics*

P.1: Title: *Scaled deployment of Wolbachia to protect the community from Aedes transmitted arboviruses*

Although there are several studies demonstrating efficient pathogen blocking effect of *Wolbachia* against several arboviruses transmitted by *Aedes aegypti*, including many from this research group, this specific manuscript only shows epidemiological data on dengue. Therefore, the title would be more precise if changed to reflect the specific arbovirus evaluated.

P.4: Rearing (2nd paragraph): "*The wildtype colony was backcrossed for three generations*…"

Although there is data pointing towards the inexistence of *kdr* mutations in the local *Ae. aegypti* population present in Queensland, indicating susceptibility to pyrethroid insecticides, there are several recent reports indicating the sporadic detection of non-native *Ae. aegypti* mosquitoes carrying insecticide resistance alleles not found in Australia. Areas with intense international flux like seaports and airports are a point-of-entry for these alleles into the local population (Endersby-Harshman *et al*., 2017[1]). How is the WMP taking the potential risk of insecticide resistance into consideration when rearing their mosquitoes for field releases? Few sentences regarding this aspect would be helpful, given that insecticide resistance alleles could highjacks *Wolbachia* establishment in areas heavily treated with insecticides by indoor residual spraying (IRS). Are the mosquitoes selected for backcrossing checked for chemical compounds resistance? What is the level of synchrony between the WMP approach and the guidelines established by the Queensland dengue management plan 2015-2020 which indicates the use of IRS as an approach against disease vectors?

P.4: Rearing (2nd paragraph): "*In order to prevent inbreeding, 10% wildtype males were added to each generation of the 'broodstock'*."

To avoid inbreeding is of great relevance. That being said, is this value of 10% resulted from a pool of males collected across all the same 49 sites in Townsville, the same used to establish the original wildtype colony? Additionally, it is to be expected that at a certain point, given the establishment of *Wolbachia* in the field, that the males collected would harbor the bacterium. Did the authors screened a portion of the males added to the cages after the copulation period?

P.5: Rearing (7th paragraph): "*Wolbachia infection was also tested each week of production, 80 females and 80 males were screened from each broodstock cohort*…"

1. These numbers of female and males tested represents what percentage of the total population?
2. Given the potential for maternal transmission leakage of *Wolbachia* when considering the transmission dynamics of this bacterium, something speculated to be one of the factors contributing to the difficult establishment of *Wolbachia* in Cairns, another area where releases by the WMP took place (Schmidt *et al*., 2018[2]), it is somewhat unexpected that the field colony was not screened prior to release, only the broodstock. What would be the reason for that? May be the consistency by which the mosquitoes were reared under laboratory conditions?

P.5: Rearing (8th paragraph): "*The colony was therefore refreshed with 2500 males and 2500 females each week*."

The same question previously asked (**P.4**: Rearing (2nd paragraph). Are these mosquitoes obtained from a pool across all collection sites or derived from a single site?

P.5: Rearing (8th paragraph): "*This was achieved by placing suspected male pupae based on size into cups of 10;*…"

Given the difficulty of visually sexing each pupae cup without the aid of software and hardware-based engineering, what was the overall confidence level in this sex by size strategy? Although there is no risk for the community, as the CI and female-based deployment deals with the issues associated with accidental female release in this case, it would be interesting to address this aspect of the method used by the research team.

P.7: Mosquito releases (3rd paragraph): "*For stage 1, it required between 7 and 19 weeks of releases for each suburb to reach that target*."

It is known that the rate of dispersion of *Ae. aegypti* correlates with the human density in a given area. As such, how long did the authors wait to start screening field collected mosquitoes, as a way to avoid screening the same mosquitoes that were released in a particular area? It is not clear how far the BG traps were set apart from the MRC's.

P.7: Mosquito releases (2nd and 4th paragraphs): In summary, MRC's had 100 eggs / 1L of water and 5 (summer) / 6 (winter) wafers of Aqua One vege wafer fish food, while Mozzie boxes had 100 eggs/ 400mL of water and 4 (summer) / 5 (winter) wafers of Aqua One vege wafer fish food. In terms of quality assessment of the fitness of the mosquitoes being released, how was the comparison between the MRC's and the Mozzie boxes?

P.7: Mosquito releases (5th paragraph): "*Finally, in the last two suburbs of stage 3 (Kelso & Rasmussen) and across stage 4, releases of adult mosquitoes were used to fill in gaps in MRC coverage*".

Here two distinct deployment strategies were combined. As such, the release procedures used were the same as established in Hoffmann *et al*., 2011 (citation #7), in terms of number of females and release period (in weeks)? How this combined approach compares to the deployment of *Wolbachia* through a single release method in terms of establishment efficiency?

P.8: School releases (2nd paragraph): "*…but made of clear plastic to encourage student observation (Figure 2B)*".

No suggestion here. Just a praise to the attention to the details contributing to the community engagement. WMP is not only deploying their method, but also educating the community.

P.8: School releases (3rd paragraph): "*…who gave presentations encouraging participation prior to each of the three mosquito release cycles*."

How was the level of engagement along the three cycles? Given the author's interesting approach, this information could be helpful as a proxy for the predicted efficacy of this strategy in release areas worldwide.

P.12: Results and discussion (3rd paragraph): "*In general, establishment of Wolbachia occurred reliably after releases stopped once the 50% threshold was met.*"

To date, does the WMP continues to screen these areas where *Wolbachia* was released roughly 2-3 years ago? If the answer is yes, does the infection frequency still high in these areas? I am particularly interested in the Condo area, where the last data point shows an infection frequency of 51.79%.

Final thoughts and suggestions:

The following questions are not within the scope of what is proposed for this manuscript, just a couple suggestions.

1. Have the authors considered screening areas where *Wolbachia* deployment did not occur but were adjacent to release zones? For instance, given the low human density in Townsville, a key factor associated to mosquito dispersal, as previously stated, and recent data showing challenges in *Wolbachia* establishment in Cairns, having long-range dispersal as one potential reason, would be interesting to see how contained *Wolbachia* deployment is, in my opinion this information would be particularly relevant in areas where *Wolbachia* is soon to be deployed, as it can avoid legal conflicts within the context of community acceptance in neighborhoods where the use of the bacterium has not been yet approved or assessed.

2. Would be interesting to see data based on mitochondrial DNA screening in *Wolbachia*-harboring mosquitoes. The fine-tune of such approach using Townsville as a model would benefit the program as a whole, providing information related to mosquito immigration events and potential imperfect transmission of the bacterium.

3. A future genomic analysis of the mosquito population pre- and post- release of *Wolbachia* would provide a unique opportunity to evaluate the intrinsic impact of the bacterium on the mosquito population structure as a whole.

Finally, I want to take this opportunity to congratulate the authors and the team personnel for the massive work necessary to obtain the results described here.

References

1. Endersby-Harshman NM, Wuliandari JR, Harshman LG, Frohn V, Johnson BJ, Ritchie SA, Hoffmann AA: Pyrethroid Susceptibility Has Been Maintained in the Dengue Vector, Aedes aegypti (Diptera: Culicidae), in Queensland, Australia.*J Med Entomol*. 2017; **54** (6): 1649-1658 PubMed Abstract | Publisher Full Text
2. Schmidt TL, Filipović I, Hoffmann AA, Rašić G: Fine-scale landscape genomics helps explain the slow spatial spread of Wolbachia through the Aedes aegypti population in Cairns, Australia.*Heredity (Edinb)*. 2018; **120** (5): 386-395 PubMed Abstract | Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
No source data required

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Reviewer Report 10 August 2018

https://doi.org/10.21956/gatesopenres.13925.r26599

Gregor J. Devine
Mosquito Control Laboratory (MCL), QIMR Berghofer Medical Research Institute, Herston, Qld, Australia

Summary:

Wolbachia-mediated control is one of the most exciting recent developments in the struggle against dengue and other mosquito borne diseases. We already know from this group's previous work in Cairns that the replacement of wild-type *Aedes aegypti* populations with Wolbachia-infected forms is both feasible and sustainable in towns of around 150,000 people.

Epidemiologically, it would have been more interesting to see a summary of impacts on dengue transmission in Cairns as, historically, that town experiences far more dengue transmission and more dengue imports than Townsville.

The novelty in the current report is that this is the first "citywide scaled deployment". This scaling refers to the direct involvement of the community and local school children in executing the releases.

Comments:

I would have liked a little more background and explanation on the following:

1. What is the rationale for backcrossing the wMel strain with a local wildtype for three generations prior to release? Is there any underlying empirical basis for this in terms of fitness and/or genetic homogeneity?

2. It appears from Fig 3 that, by the end of the monitoring period (mid 2016), very few mosquitoes were being captured and that almost all were Wolbachia-infected. Was that very low density a result of a hostile climate? Is it possible that mosquito suppression as well as replacement is having an impact on transmission here? Is declining mosquito density a feature of wMel establishment? Were any Aedes endemic, non-release areas monitored for comparison?

3. The authors state that the outcomes of releases by schools-based programs were of secondary importance to their value as instruments of engagement. In this paper, it is flagged as a major component of "scaled deployment" so it would have been interesting to report on the operational success of the schools-based program. Was there any evaluation of compliance (observation of

the releases made by children) or of the rates of Wolbachia replacement in areas specifically targeted by schools?

4. It seems likely that Wolbachia is reducing transmission risks in Townsville, but other contributing factors may be being ignored. My understanding is that releases of Wolbachia in Townsville have coincided with some of the hottest and driest years on record. Climate has a direct impact on mosquito survival and is strongly correlated with transmission.

5. There's no real discussion of limitations or challenges here. The authors state that economies of scale, in regions of high population density, will result in successful deployments costing less than $1 per capita. The assumption is that population replacement and dengue-blocking across hyperendemic urban sprawls will be cheap and simple.

   Townsville has very limited dengue transmission and adult indices appear lower than for many tropical cities. Does the WMP not see some issues with extrapolating successes in Townsville to the rest of the world?

6. The authors dismiss work on Wolbachia loss and heat stress (Ulrich et al PLoS NTDs, Ross et al PLoS Pathogens) but Aedes do demonstrate fast and successful development in the field at water temperatures ≥ 30^{o}C and the truth is that we don't know much about the operational impacts of those conditions on Wolbachia stability.

   This paper certainly suggests that, in Townsville, Wolbachia-infection is stable. But in Townsville monthly average high temperatures range between 31.5 and 25.1^{o}C. In Bangkok, they range from 35.4 to 31.7^{o}C. A global Wolbachia operation will at least need to consider the potential impacts of high water temperatures in aquatic habitats.

7. What are the factors that result in a minority of sites hovering around 70% Wolbachia coverage? Is it immigration, or the presence of some protected, wild-type reservoir. Or is it related to mosquito density and a relatively small number of dispersal events?

Overall, this paper represents a keenly awaited progress report on the stability and feasibility of Wolbachia releases in Australia and the ways in which those releases might be made more cost effective.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Vector ecology, vector control, vector competence, dengue interventions, characterisation of Wolbachia impacts

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 16 Aug 2018

Scott O'Neill, Monash University, Clayton, Australia

Response to reviewer 1

The reviewer's statements around novelty are incorrect in stating that we already know that we can deploy in cities of 150,000 by referring to deployments around Cairns, Australia. The only studies published from Cairns relate to small pilot deployments. Many small pilots have been undertaken in Cairns and surrounding areas since 2011 that have focused on obtaining data on the best ways to deploy mosquitoes. In the last 2 years, we have "filled in" around these pilot areas to provide broad coverage to all of North Queesnland's major dengue risk areas. This study is actually the first published study of a citywide deployment being undertaken as a single project which shows that a "greenfield" city can be engaged and the intervention deployed at scale, cost effectively and quickly. The use of the community to deploy is an additional component of the paper but not the primary aim.

We have a companion paper in preparation that will report the results from deployments in the city of Cairns that will reinforce the findings of this study.

Specific Comments

1. We undertook three generations of backcrossing to make sure our release strain closely approximated the genetic background of the Townsville target population. This was done as a precautionary measure and was not based on specific empirical data that aimed to characterise any differences between the Cairns and Townsville genetic backgrounds of the resident mosquitoes.

2. Figure 3 shows total counts of mosquitoes that were run through diagnostics in a given sampling period. This is presented to help provide an estimate of sample size that underpins a given frequency estimate. There is a correlation between this number and actual mosquitoes caught but it cannot be used to estimate population size because the number of traps contributing the sample was variable. We reduced BG traps in a given area after Wolbachia was considered established (often by more than 60%) and these traps were moved to areas where active releases were being undertaken. As a result, the impression that mosquito populations declined after release is not accurate. Modelling predicts that mosquito populations should reduce slightly after the introduction of Wolbachia but we did not attempt to measure that in this study

3. Because the schools program used volunteer students to undertake the releases and these

children were scattered geographically they were supplemented with programmatic releases in adjoining areas which made it impossible to compare their effectiveness from an entomological perspective with purely programmatic releases. We did undertake QA procedures on a sample of student release containers to evaluate the program. The major consideration for us was that this form of release was quite expensive compared to other forms so from a purely economic perspective it was inefficient. However, its value to us was more from a community engagement perspective as Schools are fundamentally trusted in the community and it was an indirect method to engage parents of school children through undertaking the releases and increase community awareness of the program.

4. Temperatures between 2015-18 in Townsville have been above average and rainfall below average but certainly not the hottest or driest years on record. Indeed, if you examine the meteorological data for the period in which we analysed dengue cases you can see that similar climatic conditions were experienced in Townsville between 2001 -2006 and during these years significant local dengue transmission events occurred, indicating no obvious correlation between temperature/rainfall variability in Townsville and dengue transmission. See figure below for data

Yearly rainfall (A), maximum temperature (B) and minimum temperature (C) anomalies, and locally acquired dengue cases (D) for Townsville from 2001-2018. Yearly rainfall anomaly values were calculated by comparing yearly, northern wet season rainfall totals (cumulative rainfall totals from October to April each year) to the long-term, 30-year wet season yearly average (October 1961 to April 1991; Standard Reference Period as defined by World Meteorological Organisation) (Bureau of Meteorology, Retrieved 15 August 2018 from http://www.bom.gov.au/climate/how/newproducts/map-periods.shtml); yearly maximum temperature anomaly values were calculated by comparing yearly, northern wet season maximum temperature mean values (daily maximum temperatures from October to April) to the long-term, 30-year wet season average (October 1961 to April 1991); yearly minimum temperature anomaly values were calculated by comparing yearly, northern wet season minimum temperature mean values (daily minimum temperatures from October to April) to the long-term, 30-year wet season average (October 1961 to April 1991); yearly cumulative dengue cases from October in the previous year through to September in the current year (Note: 2001 cumulative cases from January to September 2001; 2018 cumulative cases from October 2017 to June 2018). Weather/climate data obtained from Bureau of Meteorology (Station: TOWNSVILLE AERO, 032040). Green shading represents period of *Wolbachia* mosquito release and establishment (commenced October 2014).

5. The main assumption underlying reduction in costing in based on the fact that major urban tropical cities have much higher population densities than Australian cities but many of the costs in deployment relate to the area of deployment. This means that costs will automatically decline in areas of higher population density and that is what we have seen in other work of the WMP where deployments have already been undertaken successfully as part of clinical trials that are underway in other countries.

6. Our criticism is that the heat stress experiments have been undertaken either in incubators or in semi-field settings and then the results extrapolated to infer a potential problem in stability of *w*Mel in the field. However, the field is much more complex than an incubator and many breeding sites are cryptic and difficult to assay, so the true water temperature experienced by a population of

mosquitoes in the field cannot be accurately measured. What we do know from this and unpublished work in other cities is that *w*Mel appears unaffected in locations with ambient temperatures that might indicate problems for *Wolbachia* transmission based on these studies. We can only assume from this that the studies predicting breakdown of wMel are not actually predictive of field settings.

7. Our current hypothesis is that *Aedes aegypti* can often have a relatively large number of eggs in a dried state in a given area and over time these eggs will hatch depending of factors such as rainfall events or human assisted wetting. This mimics the effect of *Wolbachia* uninfected individuals immigrating into an area. Over time this egg bank becomes depleted and frequencies then move to stable rates closer to 90% or above.

Competing Interests: No competing interests were disclosed.

Author Response 17 Aug 2018

Scott O'Neill, Monash University, Clayton, Australia

For access to temp and rainfall data in Townsville see

https://s3-eu-west-1.amazonaws.com/gatesopenresearch/linked/180566.0%27NeillFigure.docx

Competing Interests: No competing interests were disclosed.

Author Response 25 Oct 2018

Scott O'Neill, Monash University, Clayton, Australia

Response to reviewer 2

Response to General comments

1. Unfortunately similar work as reported here was being undertaken in other population centres in Australia at the same time and as a result there is no suitable site to use as an external control that is of equivalent size and not having Wolbachia mosquitoes released. We provided a detailed response to the other reviewer indicating that weather conditions across the monitoring period before and after releases did not correlate with the observed reduction in dengue cases.

2. Discussion addressing this point has been added to the revision.

Detailed points
P1. Title changed to explicitly reference dengue

P4. In releases in Australia our approach was just to backcross to local populations in order to attempt to match the local population in various traits including insecticide resistance. We did not do more than this in Australia since early pilot studies (eg Hoffmann et al 2011) showed that this strategy was adequate. As shown by the results in this setting this was a sufficient approach to obtain success. In other countries, we have had to develop different strategies to deal with high

levels of insecticide resistance. These approaches and results will be presented in future papers describing results from those study sites. We feel that it would be better to address those topics in those papers rather than in this paper that did not examine insecticide resistance deeply.

P4. Our collections of wild type material were made from pooling across many ovitraps placed in areas where Wolbachia had not been released as we did for the initial backcrossing. Our goal was to generate genetically diverse material so it was pooled. Given that we could store the eggs we were able to maintain material for outcrossing during the release period as described in methods. As a result, we did not monitor for Wolbachia in the males. In future studies, there is no reason to exclude already treated areas or for the included males not to be Wolbachia infected. We have added text to the revised paper to improve clarity.

P5. Wolbachia infection testing – 1. We tested 160 individuals from each cohort. A cohort was on average around 7500 individuals so around 2% of individuals. The sample size of 160 individuals was determined from a prior power analysis. 2. We have seen no evidence for reduced maternal transmission in our mass production.

P5 – Yes these mosquitoes were derived from the same material used to outcross the colony in our Melbourne labs as well as backcross.

P7 – Screening started prior to releases starting and was undertaken weekly. MRCs had variable placement due to the dependence f volunteers to host MRCs. It was not possible with that system to designate fixed differences between MRC's and BGs. However, if you examine the shape of the establishment curves you can see that in most suburbs catching back of release material was not a big issue. If it was then you would expect to see Wolbachia frequencies climb quickly and then potentially decay after releases stopped. We only see those patterns in areas where adult releases took place.

P7 – No fitness comparisons were made between the two but regular QA procedures indicated that emergence rates were similar between the two.

P7 – The mixed approach used in the final experiments was undertaken due to operational issues with the completion of the projects and staff contracts completing. This was done to complete the study in time to coincide with staffing contracts finishing and was not set up as a comparison – it can't really be analysed that way as a result. Comparisons between deployment methods are being undertaken in other sites in a more rigorous manner and these will be able to report on this comparison.

P8 – Generally we found difficulty in sustaining interest in repeating the releases by the third round and our engagement officers needed to work harder to maintain interest levels in students for later round releases. As indicated in the paper we felt that school releases were better primarily as an engagement tool and direct public releases more effective from an establishment and cost perspective.

P12 we have updated the figures with the latest monitoring data across the city to provide a more recent view of establishment success and dengue cases. Later monitoring has involved LAMP diagnostics and this protocol has also been included in the methods description. We have also updated the epidemiological figure with the latest data showing an extension of post release

monitoring till Oct 2018 and continued impact on locally acquired dengue cases.

Final thoughts
1. Work on dispersal is underway in our Indonesian RCT site which will provide detailed information on Wolbachia dispersal between adjacent geographic areas. In generally natural dispersal rates are low as described already in Cairns.

2. mtDNA as a marker for imperfect transmission or immigration cannot be done in Australia as the release strain carries the same mtDNA genotype as the wild population. It can be done however in other countries and is being done in different sites.

3. Agreed and something for future work.

Competing Interests: No competing interests were disclosed.