BMC Medicine

RESEARCH

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Adjacent spillover efficacy of *Wolbachia* for control of dengue: emulation of a cluster randomised target trial

Jue Tao Lim[1,2†], Diyar Mailepessov[2†], Chee Seng Chong[2†], Borame Dickens[3], Yee Ling Lai[2], Youming Ng[2], Deng Lu[2], Caleb Lee[2], Li Yun Tan[2], Grace Chain[2], Soon Hoe Ho[2], Chia-Chen Chang[2], Pei Ma[3], Somya Bansal[3], Vernon Lee[4], Shuzhen Sim[2*], Cheong Huat Tan[2] and Lee Ching Ng[2,3,5]

Abstract

Background Matings between male *Aedes aegypti* mosquitoes infected with *w*AlbB strain of *Wolbachia* and wild-type females yield non-viable eggs, thereby suppressing *Ae. aegypti* abundance in the field. We evaluated the spillover efficacy of releasing *w*AlbB-infected *Ae. aegypti* male mosquitoes to suppress dengue in sites adjacent to release sites (spillover sites).

Methods The protocol of a two-arm cluster-randomised test-negative controlled trial (cRCT) was specified and emulated using a nationally representative dengue test-negative/positive database of 454,437 individuals reporting for febrile illness to primary or secondary care in public healthcare institutions. Spillover intervention sites were defined by geolocating locations which were adjacent to, i.e. shared geographical borders with, actual *Wolbachia* intervention sites. We built a cohort of individuals who resided in spillover sites versus a comparator control group who resided in sites which did not receive *Wolbachia* interventions. We emulated a constrained randomisation protocol used in cRCTs to balance dengue risk between spillover and control arms in the pre-intervention period. We matched individuals reporting for testing in intervention and control groups by calendar time and a high-dimensional battery of sociodemographic, environmental and anthropogenic variables. Intention-to-treat analysis was conducted to estimate the protective efficacy against dengue given spillover *Wolbachia* exposure.

Results The final cohort consisted of 2354 matched individuals residing in *Wolbachia* spillover and control sites for at least 3 months in the study period. Compared to the controls, individuals residing in spillover sites for 3 or more months were associated with a 45% (OR: 0.55, 95% CI: 0.42–0.74) reduction in risk of contracting dengue. Higher durations of spillover *Wolbachia* exposure also modestly increased protective efficacies. Compared to the control arm, the proportion of virologically confirmed dengue cases was lower in the spillover arm overall and across each subgroup. Protective efficacies were found across all years, age and sex subgroups.

Conclusions Our results demonstrated the potential of *Wolbachia*-mediated sterility for reducing the risk of contracting dengue even in sites which were not directly treated by the intervention.

Keywords *Wolbachia*, Incompatible insect technique, Adjacent spillover, Dengue, Singapore

† Jue Tao Lim, Diyar Mailepessov and Chee Seng Chong contributed equally to this work.

*Correspondence:
Shuzhen Sim
Sim_Shuzhen@nea.gov.sg
Full list of author information is available at the end of the article

Background

Dengue is the predominant arboviral disease globally and has shown sustained increases in burden over the years [1], with the Americas and Southeast Asia routinely accounting for the largest proportion of global cases [2]. While the two licenced vaccines against dengue were reported to be somewhat efficacious against certain serotypes and could be recommended for settings with high transmission intensity, there is incomplete evidence of protection against all four dengue serotypes [3, 4]. Vector control remains the primary tool for mitigating dengue due to the lack of available therapeutics and highly effective vaccines globally. Conventional vector control measures can comprise environmental management, source reduction and insecticide use [5, 6]. While these measures effectively reduce the burden of dengue, some may be resource intensive and yield diminishing returns.

Few randomised controlled trials have examined the efficacy of vector control tools to reduce dengue burden. One multi-site trial has shown that community mobilisation led to fewer reports of *Aedes* mosquito infestations in Nicaragua and Mexico [7] and another has used the endpoint of virologically confirmed dengue to examine the impact of introgressing "virus-blocking" strains of *Wolbachia* into field *Aedes aegypti* populations on dengue incidence in Yogyakarta, Indonesia [8]. Both trials estimated the direct effect of interventions on intervened locations, but field-based interventions such as vector control can have spillover effects to control units and may generate noticeable reductions in dengue burden even in locations which were not the designated intervention sites. To the best of the authors' knowledge, no trial has examined the potential spillover effect of these interventions on dengue risk in nearby sites, and current work may have severely underestimated the true intervention effect of vector control, especially when designated control locations were located close to intervention locations.

To augment vector control in Singapore, an equatorial city-state where dengue transmits year-round, extensive field trials of *Wolbachia*-mediated incompatible insect technique (IIT) targeting *Ae. aegypti* have been conducted in 4 towns comprising over 600,000 residents. IIT is an alternative vector control measure where only *Wolbachia*-infected male mosquitoes are released. Through cytoplasmic incompatibility [9, 10], matings between *Wolbachia*-infected males and uninfected females yield infertile eggs, and repeated releases of *Wolbachia*-infected males have been shown to substantially suppress wild-type mosquito populations, even in nearby locations which were not designated intervention sites [11]. We therefore hypothesise that significant protection against dengue can be expected in these locations.

In the study setting, 4 towns were subject to interventions being adopted in either a staggered or targeted fashion [12, 13], which provided a rare opportunity to ascertain the spillover epidemiological efficacy of *Wolbachia* interventions to reduce dengue risk. Though data from randomised trials are currently not available, observational analyses can be used to ascertain epidemiological efficacies by adopting a target trial emulation approach [14–16]. This study used a nationally representative test-positive/negative cohort comprising individuals who were tested for dengue to emulate a cluster-randomised test-negative target trial to ascertain the epidemiological efficacy of *Wolbachia*-mediated sterility to reduce the incidence of virologically confirmed dengue in individuals residing in areas which were not directly treated by *Wolbachia* interventions.

Methods

Specification of the cluster-randomised test-negative target trial

We specified a cluster-randomised test-negative target trial [14] to evaluate the spillover efficacy of releasing *w*AlbB-infected *Ae. aegypti* male mosquitoes for dengue control via vector population suppression, from epidemiological week (EW) 1 2019 to EW26 2022 in Singapore. The target trial was emulated using a nationally representative test-positive/negative database which comprised all patients who report to any general practitioner clinic, polyclinic or public/private hospital and were suspect of dengue illness during the study period in Singapore (Additional file 1: S1, Table S1).

Characterisation of *Wolbachia* interventions

Male *Wolbachia*-infected *Ae. aegypti* were released twice weekly (weekdays, 0630–1030 h) at four townships in high-rise public housing estates covering 607,872 individuals as of EW26 2022. Bukit Batok, Choa Chu Kang and Yishun towns were subjected to interventions which combined IIT with SIT. Tampines town used the high-fidelity sex-sorting methodology and also progressively adopted sterile insect technique (SIT) protocols to release irradiated mosquitoes from January 2020 [16] (Additional file 1: S2, [15]). To trial whether *Ae. aegypti* population suppression could be sustained over increasingly larger areas, an expanding release approach was adopted in two large towns (Yishun, Tampines), where release sites were gradually expanded to adjacent neighbourhoods. In Bukit Batok and Choa Chu Kang towns, a targeted release approach was adopted, which focused releases on areas with high *Ae. aegypti* abundance and persistent dengue transmission (Table 1, Additional file 1: S3, Figs. S1–S4).

Direct and spillover efficacy of *Wolbachia* interventions to reduce *Ae. aegypti* abundance was measured using Gravitrap index [11, 17], which was derived from Gravitrap surveillance data (see Additional file 1: S4, [17]). Gravitraps were deployed in all public housing apartments in Singapore and were measured on a bi-weekly basis. The Gravitrap index was defined as the mean number of female adult *Ae. aegypti* caught per functional Gravitrap. It estimates the *Ae. aegypti* population by normalising the total number of female adult *Ae. aegypti* caught with the total number of functional Gravitraps recovered.

Emulating randomisation protocols from cluster-randomised trials for *Wolbachia* interventions

Twenty-six townships in Singapore which were never subject to *Wolbachia* interventions during the study period were considered potential control site locations. Towns were demarcated planning areas used by government ministries and departments for administrative purposes. Towns were further divided into sectors, which comprised 10 or more public housing apartment blocks and with area spanning around 0.088 km^2 on average and were used for planning of surveillance and control for environmental infectious diseases in Singapore, as well as the release schedule of *Wolbachia* interventions. While four towns which were subject to long-term *Wolbachia* releases were not randomly pre-selected, we emulated constrained randomisation protocols for cluster-randomised trials by generating 1000 iterations of randomly selected combinations of 12 townships and retaining only those that had almost identical average historical dengue incidence to the towns within the intervention arm in the pre-intervention period, before randomly selecting one of the combinations [8, 18, 19]. This was done to prevent imbalance in baseline dengue risk due to the small number of intervention ($n=4$) locations considered (Additional file: S5). All locations practised the same baseline dengue control protocol before and during the intervention period [5, 6].

Cohort

Under the Infectious Diseases Act, all laboratory-confirmed cases of dengue are legally mandated to be reported and are collated into the national dengue surveillance system. Approval from the Director General of Health, Ministry of Health, was obtained to collect and use the data of dengue-suspected patients, whose blood samples are sent for dengue tests, through a national network of diagnostic laboratories that support private clinics, public polyclinics and public/private hospitals.

We collated test results of all individuals who were suspects of dengue illness and reported to any general practitioner clinic, polyclinic or public/private hospital in the study period. A total of 133,821 individuals reported for febrile illness and were tested for dengue nationally from EW1 2019 to EW26 2022. All samples from dengue-suspect patients were tested by licenced diagnostic test laboratories using either an internally controlled quantitative RT-qPCR assay, rapid test kits or enzyme linked immunoassays (ELISA) to detect dengue non-structural protein 1 (NS1) or IgM [6, 18–21]. We excluded individuals

Table 1 Summary of *Wolbachia* intervention approaches over 4 townships

Township	Bukit Batok	Choa Chu Kang	Tampines	Yishun
Intervention start date	EW23 2020	EW20 2020	EW39 2018	EW27 2018
Study end date	EW26 2022	EW26 2022	EW26 2022	EW26 2022
Intervention time (weeks)	109	112	197	209
Total township size (m^2)[a]	627,441	1,145,559	5,088,046	3,473,690
Production approach[a]	IIT-SIT	IIT-SIT	High-fidelity sex-sorting[f]	IIT-SIT
Frequency of release	Twice weekly	Twice weekly	Twice weekly	Twice weekly
Release strategy[c]	Targeted[d]	Targeted	Expanding[e]	Expanding
Number of mosquitoes released	1–7 *w*AlbB-SG males were released per study site resident per week			
Total number of mosquitoes released (rounded to thousands)	17,139,000	14,598,000	109,432,000	77,659,000
Township population covered by release over study period	40,132	64,672	272,048	231,020

[a] Total area of public housing estates subject to release in respective townships in EW 26 2022

[b] The IIT-SIT approach and high-fidelity sex-sorting were detailed above and has been previously characterised [16, 17]

[c] Denotes approach to releasing *Wolbachia*-infected *Ae. aegypti* males

[d] Targeted approach which focused releases on areas with high *Ae. aegypti* abundance and persistent dengue transmission

[e] Expanding ("rolling") approach where release sites were gradually expanded to adjacent neighbourhoods

[f] IIT-SIT increasingly adopted into production protocol from August 2020 onwards

(9%) who were tested on more than one occasion in 4 weeks, individuals who had more than one residential address in different control or intervention townships and individuals who had been tested at different labs with conflicting dengue results. We also excluded individuals who had residential addresses at or around intervention sites at the time of the test but were not spillover-exposed to *Wolbachia* interventions for at least 3 months, based on exposure criteria described below. The 3 or more months exclusion criteria was based on the time taken for *Wolbachia* interventions to induce suppression of wild-type mosquitoes in areas which have been spillover exposed to the intervention [11].

Four intervention townships (Bukit Batok, Choa Chu Kang, Tampines, Yishun) had 2354 tested individuals residing in areas which were adjacent to directly treated areas in the study period. After constrained randomisation, we selected 12 control townships (Ang Mo Kio, Bukit Merah, Hougang, Jurong East, Jurong West, Kallang, Pasir Ris, Punggol, Sembawang, Sengkang, Serangoon, Toa Payoh) with 65,287 tested individuals in the study period. Post-selection, the control arm had an average dengue incidence rate normalised by population size which was less than 5% different from the intervention arm in the pre-intervention period of EW1 2010 to EW52 2016, indicating good balance in historical dengue risk between arms. The locations of intervention and control sites are depicted in Fig. 1.

We further employed exact matching for calendar time among individuals who were spillover-*Wolbachia* exposed and unexposed (from control sites). This ensured that baseline transmission risks were balanced between the temporally matched exposed/unexposed groups. Furthermore, exact matching of calendar time ensured that the probability of being a dengue case versus test-negative control is the same under the null, across the study period, mitigating calendar time bias which may stem from the staggered adoption of spillover-sites. Moreover, we employed nearest neighbour matching for all other environmental and anthropogenic characteristics to control for differences in baseline characteristics between spillover-*Wolbachia* exposed and unexposed groups. Each spillover-*Wolbachia* exposed individual is matched to 1 unexposed individual. Standardised mean differences (SMDs) for matching variables were then computed to assess covariate balance after matching.

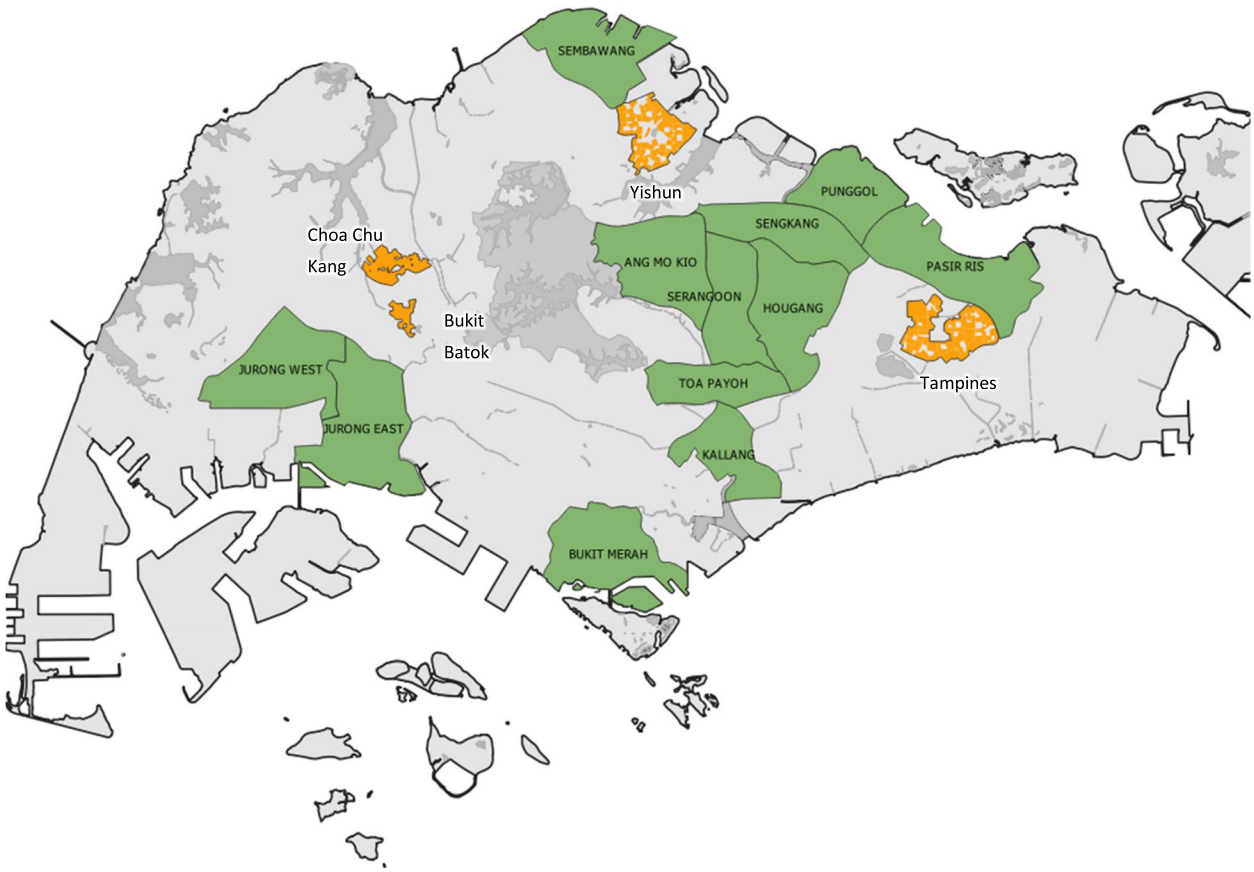

Fig. 1 Intervention and randomly selected control sites. Control sites are labelled in green while 4 intervention sites in yellow

Exposures and outcomes of interest

The protective effect of spillover *Wolbachia* exposure (Fig. 2) was studied and was taken as a binary classification based on the tested individual residing in a township which was designated as a *Wolbachia* intervention town, but in a sector which did not experience *Wolbachia* releases at the point of the individual's test and shared a geographical border with a sector which had *Wolbachia* releases (spillover *Wolbachia*-exposed), or a control sector within the selected control townships (*Wolbachia*-unexposed).

We subcategorised spillover *Wolbachia* exposure based on whether an individual resided in a sector which was adjacent to a sector which experienced sustained *Wolbachia* releases for at least 3, 6 or 12 months. The criteria was used following previous work which demonstrated the time required for releases to induce noticeable vector suppression in locations which did not experience releases [11]. Home address was defined as the primary place of residence reported on the diagnostic test date. 2354, 1755 and 1066 residents were spillover-exposed in the study period for at least 3, 6 and 12 months respectively.

The spillover intervention effect was estimated from an odds ratio comparing the exposure odds (residence in a spillover intervention location for at least 3, 6 or 12 months versus unexposed controls) among participants who were dengue test-positive versus test-negative controls, estimated using G-computation on a matched sample after fitting the outcome using logistic regression as described in statistical analysis below. The null hypothesis was that the odds of residence in a spillover intervention sector would be the same among participants who test-positive as that among test-negative, versus control sectors. Spillover intervention efficacy was calculated as $100 \times (1 - \text{odds ratio})$.

Covariates

We compiled a comprehensive array of high-resolution variables with specific spatial and temporal characteristics to delineate the environmental diversity across sectors during the study period. Prior to selecting these variables, we considered various covariates that may act as confounders, including (1) vegetation maps from satellite imagery classified into types that represent different ecological niches and availability of feeding, classifying areas into multiple vegetation types such as forests and managed vegetation to indicate the presence of natural breeding sites and nectar availability for male mosquitoes, (2) averaged Normalised Difference Vegetation Index per sector serving as a general indication of greenness, (3) population data on public housing estates where over 75% of the resident population resides describing host density and urban breeding habitat availability, (4) average age of housing from transaction datasets as older building estates are a known risk factor for

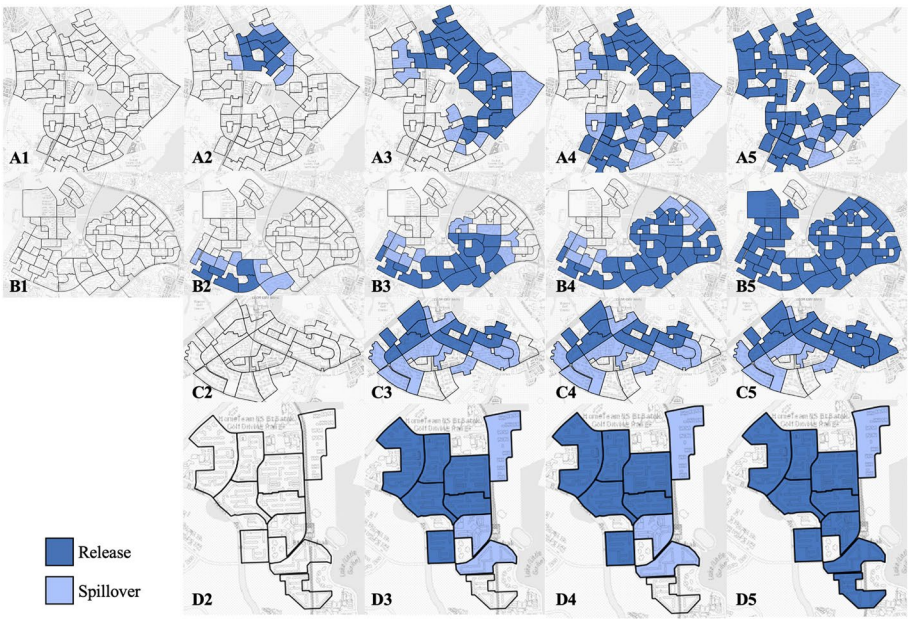

Fig. 2 Release and spillover sectors in (**A**) Yishun, (**B**) Tampines, (**C**) Choa Chu Kang and (**D**) Bukit Batok in June of (**1**) 2018, (**2**) 2019, (**3**) 2020, (**4**) 2021 and (**5**) 2022. Release sectors were sectors where sustained *Wolbachia* releases have occurred at that point of time; spillover sectors are locations with borders touching actual release sectors

increased mosquito breeding rates, (5) average house price over the study duration acting as a socioeconomic status indicator, (6) building height where taller buildings may cause more disruptions in wind movement and mosquito movement, (7) number of condominium and landed properties within each sector representing more affluent housing and alternative habitat and host availability, (8) percentage cover of built area inclusive of all residential, commercial and industrial building types as concrete surfaces can be more conducive for *Ae. aegypti* breeding and (9) major open drainage network data from the Public Utilities Board which is a known area of mosquito breeding where average distance to housing and total length of open drain is measured within each sector. In addition to these ground variables, well-established meteorological variables known to affect mosquito survival or fecundity such as daily mean, maximum and minimum temperature, total rainfall and wind speed were collected from 21 local weather stations. Hourly dewpoint and ambient ground air temperature were also obtained from remote sensing measurements to estimate relative humidity over the time period using a standard formula. These values were aggregated at a weekly level to align with the temporal frequency of dengue test-positive/negative data. Detailed information about data sources and processing procedures is provided in Additional file 1: S6 [18, 22–30].

Statistical analysis

Baseline characteristics of the cohort were presented as mean and standard deviation or as frequency and percentage. SMDs were used to evaluate balance between intervention and control arms, with SMD < 0.1 indicating good balance.

To estimate the spillover effect of *Wolbachia* interventions on the risk of dengue, we employed G-computation using trained logistic regression models. We specified logistic regression models taking *Wolbachia* exposure status as the variable of interest, with the cohort matched on calendar time and other environmental/anthropogenic characteristics. We then estimated the marginal odds ratios (ORs) of being dengue test-positive between the matched spillover and control groups using G-computation, which compared the expected potential outcome under spillover *Wolbachia*-exposure and the expected potential outcome under no exposure across the dataset.

To account for between-town dependencies, we relied on cluster bootstrapping based on 1000 clustered resamples. In each iteration, we resampled townships with replacement and repeated the matching and estimation procedure of ORs for each subsample to construct the bootstrapped distribution of ORs. Balanced bootstrap resampling based on town membership can account for within-town dependencies and has been used as a competitive approach to analyse hierarchical data [31]. The associated bootstrap percentile-based confidence interval was used to construct the 95% confidence interval for odds ratios, and findings were considered to be statistically significant when the 95% confidence intervals for ORs did not cross 1. Additional information on statistical analysis plan can be found in Additional file 1: S7.

Subgroup analysis

We repeated all analysis by subsetting the exposed group to years (2020, 2021, 2022) to examine any potential differences in spillover intervention effect between epidemic and inter-epidemic years. We also repeated analysis by age (< 20, 20–65, 65 +) and sex (male, female) groupings. Here, we conducted subgroup analyses by re-estimating odds ratios using the aforementioned statistical procedures, but only including individuals enrolled or comprising that specific subgroup.

Robustness checks

We conducted a battery of sensitivity analyses to ensure the robustness of our model estimates. We (1) re-randomised the allocation of controls 1000 times using the constrained randomisation approach and repeated our analysis by using the newly allocated controls arm, and compared our primary estimate of spillover intervention efficacy against the empirical distribution of re-randomised intervention efficacies; (2) used the algorithmically selected set of covariates for matching based on nearest neighbour instead of all covariates in our main analysis, to recompute odds ratios and spillover intervention efficacies; (3) controlled for the same variables used in matching to provide additional robustness to slight imbalances remaining after matching; (4) conducted in-space placebo checks on control sites, taking each allocated control site as the allocated placebo-intervention site and re-estimated odds ratios and intervention efficacies by comparing test-negative and positive individuals in the placebo-intervention versus other control sites; (5) conducted placebo checks on spillover intervention sites, taking each spillover intervention site in 52 and 104 weeks before the actual spillover intervention as the intervention period, and recomputed spillover intervention efficacies among allocated controls and spillover intervention sectors based on this placebo-intervention period; and (6) recomputed spillover

intervention efficacies using sectors instead of towns for clustered bootstrapping procedure.

Code availability statement

All analyses were conducted in R version 4.2.3 and can be shared upon request.

Permission to use anonymised dengue test-negative/positive data was obtained from the Ministry of Health, Singapore for the sole purpose of evaluating *Wolbachia* interventions. Third party sharing of these data are precluded.

Results

Suppression of *Ae. aegypti* populations in spillover sites

Suppression of adult wild-type *Ae. aegypti* populations was demonstrated across spillover sites which were adjacent to actual *Wolbachia* release sites, with the Gravitrap *Ae. aegypti* index (GAI) reduced as the duration of being an adjacent site increased. This is most noticeable across townships (Tampines, Yishun) where interventions were adopted in a staggered setting (Fig. 3).

Study characteristics

Among 137,806 individuals who reported for febrile illness from EW1 2019 to EW26 2022, in the intervention and control arms, 58,640 (42.55%) were included for matching in the study. Baseline demographic and spatial–temporal characteristics between *Wolbachia* spillover-exposed and unexposed groups before and after matching was employed were presented in Table 2. Characteristics were well-balanced after the matching procedure (Table 2) with negligible differences in baseline characteristics between both groups.

Efficacy of *Wolbachia*-releases in reducing risk of dengue in spillover areas

Among dengue-tested individuals residing in areas which were *Wolbachia* spillover-exposed for at least 3 months, the percentage of individuals who tested positive for dengue (12.79%, 301 of 2354 individuals) was lower compared to the *Wolbachia*-unexposed (21.07%, 496 of 2354 individuals) group. The pattern of lower test-positivity rate was consistent across all spillover exposure durations and subgroups.

In primary analysis, *Wolbachia* spillover-exposure of at least 3, 6 or 12 months was associated with a lower risk of being tested positive for dengue. Spillover exposure was associated with varying levels of protective efficacy, with no increasing trend as spillover exposure time increased—at 45% (OR: 0.55, 95% CI: 0.42–0.74), 46% (OR: 0.54, 95% CI: 0.42–0.80) and 47% (OR: 0.53, 95% CI: 0.42–0.81) protective efficacy for at least 3, 6 and 12 months of spillover *Wolbachia* exposure respectively (Table 3).

By age groups, the highest level of spillover protective efficacy was in adolescents (57–76% protective efficacy) versus adults (48–49%) and the elderly (39–45%). Females had higher protective efficacy compared to males. Spillover protective efficacies were found across majority of subgroups and considered durations of exposure, supporting consistent biologic replication (Table 3). Higher durations of spillover-*Wolbachia* exposure appeared to marginally improve protective efficacies on aggregate and across subgroups (Table 3).

Sensitivity analyses

We conducted a battery of sensitivity analyses to ensure the robustness of our protective efficacy estimates (Additional file 1: S8). Re-randomising our allocation of controls into the control arm 1000 times using the aforementioned constrained randomisation procedure and repeating the main analysis by using each newly allocated controls arm did not change spillover intervention efficacy estimates versus our primary estimate of spillover intervention efficacy (3 months + mean OR: 0.56, 3 months + minimum OR: 0.46, 3 months + maximum OR: 0.69) (Additional file 1: Fig. S5, Table S2). Inclusion of algorithmically selected covariates instead of all available covariates in the matching step did not change spillover intervention efficacy estimates (3 months + OR: 0.55, 95% CI: 0.43–0.74) (Additional file 1: Table S3). Using the full set of all available covariates in matching and adjusting for these same variables in regression step to alleviate any residual imbalances in baseline characteristics between arms produced similar intervention efficacy estimates as the main analysis (3 months + OR: 0.54, 95% CI: 0.22–0.74) (Additional file 1: Table S4). We did in-space placebo checks on control sites by taking each allocated control site as the allocated placebo-intervention site and

(See figure on next page.)

Fig. 3 Weekly average, town level Gravitrap *Ae. aegypti* index (GAI) by the time-since-intervention (event time) in spillover intervention sites of **a** Bukit Batok, **b** Choa Chu Kang, **c** Tampines and **d** Yishun. GAI is defined as the mean number of female adult *Ae. aegypti* caught per functional Gravitrap per week, hence proxies for adult wild-type *Ae. aegypti* abundance in public housing areas in the spillover area of each town. The corresponding ratios of pre- and post-GAIs at the sector is also plotted in **e**, **f**, **g** and **h**, per year, on a per spillover-sector basis on a log scale. The threshold of 1 indicates no difference between pre- and post-GAIs for a specific sector in that specific year versus the pre-intervention period, and values above 1 indicate lower GAIs in the post-intervention period

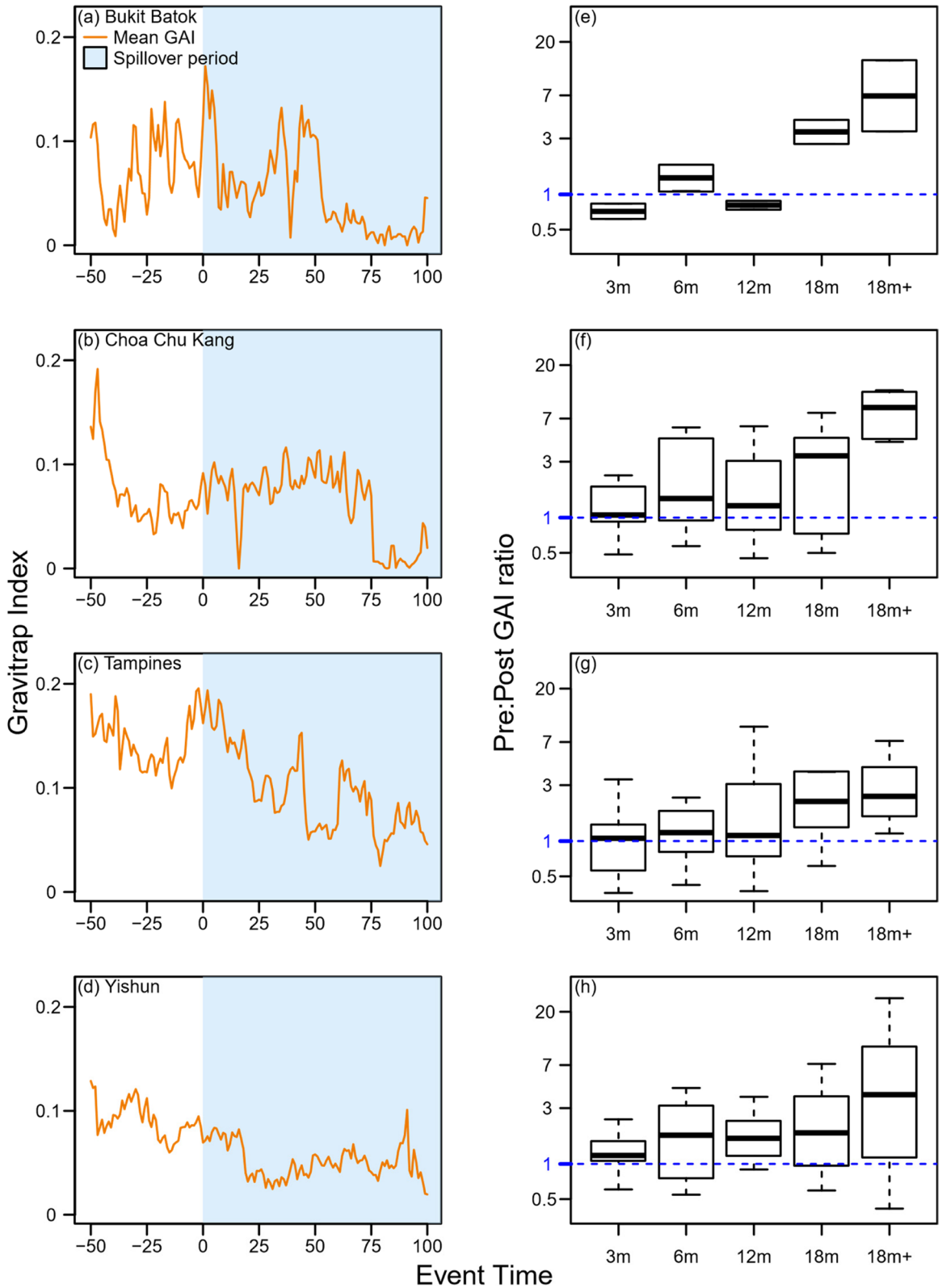

Fig. 3 (See legend on previous page.)

Table 2 Baseline characteristics of study population pre- and post-*Wolbachia* releases in intervention and pre-selected control group, at the sector resolution

	Intervention		Control		SMD[d]
	Observed	Matched	Observed	Matched	
Pre-intervention dengue incidence per 100,000[a]	167.83 (21.61)	–	136.30 (65.84)	–	
Post-intervention dengue incidence per 100,000[a]	158.37 (31.04)	NA	134.57 (63.46)	NA	
Dengue test positive in test-negative data set (%)[b]	12.79 (0.33)	12.79 (0.33)	20.73 (0.41)	21.07 (0.41)	
Covariates[c]					
Male (%)	53.82 (0.50)	53.82 (0.50)	50.82 (0.50)	54.08 (0.50)	> 0.01
Age	48.94 (23.60)	48.94 (23.60)	45.53 (24.15)	50.53 (30.98)	> 0.01
NDVI (Vegetation Index)	0.33 (0.05)	0.33 (0.05)	0.33 (0.05)	0.33 (0.05)	0.05
Area within 300 m of a waterbody (%)	0.14 (0.26)	0.14 (0.26)	0.47 (0.41)	0.18 (0.31)	> 0.01
Average public housing height (m)	34.03 (7.50)	34.03 (7.50)	38.92 (10.13)	34.85 (11.29)	> 0.01
Average public housing age (years)	26.23 (12.46)	26.23 (12.46)	28.92 (11.13)	27.15 (10.68)	> 0.01
Average house price (SGD$)	2,558,722 (1,821,342)	2,558,722 (1,821,342)	823,451 (881,903)	1,633,889 (1,554,188)	0.51
Distance of centroid to drainage network (m)	560.99 (246.95)	560.99 (246.95)	412.53 (248.70)	534.75 (238.03)	0.11
Length of drainage network inside spatial unit (m)	15.92 (59.77)	15.92 (59.77)	54.76 (136.84)	17.42 (76.77)	> 0.01
Forest area (%)	0.01 (0.04)	0.01 (0.04)	0.001 (0.01)	0.003 (0.01)	0.12
Grass area (%)	0.02 (0.06)	0.02 (0.06)	0.01 (0.04)	0.01 (0.02)	0.21
Total vegetation area (%)	0.04 (0.06)	0.04 (0.06)	0.02 (0.04)	0.03 (0.05)	0.03
Building area (%)	0.25 (0.05)	0.25 (0.05)	0.24 (0.05)	0.26 (0.04)	> 0.01
HDB residential units	690.35 (810.20)	690.35 (810.20)	598.10 (701.12)	914.07 (753.49)	> 0.01
Mean temperature (°C)	27.84 (0.86)	27.84 (0.86)	28.09 (0.84)	27.95 (0.84)	> 0.01
Maximum temperature	31.83 (1.02)	31.83 (1.02)	31.98 (1.05)	31.88 (1.02)	> 0.01
Mean wind speed	8.60 (2.05)	8.60 (2.05)	8.96 (2.47)	8.61 (2.31)	> 0.01
Relative humidity	79.89 (3.04)	79.81 (3.04)	79.57 (3.28)	79.96 (2.97)	> 0.01
Rainfall (mm)	7.28 (5.76)	7.28 (5.76)	6.46 (5.16)	7.24 (5.53)	> 0.01

The numbers in bracket represent standard deviation for each characteristic

[a] Pre-intervention period dengue incidence denotes number of dengue cases per 100,000 per sector annually

[b] Post-intervention percentage of dengue test positives compared to total number of tests per sector. Data on dengue tests were only available 2016 onwards

[c] Maximum temperature was calculated by taking maximum of temperature across all sectors within intervention or control groups. Length of drainage network and number of public housing units were calculated by taking sum across all sectors within intervention or control groups. The remaining characteristics were calculated by averaging across all sectors within intervention or control groups. All the calculations were done for the specified time period

[d] Standardized mean differences (SMD) after matching procedure of treated (*Wolbachia* spillover-exposed) and controls (*Wolbachia*-unexposed) individuals. Tested individuals were considered *Wolbachia* spilover-exposed here if they reside in a place of residence adjacent to sectors which has sustained *Wolbachia* interventions for 3 or more months

re-estimating protective efficacies by comparing test-negative and positive individuals in the placebo-intervention versus other control sites. There were only 12 positive and 18 negative statistically significant spillover intervention efficacies demonstrated in the placebo-control sites out of 100 with the grand mean of spillover protective efficacy estimates centred closely to 1 (OR mean: 0.99, range: 0.74–1.35) (Additional file 1: Fig. S6, Table S5), further indicating that our control selection approach did not generate spurious intervention efficacy estimates. We conducted in-time placebo checks on spillover intervention sites, where we took placebo-interventions in the actual intervention site before the spillover intervention began and demonstrated that there were no significant/positive spillover protective effects as pre-trends in the spillover intervention sites in the placebo-intervention period (3 months + OR: 0.93, 95% CI: 0.43–1.24) (Additional file 1: Table S6). Re-running statistical inference using the sector-level resolution rather than the town-level resolution using the cluster bootstrap approach also demonstrated that we could recover significant spillover intervention efficacy point estimates (3 months + OR: 0.60, 95% CI: 0.45–0.78) (Additional file 1: Table S7). Full set of results on all sensitivity analyses are provided in Additional file 1: S8.

Discussion

Individuals residing in sectors adjacent to *Wolbachia* release sectors for at least 3–12 months were associated with a 45–47% protective efficacy against dengue, versus control sectors. Across all years, age and sex subgroups, residing in *Wolbachia* spillover areas was associated

Table 3 Odds ratios (OR) and test positive percentages for different *Wolbachia* spillover-exposure categories and across year, age and sex subgroups

Exposure time[b]	OR (95% CI)[a]	Test-positive %[c]		OR (95% CI)	Test-positive %		OR (95% CI)	Test-positive %	
		Exposed (total sample)	Unexposed (total sample)		Exposed (total sample)	Unexposed (total sample)		Exposed (total sample)	Unexposed (total sample)
By year	**2020**			**2021**			**2022**		
3 months +	**0.46* (0.31–0.70)**	19.21% (781)	33.93% (781)	**0.70* (0.39–0.86)**	7.22% (886)	10.05% (886)	**0.60* (0.34–0.95)**	14.84% (438)	22.37% (438)
6 months +	**0.43* (0.21–0.66)**	15.15% (396)	29.29% (396)	**0.63* (0.37–0.84)**	6.98% (831)	10.59% (831)	0.62 (0.36–1.00)	15.4% (422)	22.75% (422)
12 months +	**0.61* (0.48–0.75)**	22.9% (131)	32.82% (131)	**0.40* (0.34–0.76)**	5.68% (546)	13.00% (546)	0.61 (0.34–1.04)	15.42% (389)	23.14% (389)
By age	**0–20**			**20–65**			**65 +**		
3 months +	**0.43* (0.16–0.72)**	7.56% (238)	15.97% (238)	**0.52* (0.41–0.70)**	14.58% (1420)	24.86% (1420)	**0.61* (0.47–0.96)**	11.00% (691)	16.93% (691)
6 months +	0.63 (0.23–1.32)	9.09% (176)	13.64% (176)	**0.51* (0.42–0.76)**	12.8% (1055)	22.18% (1055)	**0.55* (0.39–0.79)**	7.87% (521)	13.44% (521)
12 months +	**0.24* (0.00–0.73)**	5.61% (107)	19.63% (107)	**0.51* (0.39–0.85)**	13.88% (663)	24.13% (663)	0.56 (0.45–1.16)	7.8% (295)	13.22% (295)
By gender/ aggregate	**Male**			**Female**			**Aggregate**		
3 months +	**0.64* (0.47–0.83)**	14.05% (1267)	20.36% (1267)	**0.53* (0.32–0.75)**	11.32% (1087)	19.41% (1087)	**0.55* (0.42–0.74)**	12.79% (2354)	21.07% (2354)
6 months +	**0.61* (0.42–0.79)**	11.76% (961)	18.00% (961)	**0.55* (0.40–0.76)**	9.95% (794)	16.62% (794)	**0.54* (0.42–0.80)**	10.94% (1755)	18.46% (1755)
12 months +	**0.62* (0.43–0.83)**	12.36% (607)	18.45% (607)	**0.50* (0.36–0.74)**	10.02% (459)	18.08% (459)	**0.53* (0.42–0.81)**	11.35% (1066)	19.61% (1066)

[a] Denotes an OR < 1 with 95% confidence intervals (CI) which are not bounded by 1 denotes a significant spillover protective effect of *Wolbachia* interventions on the risk of dengue. ORs are estimated using logistic regression with matched exposed/unexposed individuals. Cluster bootstrap at the town resolution was used to obtain CIs to account for spatial clustering of data and the intervention

[b] An individual testing for febrile illness is considered *Wolbachia* spillover-exposed if the individual resides in a sector adjacent to one with at least 3, 6 or 12 months of sustained *Wolbachia* release

[c] Percentages of individuals testing positive in *Wolbachia* spillover-exposed and *Wolbachia* spillover-unexposed sectors in matched cohort

with a substantial level of protective efficacy against dengue. Efficacy was found to be highest among adolescents (0–20) versus adults (20–65) and the elderly (65 +), which may be due to pre-existing high seroprevalence and lower incidence among older individuals, and consequentially, a lower risk of re-contracting dengue of the same serotype in any period relative to younger individuals [32, 33]. Females had a higher protective efficacy compared to males consistent with findings which estimated the direct protective efficacy of *Wolbachia* across the same subgroups [12]. In the epidemic year (2020) where recorded dengue cases were historically the highest in the study setting, the spillover protective effect was also higher, compared to 2021 and 2022, demonstrating that *Wolbachia* may be most efficacious in preventing dengue cases when transmissibility is high.

The estimated spillover protective efficacies were also more modest compared to the direct protective efficacies of *Wolbachia* when individuals reside within *Wolbachia* release sites. In previous work using the same trial emulation framework and study setting, individuals residing in directly treated areas experienced up to 77% of protective efficacy [12]. This is consistent with the higher level of *Ae. aegypti* suppression observed in directly treated sites relative to spillover locations [11], and the longer duration that individuals were subject to direct *Wolbachia* interventions due to the manner that releases expanded over time. The protective efficacy of *w*AlbB-infected *Ae. aegypti* male mosquitoes releases in spillover locations is consistent with previous entomological and epidemiological investigations [11–13]. Release of *w*AlbB-infected *Ae. aegypti* male mosquitoes can drive substantial suppression of wild-type *Ae. aegypti* mosquitoes even in nearby sites which were not directly treated [11, 15, 34–36], leading to reductions in dengue risk in these spillover locations, which can possibly converge in protective efficacy to directly treated sites, if spillover exposure was sustained over time [37].

This study used a nationally representative dengue test database to emulate a cluster-randomised controlled

trial, to examine the spillover epidemiological efficacy of releasing *w*AlbB-sg infected male *Ae. aegypti* mosquitoes to protect against dengue. We used a large battery of robustness checks, which included placebo checks, different balancing approaches, re-randomisation of controls and covariate selection procedures to ascertain the internal validity of our analytical framework. By design, the emulation of a cluster-randomised controlled trial also enabled us to adopt important characteristics of real trials which improved upon the validity of our study, which included (1) constrained randomisation and the use of temporally matched intervention and control arms, enabling both arms to have balanced historical dengue risk, covariate profiles and enrolment times, (2) use of test positive and negative comparator groups, to avoid reporting and selection bias at the point of testing and enable detection of laboratory confirmed dengue cases. (3) By matching unexposed/exposed individuals exactly to calendar time, under the null hypothesis, we increased the likelihood that test-positive and negative individuals come from the same source-population at enrolment—a key assumption of the test-negative design. These facets thereby strengthen causal identification of the spillover protective efficacy of *Wolbachia* IIT on dengue.

Incompatible insect technique coupled with sterile insect technique has several key advantages. (1) While we showed that the technology can protect individuals from dengue even away from directly treated sites, this spillover efficacy should be similar against other diseases transmitted by *Ae. aegypti*, such as Zika, chikungunya and yellow fever, as the strategy aims to suppress vector populations rather than block disease transmission under the *Wolbachia* introgression approach. However, evaluation of spillover protective efficacies cannot be conducted for other *Aedes*-borne diseases as those diseases are sporadic in the study setting. (2) Baseline studies demonstrated high public knowledge and acceptance towards the studied intervention [38], which involves releases of non-biting males only and reduces biting pressure. (3) While dengue virus may evolve resistance to *Wolbachia* under the *Wolbachia* introgression approach [39], our approach suffers no drawbacks related to *Wolbachia*-associated selective pressure of viruses [4]. The *Wolbachia* technology, which has previously been demonstrated to be cost-effective at preventing dengue at a efficacy threshold of 40% in the study setting [40], had the efficacy threshold exceeded in studies estimating intervention efficacy in spillover and directly treated locations [12, 13].

Our study provides a twofold contribution to the literature. Current field studies for vector control interventions have demonstrated the field efficacy of only directly treated sites. Our study advances these results by (1) designing a robust quasi-experimental analytical framework to estimate the effects of releasing *w*AlbB-sg infected male *Ae. aegypti* mosquitoes interventions to protect against dengue and consequentially (2) providing the first empirical evidence of how IIT-based vector control measures could generate spillover protective effects in areas away from actual intervention sites, using a nationally representative cohort of individuals who test-negative/positive for dengue.

Conclusions

The release of *Wolbachia*-infected male *Ae. aegypti* is a novel method for the control of dengue. The technology can complement conventional approaches, such as source reduction, community engagement in further reducing dengue transmission. In our experience, the protective efficacy of the intervention on dengue, as well as its entomological efficacy, is likely to be maximised if it is used to complement and enhance conventional vector control measures, rather than replace them.

Abbreviations

ATT	Average treatment effect on the Treated
CI	Confidence interval
cRCT	Cluster-randomised controlled trial
GAI	Gravitrap *Aedes aegypti* index
EW	Epidemiological week
ELISA	Enzyme linked immunosorbent assay
IIT	Incompatible insect technique
IgM	Immunoglobulin M
NS1	Non-structural protein 1
OR	Odds ratio
SIT	Sterile insect technique
SMD	Standardised mean difference

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12916-025-03941-2.

Additional File 1: Table S1. Summary of the protocol for target trial emulation. Figure S1. Visualization of the phased release approach in Yishun township from Sep 2018 to March 2022. Figure S2. Visualization of the phased release approach in Tampines township from Sep 2018 to March 2022. Figure S3. Visualization of the targeted release approach in Bukit Batok township from Sep 2018 to March 2022. Figure S4. Visualization of the targeted release approach in Bukit Batok township from Sep 2018 to March 2022. Figure S5. Histogram of protective efficacies for 1000 eligible potential control arms compared against the point estimate of the randomly selected control allocation. Table S2. Summary statistics for Odds Ratios for 1000 eligible potential control arms. Table S3. Odds ratios and test positive percentages for different Wolbachia spillover-exposure categories. Table S4. Odds ratios and test positive percentages for different Wolbachia spillover-exposure categories. Figure S6. Histogram of placebo protective efficacies for 100 random placebo-intervention and control arms compared against the point estimate of the randomly selected control allocation. Table S5. Summary statistics for Odds Ratios for 100 random placebo-intervention and control arms. Table S6. Summary statistics for Odds Ratios for in-time placebo check. Table S7. Odds ratios and test positive percentages for different *Wolbachia* spillover-exposure. Table S8. Summary statistics for mean annual dengue incidence rate and standard deviations by town and by NEA operational case definitions.

Acknowledgements
We would like to acknowledge colleagues at National Environment Agency, Singapore and Ministry of Health, Singapore who are involved in *Wolbachia* program.

Authors' Contribution
Conceptualization and design of study: JTL. Formal analysis: JTL, CSC, DM. Interpretation of data: JTL, DM. Visualization: JTL, SB, LYT, GC. Data collection: YLL, DL, CL, YN, BD, SHH, SS, CSC, PM. Project administration: VL, CCC, CHT, LCN. Writing (original draft): JTL, DM. All authors read and approved the final manuscript.

Funding
This study was supported by funding from Singapore's Ministry of Finance, Ministry of Sustainability and the Environment, National Environment Agency, and National Robotics Program. JTL is supported by the Ministry of Education (MOE), Singapore Start-up Grant. SB is supported by an MOE Tier 2 grant.

Data availability
Permission to use anonymised dengue test-negative/positive data was obtained from the Ministry of Health, Singapore for the sole purpose of evaluating *Wolbachia* interventions. Third party sharing of these data are precluded..

Declarations

Ethics approval and consent to participate
This project was exempted from formal bioethics review by National Environment Agency Bioethics Review Committee (IRB024) as it is not considered human biological research, as advised by the Ministry of Health, Singapore. All laboratory tests were performed for clinically directed reasons, and the data from these tests is routinely collected as part of routine dengue surveillance under the Infectious Disease Act, which exempted the need for informed consent. All data was anonymised by a designated analyst authorised to receive identifiable information. Statisticians who were responsible for the analysis only received de-identified data through a secure transfer platform.

Consent for publication
All authors read and agreed to publish the final version of the manuscript.

Competing interests
The authors declare no competing interests.

Author details
[1]Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore, Singapore. [2]Environmental Health Institute, National Environment Agency, Singapore, Singapore. [3]Saw Swee Hock School of Public Health, National University of Singapore and National University Health System, Singapore, Singapore. [4]Ministry of Health, Singapore, Singapore. [5]School of Biological Sciences, Nanyang Technological University, Singapore, Singapore.

Received: 2 August 2024 Accepted: 10 February 2025
Published online: 28 March 2025

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