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Efficient production of male *Wolbachia*-infected *Aedes aegypti* mosquitoes enables large-scale suppression of wild populations

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The range of the mosquito *Aedes aegypti* continues to expand, putting more than two billion people at risk of arboviral infection. The sterile insect technique (SIT) has been used to successfully combat agricultural pests at large scale, but not mosquitoes, mainly because of challenges with consistent production and distribution of high-quality male mosquitoes. We describe automated processes to rear and release millions of competitive, sterile male *Wolbachia*-infected mosquitoes, and use of these males in a large-scale suppression trial in Fresno County, California. In 2018, we released 14.4 million males across three replicate neighborhoods encompassing 293 hectares. At peak mosquito season, the number of female mosquitoes was 95.5% lower (95% CI, 93.6–96.9) in release areas compared to non-release areas, with the most geographically isolated neighborhood reaching a 99% reduction. This work demonstrates the high efficacy of mosquito SIT in an area ninefold larger than in previous similar trials, supporting the potential of this approach in public health and nuisance-mosquito eradication programs.

Aedes aegypti (Linnaeus) is the primary vector of dengue, chikungunya, Zika, and yellow fever. Native to Africa, *A. aegypti* has invaded much of the tropics and subtropics over the past four centuries[1–3], putting more than two billion people at risk of arboviral infection[4]. Although effective on a small scale, traditional control methods such as source reduction and chemical insecticides, as currently implemented, have not prevented the proliferation and spread of this species (although see ref. [5]). SIT is an alternative control strategy that exploits the fact that female mosquitoes normally mate only once[6]. If that mating is with a sterile male, the female will not produce viable progeny. For agricultural pests, large-scale, inundative releases of sterile males over many generations have resulted in population crashes and, in some cases, local or widespread elimination[6,7]. SIT avoids many of the pitfalls of traditional mosquito abatement techniques, such as off-target effects, insecticide resistance, and difficulties treating cryptic breeding sites, but its efficacy in controlling wild populations of *A. aegypti* remains unproven, with small field studies of typically less than 100 hectares (ha) in size showing highly variable suppression results[6,8–11].

A common way to sterilize males is by altering their genomes in either a non-targeted manner (irradiation) or a targeted manner (genetic engineering). However, the impaired ability of genetically altered males to compete for female mates in the wild and public resistance to the release of genetically modified mosquitoes remain barriers to widespread use of these techniques[12,13]. Alternatively, the maternally inherited, intracellular bacterium *Wolbachia pipientis* can be used to create conditional sterility between released males and wild-type females through a phenomenon termed cytoplasmic incompatibility[14]. *Wolbachia* infects over half of all insects[15] but not wild *A. aegypti* populations[16,17]. However, egg microinjection has been used to establish multiple infected lines of *A. aegypti* with stable transfections of *Wolbachia* strains native to other dipteran insects[18–21]. In the case of the *w*AlbB *Wolbachia*-infected *A. aegypti* WB1 colony[18] used for this work, when an uninfected female mates with a WB1 male, incompatibility between the maternal cytoplasm and sperm results in undeveloped zygotes. However, infected WB1 females produce viable, *Wolbachia*-positive progeny regardless of the infection status of the male (Fig. 1a). Males from transfected colonies like WB1 are incompatible with uninfected, wild-type females but do not suffer the same drawbacks as genetically altered males (for example, refs. [10,22–25]), making them an attractive tool for mosquito control. As *Wolbachia*-infected males are not sterile in the

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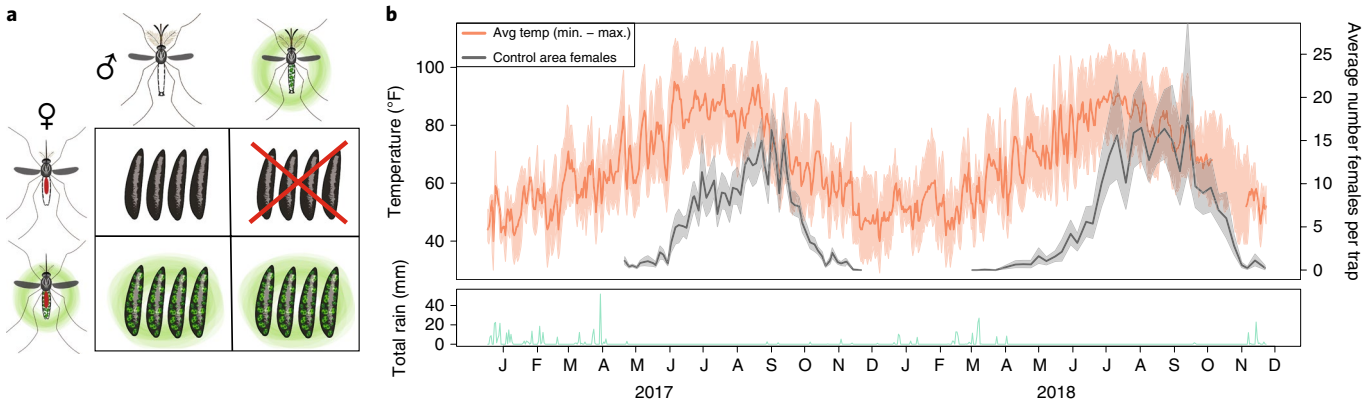


Fig. 1 | Cytoplasmic incompatibility and mosquito seasonality in Fresno County. a, The outcome of mating between males and females with (green shading) and without *Wolbachia* (no shading). Infected females (bottom) always lay viable *Wolbachia*-infected eggs. Uninfected females (top) lay viable, uninfected eggs when mated with uninfected males, but lay inviable eggs when mated with *Wolbachia*-infected males. **b**, Red plot, average daily temperature at Fresno Yosemite International Airport with shading indicating minimums and maximums during 2017 and 2018. Gray plot, average number of females per trap night scaled according to the right *y* axis with 95% CIs shaded ($n = 38$ independent trap samples per collection day in 2017 and $n = 28$ in 2018). The bottom plot indicates total daily rainfall (mm) during 2017 and 2018. Data retrieved from http://ncdc.noaa.gov.

classic sense, this approach is sometimes referred to as the incompatible insect technique[26].

All mosquito SIT programs aim to minimize the release of females to avoid increasing nuisance biting and disease transmission. However, preventing the release of females is particularly important with *Wolbachia*-based programs because they have the potential to establish and replace the wild population of mosquitoes, eliminating the utility of *Wolbachia*-infected males for control. Although such population replacement is unlikely when there is a large population of wild mosquitoes, the chances of population replacement increases when wild populations are small, making high-accuracy sex sorting ever more important[18,27,28]. To minimize the likelihood of population replacement, two groups recently treated pupae with low-dose irradiation to sterilize residual *Wolbachia*-infected females[29,30]. While promising, as implemented this technique still reduces the competitiveness of males, albeit less than traditional high-dose irradiation, and does not always result in complete female sterilization.

Regardless of the sterilization technique, large-scale control of mosquito populations with SIT is a challenging operational problem requiring industrialization of rearing, sex sorting, and release. Groups around the world began to tackle these challenges in the middle of the 20th century, with several notable successes. In the late 1970s, the United States Department of Agriculture (USDA)-backed SIT program targeting *Anopheles albimanus* in the Lake Apastepeque region of El Salvador achieved near elimination across a 1,500 ha valley due to a combination of mass-rearing and release innovations, careful execution, and spatially limited wild mosquito reproduction[31–35]. In 1967, *Culex quinquefasciatus* was temporarily eliminated from a small town in southern Burma, although it rapidly re-established owing to the relatively long flight distance of *Culex* mosquitoes[36]. Unfortunately, difficulties in sustaining production of competitive males and in obtaining funding led to the dissolution of all major mosquito SIT programs by the early 1980s. Recent technological advancements, including genetically modified sterile males[12] and *Wolbachia*-transfected mosquito colonies[18], have led to renewed interest and investment in mosquito-targeted SIT.

Here, we develop tools to automate the production and distribution of male mosquitoes infected with *Wolbachia* and test them on field populations in Fresno County, which lies in the Central Valley of California. *A. aegypti* was first detected in this region in 2013, with genetic analysis suggesting the South Central region of the United States as the most likely source population[37]. Although efforts were made to eliminate the nascent population with traditional control tools, the mosquito became established and continues to expand its range in the Central Valley, invading new cities at a rapid rate[3]. Establishment in Fresno is part of a recent, larger range expansion of *A. aegypti* into dry, hot metropolises across the southwestern United States, including Los Angeles, Phoenix, and Las Vegas. Unlike in tropical habitats, the population of *A. aegypti* in Fresno County depends on anthropogenic water sources and is highly correlated with seasonal ambient temperatures, with adult populations increasing in June and July, peaking from August to October, and largely undetectable from December to April (Fig. 1b). We demonstrated the effectiveness and scalability of automated SIT through open releases into three neighborhoods encompassing 293 ha and over 3,000 households within the cities of Clovis and Fresno in Fresno County, California.

Results

Mosquito mass rearing. To achieve stable production of males, we developed an automated larval rearing system (LRS) that takes first instar larvae as input, and outputs pupae (Fig. 2a). Prior to loading onto the LRS, eggs are hatched overnight, after which L1 larvae are automatically counted using a COPAS 550 (Union Biometrica) large-particle flow cytometer into 50-ml conical tubes. The first step in the LRS is larval container assembly, in which disposable plastic containers are filled with water and food. Larvae are automatically transferred from the conical tube into the container by a robotic larval transfer arm. After filling and sealing, containers are automatically transferred to an incubated storage and retrieval frame (Supplementary Video 1). Larvae develop for 6 days in the frame, during which they are automatically fed. On the seventh day most larvae have developed into pupae and the containers are removed from the frame to be sex sorted (Supplementary Video 1). At maximum capacity and high rearing density, the LRS is capable of producing over 2,950,000 male pupae per week.

The LRS produced remarkably consistent numbers of synchronous, similarly sized pupae from each rearing container. To visualize the consistency of production, we calculated the daily yield of male mosquitoes over 179 production batches during our 2018 field trial. Yield was calculated as the proportion of L1 male larvae that developed into adult males and passed through the visual sex-sorting pipeline (see below). The LRS showed high temporal consistency

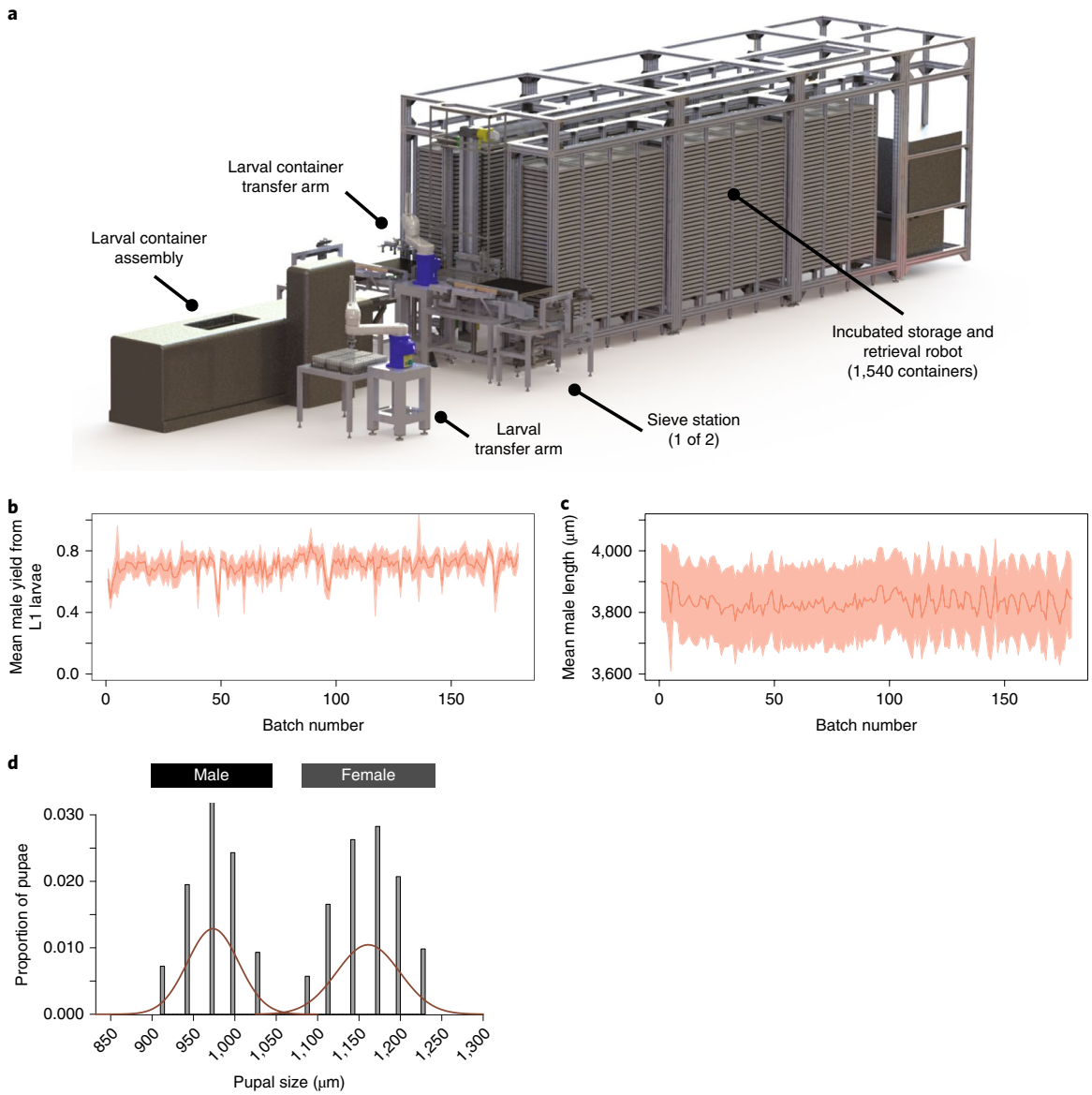
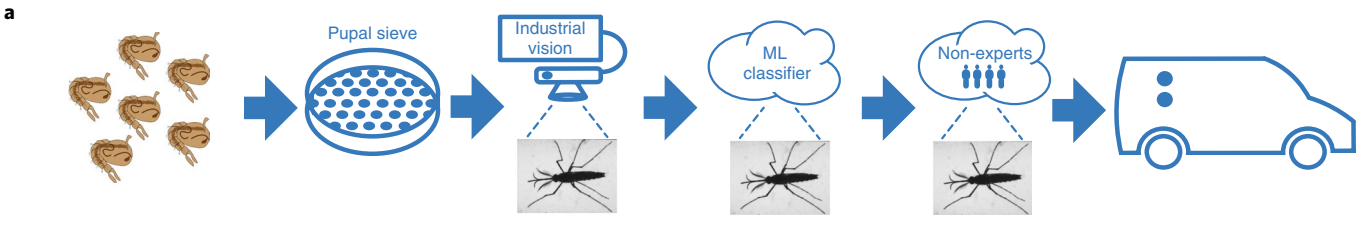


Fig. 2 | An automated LRS. a, Schematic of the LRS with major components labeled. **b**, Optimized rearing protocols resulted in a highly consistent yield, calculated as the ratio of adult males entering the release tubes relative to the number of L1 larvae introduced into larval containers. The dark line shows mean yield, shading represents the s.d., the *x* axis represents all 2018 production batches (n = mean of 96, range of 10–140 independent sex-sorter measurements per batch). **c**, Consistent mean length of adult males as measured from sex-sorter images (s.d. interval shaded, n = mean of 43,256, range of 5,827–64,282 independent male length measurements per batch). **d**, Discrete pupal size dimorphism between sexes. Histogram shows width estimates from ~18,000 pupae. Pupal width is measured in pixels resulting in bins when converted to μm. Red lines show normal distribution fit to male and female sets separately.

with an average yield of 70.39% (Fig. 2b). In addition, adult male size, estimated from the body length of male mosquitoes, was also highly consistent throughout the 6 months of production, averaging 3.8 mm ($\sigma^2 = 0.9$ mm; Fig. 2c). *A. aegypti* is sexually dimorphic for pupal size under favorable conditions[38]. Our rearing protocols (Supplementary Text) implemented on the LRS produced consistent pupal sizes resulting in clear separation between the sexes, with female pupae on average 19.26% larger than male pupae (Fig. 2d), consistent with optimized larval development[39].

Mosquito sex sorting. To minimize the chances of unintentional female release, we developed an automated, multi-step sex separation process based on known morphological differences between males and females (Fig. 3a). The first step is an automated mechanical sieve that separates based on body size, allowing male pupae to pass through while females are retained. Over the course of 2018 production, an average of 2.54% of pupae that passed through the sieve were females. Assuming a 50/50 input pupal sex ratio, we estimated that automated mechanical sieving removed 94.92% of females (Fig. 3b).

In the second step, the primarily male pupae that passed through the sieve are loaded onto a real-time visual sex-sorter where they eclose and — of their own volition — walk down a narrow path over which a camera is mounted (Supplementary Video 2). Custom industrial vision software recognizes each ambulatory mosquito as an object, attempts to physically isolate them using air jets and a shutter, and then takes at least one image. If multiple mosquitoes make it into the imaging area they are always rejected. Images with a single mosquito are inspected for male-specific body parts (Fig. 3c), including genitalia and antennal features, using a template matching



Probability of a female contaminant (95% CI)	0.50 (–)	0.03 (0.00 – 0.08)	2.39×10^{-5} (1.30×10^{-5}) – (3.79×10^{-5})	1.82×10^{-3} (3.76×10^{-4}) – (4.38×10^{-3})	1.13×10^{-4} (3.08×10^{-9}) – (4.83×10^{-4})	1.13×10^{-9} (3.88×10^{-11}) – (4.44×10^{-9})

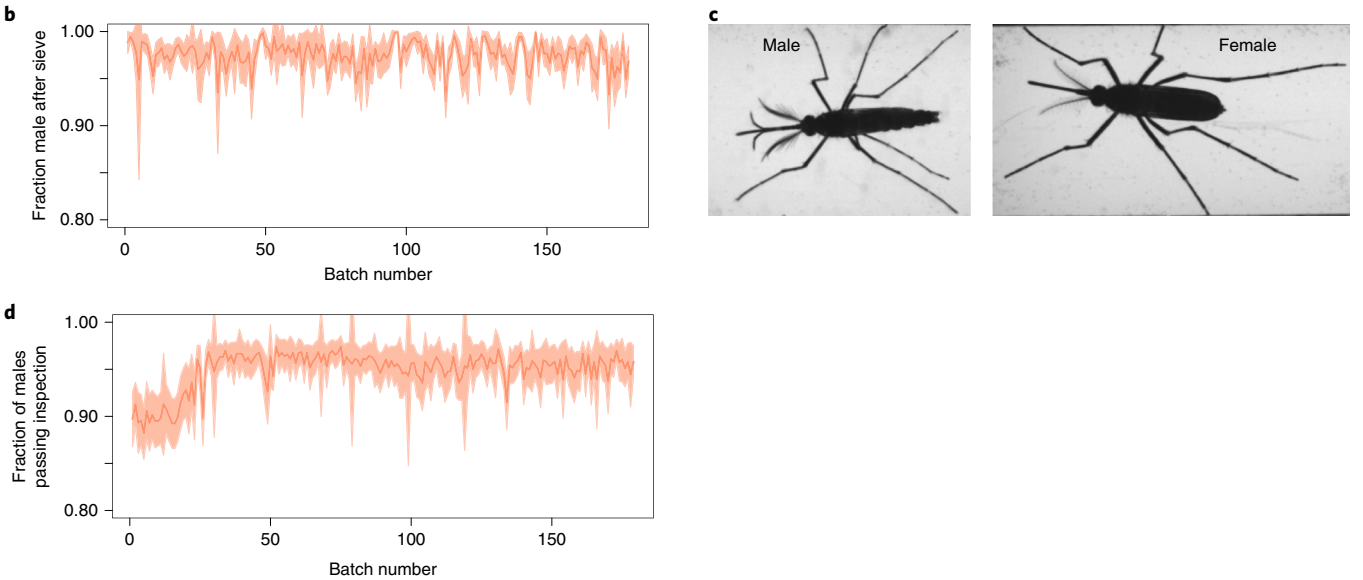


Fig. 3 | Sex-sorting pipeline. a, Illustration of the entire sex-sorting pipeline, including the mechanical pupal sieve, real-time adult visual inspection, cloud-based machine learning classifier, and non-expert review. The probability of a female contaminant with 95% CIs for each step is shown along with the estimated overall female contamination rate for the entire pipeline in the final column. **b**, The fraction of mosquitoes imaged by the sex sorter after the pupal sieve that were male with s.d. intervals shaded for 179 production batches. **c**, Example images from the adult sex sorter (male on the left and female on the right) used by both the industrial vision system and machine learning classifier. **d**, The fraction of true males that were correctly labeled and accepted by the Industrial Vision system with s.d. interval shaded (n = mean of 96, range of 10–140 independent sex-sorter lane measurements per batch).

algorithm. Individuals with male morphology are puffed into a container used to distribute mosquitoes in the field, called a 'release tube', while individuals failing inspection are rejected. At the start of the 2018 field season, an average of 89.85% of males passed inspection (Fig. 3d). After implementing improved traffic management algorithms to better isolate individuals, 95.59% of males passed inspection, resulting in consistently high male yield through the adult sex sorters.

In the third step, we submit all images of individuals labeled male by the industrial vision system for scoring by a machine learning classifier. The classifier is a deep neural network built upon the open source Inception-v3 architecture[40] and trained using 2.1 million manually labeled images. The classifier computes the probability that the individual is male and the images are ranked based on their maleness score, subsampled, and sent to a panel of five trained, but non-expert, reviewers via an online micro-task platform for inspection and labeling. We sent two samples for review: the 1% of images with the lowest male probability, and a 1% random sample of all male images. If the non-experts identified a female or if there was any inconsistency in their labels, an expert reviewed the images in question. If the expert confirmed any females, we located and purged the part of the release tube with the female before the tube left the factory.

Based on data from 2018, we estimated the probability of a female contaminant at each step of the sex-sorting pipeline (Fig. 3a). Assuming independence between the different steps in the pipeline, the combined system is expected to release 1 female for every 900 million males with a 95% CI of 1:200 million to 1:26 billion (Fig. 3a and Supplementary Text). For additional validation of the sex-sorting pipeline, we screened larvae obtained from ovitraps in our treatment areas and found no *Wolbachia*-positive larvae (Supplementary Text), confirming that we did not unintentionally establish a *Wolbachia*-infected population in the field as would be expected if we released infected females into an area in which the wild-type population had been suppressed.

Automated male mosquito releases. For a SIT intervention to be successful, released males must permeate the landscape to find unmated females. We developed an automated male mosquito release system to ensure complete and calibrated distribution of *Wolbachia*-infected males into treatment areas. The system includes transport and release tubes, automated release devices mounted inside customized vans (Fig. 4a), map-based release plan generation and triggering software (Fig. 4b), and a structured light mosquito counter (Supplementary Text).

Fig. 4 | Field sites and automated releases. a, *Wolbachia*-infected males were released into field sites using two sprinter vans equipped with automated release devices that blew males through release outlets on the rear passenger side. **b**, Release map indicating a planned route for van drivers to follow, triggering the release device. Each orange vector indicates the GPS location and direction of travel at which a segment of the release tube was released. **c**, Map of treatment areas (T1–T3) in shades of orange and control areas (C1–C3) in shades of yellow. The C1 control buffer area is shaded in purple, and the T2 treatment buffer is shaded in turquoise. These areas were monitored but not included in analysis. Only the T2 treatment buffer was treated with sterile males. **d**, Representative placement and density of adult BG-Sentinel traps (black dots) and egg traps (gray dots). Trap density was similar between treatment and control areas (Supplementary Table 1).

After a preliminary study in 2017 (see Supplementary Text for details), starting on 16 April 2018 we conducted daily releases of *Wolbachia*-infected *A. aegypti* males over a period of 26 weeks into 3 treatment sites (labelled T1, T2, and T3 in Fig. 4c), which include 3,063 households across 293 ha (Supplementary Table 1). These sites were residential neighborhoods typical of the area, situated on the edge of the Fresno-Clovis metropolitan area with at least partial isolation, and were known to have established *A. aegypti* populations based on historical trapping data. We measured adult mosquito density using BG-Sentinel traps (V2, Biogents) placed at comparable densities in both treatment and control sites (Fig. 4d and Supplementary Table 1).

In 2018, we released *Wolbachia*-males at an average rate of 78,469 per day or 267.81 ($\sigma = 61.16$) males per hectare per day for a total of 14,376,511 male mosquitoes during the study, although release rates differed between sites according to both household counts and the number of females in traps in each site (Fig. 5a–c and Supplementary Table 2). We also varied release rates per site within the three study phases. In phase I (mid-April to mid-May), release numbers were determined exclusively by the number of households

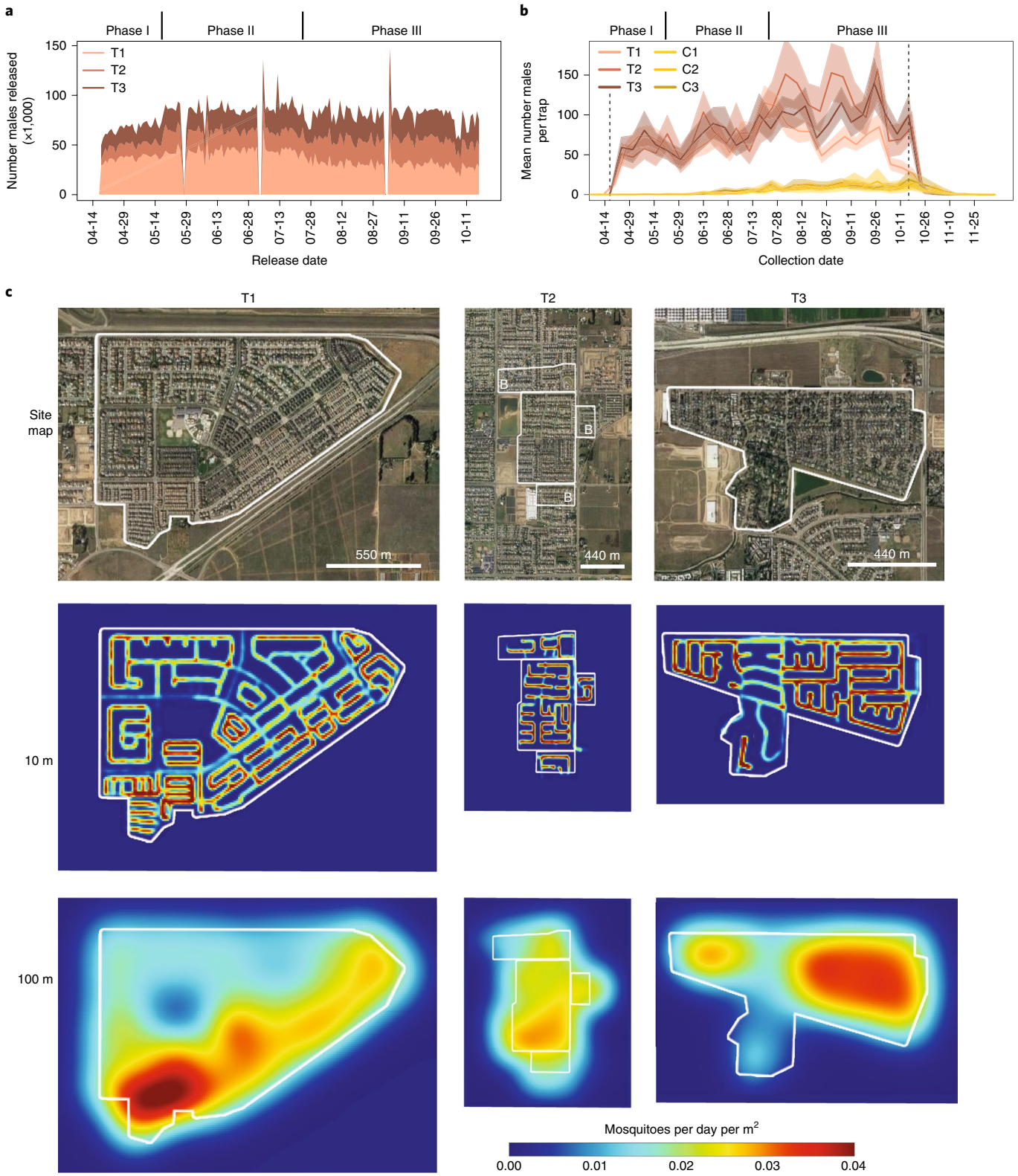


Fig. 5 | Releases of *Wolbachia*-infected males. a, Stacked sum plot showing the total number of males released into each treatment area over the 6-month study period (see the main text for a description of study phases). We released males every day, except for three pauses for US holidays. **b**, Mean number of adult males per trap in treatment areas (T1–T3) and control areas (C1–C3) with 95% bootstrap CIs ($n_{T1}=44$, $n_{T2}=24$, $n_{T3}=35$, $n_{C1}=17$, $n_{C2}=28$, $n_{C3}=15$ independent trap samples per collection day). Dotted lines indicate the first and last day of releases. **c**, Top, satellite maps with treatment areas outlined in white and treatment buffers indicated with B in T2. Middle, density of *Wolbachia*-males, as measured by the onboard structured light mosquito counter, averaged over 6 months of releases assuming a 10-m dispersal kernel revealing van path and variable release rate based on house density. Bottom, density of *Wolbachia*-males averaged over 6 months of releases assuming a 100-m dispersal kernel, suggesting nearly complete coverage of release areas.

in each site. In phase II (mid-May to late July), we increased the number of males per household for treatment sites T2 and T3, as historical data indicated that these sites have had higher wild mosquito densities and are less geographically isolated than T1. In phase III (late July to mid-October), we held the release numbers constant for sites T2 and T3, but reduced the T1 release rate in response to our monitoring data, which indicated very high ratios of *Wolbachia*-infected to wild-type males in this site (Supplementary Table 3). We monitored male mosquito densities using adult mosquito traps and found that male mosquito numbers reflected the different phases of release (Fig. 5b).

We visualized the density of released males in each neighborhood over the entire 2018 season using data from the van-mounted release device and mosquito counter. First, we modeled the density of released males assuming a 10-m dispersion kernel around GPS (global positioning system) release coordinates, which shows the van release route and highlights variations in release rate due to changes in housing density (Fig. 5c). Importantly, if we assume a more realistic dispersion kernel of 100 m, males are more evenly distributed across each site, suggesting comprehensive coverage. The only two relatively low-density spots (blue regions) correspond to a large elementary school in the center of T1 and a low-housing-density section of T3 (Fig. 5c). To evaluate the precision of releases, we compared our intended mosquito distribution targets (based on housing density) to a map of actual mosquito release density assuming a 100-m male dispersal kernel. The density of male distribution after releases largely matches the intended distribution and captures the reduction and increase in release rates in T1 and T3, respectively (Supplementary Fig. 1).

Suppression of mosquito populations in release sites. The goal of field releases was to test whether a high ratio of *Wolbachia*-infected males to wild-type males would result in enough incompatible matings to sharply reduce egg hatch and subsequently the wild-type adult population. To best isolate the effect of the *Wolbachia*-male releases, only normal mosquito abatement activity under the mandate of the California Mosquito Abatement District (CMAD) was applied in the treatment and control areas (Supplementary Table 4 and Supplementary Text).

We monitored the ratio of released to wild-type males (that is, overflooding ratio) by testing trapped adult males for *Wolbachia* using a loop-mediated isothermal amplification (LAMP) assay (Supplementary Text) and found that our releases resulted in high overflooding ratios in each of the treatment sites during the first 4 months of release, ranging from 47.53 to 557.00 (Supplementary Table 3). As overflooding ratios reached levels too high to be estimated reliably, we did not measure these for the last 2 months of releases. The overflooding ratios tended to increase month after month, consistent with both increased release rates in T2 and T3 during phase II and declines in the number of wild-type males per trap (Supplementary Table 3).

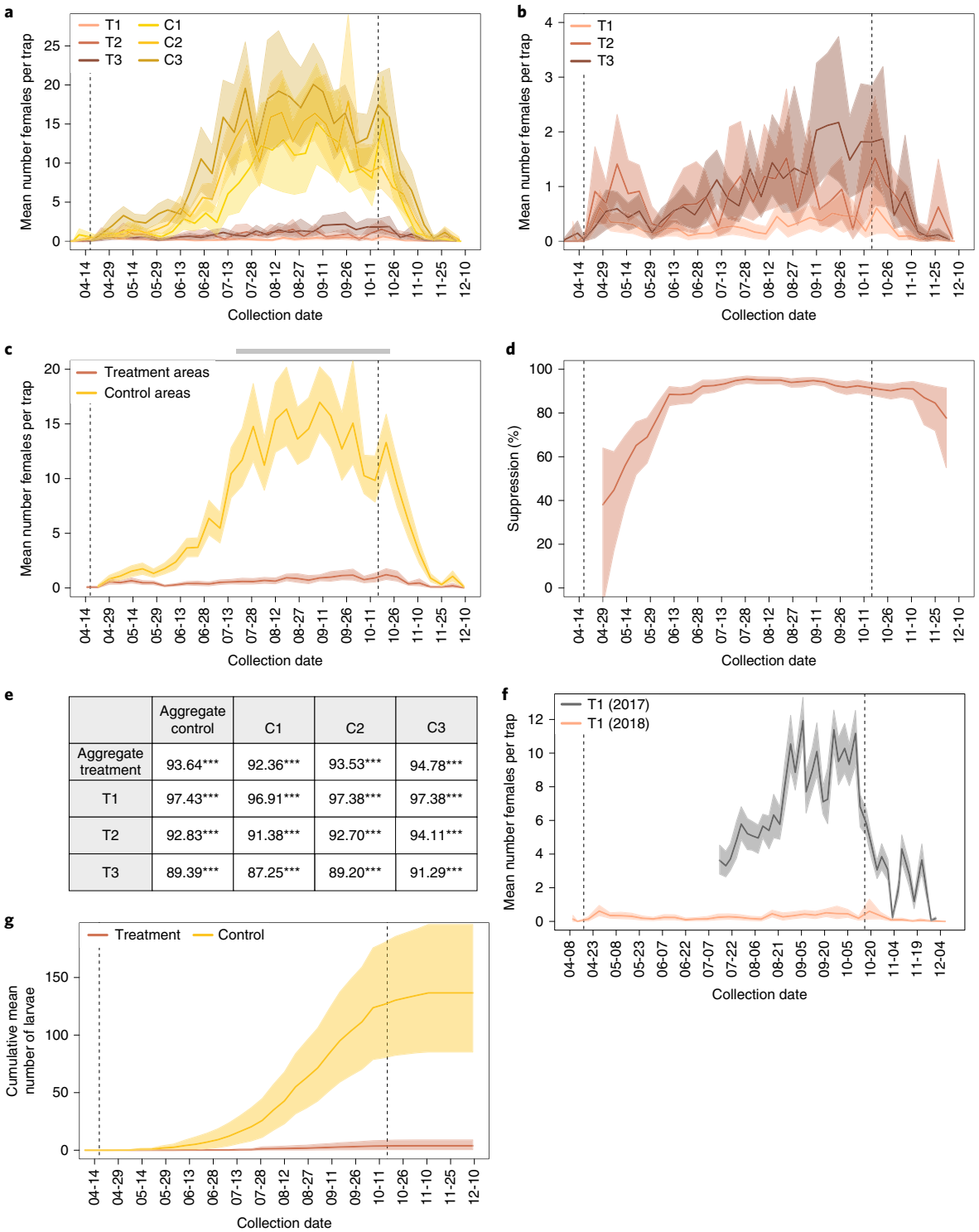
We also monitored the abundance of adult females using BG-Sentinel traps (Fig. 4d) and found that the density of adult females differed significantly between treatment and control areas during the treatment period. In each control area, the average number of females per trap night followed the expected seasonal curve, with the population increasing in June, peaking from July to September with female densities of >12 females per trap in each site, and declining in October (Fig. 6a). In contrast, female abundance in the treatment sites had a strikingly different pattern (Fig. 6a,b). T1, the most isolated site, had extremely low numbers of females in all weeks, peaking at an average of only 0.6 females per trap in the third week of October. Although sites T2 and T3 had more females than T1 as the season progressed, with peak mean females per trap of 1.52 and 2.17, respectively, the 95% confidence intervals (CIs) are fully separated from those of the control sites from mid-July to mid-November (Fig. 6a).

When comparing female abundance between aggregated treatment and control sites, there is a clear separation between the 95% CIs beginning approximately 5 weeks after the start of releases (Fig. 6c). The average number of females in aggregated treatment sites remained low for the entirety of the season with less than one female per trap night in 32 out of 36 weekly collections and a peak value of 1.2 females per trap night (95% CI, 0.78–2.47) in the third week of October. In comparison, the control sites reached a peak of 16.6 females per trap (95% CI, 13.70–19.87) in the second week of September (Fig. 6c).

Overall, release of *Wolbachia*-infected males into treatment areas resulted in 93.64% (corrected $P=1.6\times10^{-5}$) suppression of females from mid-July until the seasonal declines starting in mid-October, with a maximum 2-week suppression level of 95.5% (95% CI, 93.6–96.9%) in the fourth week of July (Fig. 6d). To test the generality of these results, we compared each treatment site individually to both the aggregate and individual control sites and found that significant suppression was achieved in all sites across the 14 weeks of peak mosquito season in all pairwise comparisons (Fig. 6e). Moreover, we found that, within 2-week windows, T1 reached a peak suppression of 98.9% (95% CI, 98.1–99.4), T2 reached 94.8% (95% CI, 92.3–96.8), and T3 reached 94.6% (95% CI, 92.0–96.4) compared to the aggregate control site. Results are similar when each treatment site is compared to individual control sites (Supplementary Table 5). We also compared female abundance in T1 in 2018 with that in 2017 (Fig. 6f and Supplementary Text), which showed a 97.1% drop in the number of mosquitoes from 2017 to 2018 (95% CI, 95.4–98.6).

Comparison of the number of larvae hatching from egg traps in treatment sites relative to control sites provides an additional view of the effect of *Wolbachia*-male releases on mosquito reproduction. We directly monitored larval production using egg traps distributed at comparable densities in both treatment and control sites (see Methods, Fig. 4d and Supplementary Table 1). For the entire season, the mean number of cumulative larvae collected per egg trap in treatment sites was 3.7 (95% CI, 0.5–8.4) compared to 126.3 (95% CI, 80.3–180.7) in control sites — a 97.1% reduction in collected larvae (Fig. 6g). Similarly, the mean number of eggs per trap was consistently lower in the treatment areas than in control areas (Supplementary Fig. 2). To infer the proportion of incompatible versus wild-type matings, we also calculated hatch rates of collected eggs. Although variable owing to small sample size, hatch rates of eggs collected in treatment sites were consistently lower than those collected from control areas (Supplementary Fig. 3). Taken together, the data demonstrate that *Wolbachia*-infected males inhibited mosquito reproduction, resulting in strong suppression of the wild population in release sites.

Mosquito migration into release sites. Despite treatment site selection intended to minimize migration through geographic isolation and treated buffer areas, several lines of evidence suggest that immigration of inseminated females from nearby untreated areas put an upper limit on achievable suppression. Although statistical support is limited by small sample sizes, more females were caught in traps on the outer edge of treatment sites (T2 and T3), as indicated by a negative correlation between the distance of a trap from the edge of the site and the average number of females it collected, whereas only one of the control sites (C2) showed this pattern (Fig. 7a–c and Supplementary Table 6). In addition, we used the LAMP assay to test for *Wolbachia*-infected males in traps from the buffer area separating T1 and C1 as well as traps within C1 (Fig. 4d). Unsurprisingly, we found *Wolbachia*-positive males in large numbers up to 200 m from the nearest release street in T1 (Fig. 7d), clearly demonstrating that our treatment sites were within the flight range of mosquitoes in untreated areas. Overall, the data are consistent with 'edge effects' driven by female mosquito migration into our treatment sites.



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	Aggregate control	C1	C2	C3
Aggregate treatment	93.64***	92.36***	93.53***	94.78***
T1	97.43***	96.91***	97.38***	97.38***
T2	92.83***	91.38***	92.70***	94.11***
T3	89.39***	87.25***	89.20***	91.29***

Fig. 6 | Wild female and larvae counts from field sites. a, Mean number of females per trap in treatment areas (T1–T3) and control areas (C1–C3) in 2018 ($n_{T1}=44$, $n_{T2}=24$, $n_{T3}=35$, $n_{C1}=17$, $n_{C2}=28$, $n_{C3}=15$ independent trap samples per collection day). **b**, Mean number of females per trap in treatment areas only, on shortened *y* axis (sample sizes are the same as in **a**). **c**, Mean number of females per trap for aggregated treatments sites and aggregated control sites ($n_{TRT}=103$, $n_{CTRL}=60$ independent trap samples per collection week). Gray bar, period defined as 'peak season'. **d**, Per cent suppression of adult females in aggregate treatment sites compared to aggregate control sites using a 2-week trailing average (see Supplementary Text for details; sample sizes are the same as in **c**). **e**, Suppression calculated across the 14-week peak-season window evaluated with a one-sided permutation test ($n_{T1}=616$, $n_{T2}=336$, $n_{T3}=490$, $n_{C1}=238$, $n_{C2}=392$, $n_{C3}=210$, $n_{TRT}=1{,}442$, $n_{CTRL}=840$ independent trap samples). $^{***}P<1.6\times10^{-5}$, Bonferroni-corrected. **f**, Year-on-year comparison of the average number of females in T1; comparison of the same period in 2018 and in 2017 ($n_{2017}=65$, $n_{2018}=44$ independent trap samples per collection day). **g**, Cumulative mean number of larvae per egg trap with treatment sites aggregated and control sites aggregated ($n_{TRT}=131$, $n_{CTRL}=77$ independent trap samples per collection week), indicating significantly different larval production between treatment and control areas. For all panels, shaded areas indicate 95% CIs, and dotted lines indicate first and last day of releases.

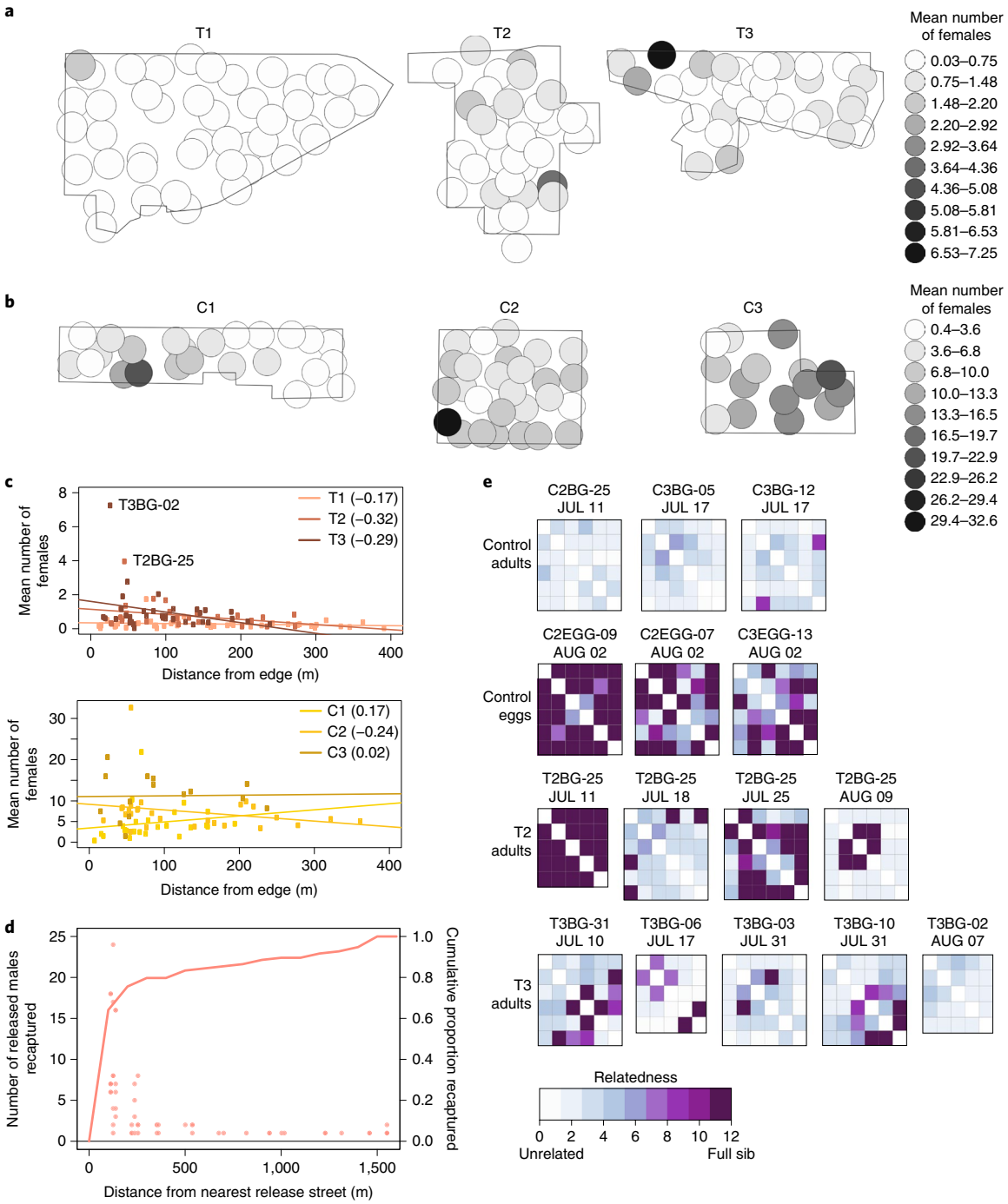

Fig. 7 | Evidence for female migration into treatment areas. a, Maps of treatment areas with a 100-m radius around each adult trap. Shading corresponds to the mean number of females per trap in 2018. **b**, Maps of control areas as in **a**, but on a different scale (as shown on the right). **c**, Top panel, correlations between mean number of females per trap in 2018 and distance from the nearest edge of treatment areas, with colors corresponding to the treatment area. Bottom panel, the same correlation in control areas. In both panels, Pearson's *r* is shown in the legend for each comparison. **d**, Dot plot showing the number of *Wolbachia*-males released in T1 and recaptured in traps in C1 on four dates during releases. The *x* axis shows the distance between the trap and the nearest release street in T1. The line shows the cumulative number of recaptured *Wolbachia*-males on the right *y* axis as a function of the same distance. **e**, Heatmaps showing genetic relatedness within trap collections from adult traps in control areas (first row), egg traps in control areas (second row), and high-female-count adult traps in treatment areas, with relatedness calculated based on HETHET/IBS0 relatedness scores, ranging from 0 to 12 (Supplementary Text). Each subpanel, labeled with trap ID and collection date, summarizes a series of pairwise comparisons between females, and the color of the tile indicates the degree of relatedness according to the scale below.

The higher numbers of females collected at the edges of treatment sites are mainly due to a small number of 'hot' traps that collected five or more females per trap collection (Fig. 7a). We sequenced individual female genomes from 'hot' traps and determined the relatedness among the sampled females (Supplementary Table 7 and Supplementary Text). Consistent with 'hot' traps being driven by nearby oviposition from inseminated female migrants, females in T2 and T3 'hot' traps had high relatedness with an average per-trap

rate of sibship of 0.47 and 0.10, respectively (Supplementary Table 7 and Fig. 7e). Similarly, larvae collected from egg traps had an average per-trap rate of sibship of 0.6. By contrast, females from C2 and C3 collections have very low rates of sibship per trap (0.00, Supplementary Table 7 and Fig. 7e), suggestive of many unrelated larval production sites (Fig. 7e). Although some larval production in treatment areas may have resulted from virgin female migrants finding a fertile mate within the treatment area or from local females evading released males, the available evidence suggests that most production is due to inseminated females migrating into the treatment areas and ovipositing.

Discussion

Our results demonstrate that efficient production of incompatible *Wolbachia*-infected males using automated systems enables strong suppression of wild populations of *A. aegypti* at scales larger than previous trials that relied on manual rearing and release methods (Supplementary Table 8). We achieved an estimated 95.55% (93.74–96.97%) reduction in the wild adult mosquito population across three replicate release sites. Suppression varied between treatment sites, with our most isolated site, T1, reaching nearly 99% reduction, while T3 reached a maximum suppression level of nearly 95% (Supplementary Table 5). One key difference between our treatment sites is that we conducted a preliminary suppression trial in T3 in 2017 (Supplementary Text), which could have impacted the results in 2018. However, we observed more females in T3 at the beginning of the 2018 season than any other site (Fig. 6b) and average suppression was lower in T3 (Fig. 6e), indicating that the 68% suppression achieved in 2017 was not sufficient for multi-year impact. Indeed, the largest differences in female densities between treatment sites developed later in the season (Fig. 6b), suggesting that immigration was a primary driver of between-treatment-site variation.

Despite maintaining very high overflooding ratios of *Wolbachia* males (>45 *Wolbachia* to 1 wild-type, Supplementary Table 3) in each treatment site, we were unable to achieve local elimination, probably due to migration of wild-type females from untreated areas. Increasing the size of release zones in future treatments should enable stronger suppression by buffering the effects of immigration over a larger area and increasing the distance between internal areas and edges. As a result, by treating larger areas and minimizing the impact of migration, we should theoretically be able to lower the number of males released per household by up to an order of magnitude, bringing overflooding ratios in the field closer to the minimum predicted to be effective by laboratory experiments[41,42]. Assuming that the male release rate can be reduced by at least half during a large-scale, phased rollout, we estimate that we could strongly suppress the mosquito population across the entire Fresno-Clovis metropolitan area (~250,000 households) in 3 years using four automated larval rearing systems operating at full capacity coupled with our sex-sorting and release technology.

A common criticism of SIT is the need for continual re-application of males each season if the target population is not fully eliminated. Close monitoring of our study sites in future years will provide insight into how quickly *A. aegypti* populations rebound after treatment and allow us to directly test whether lower release numbers can sustain suppression in previously treated areas. We expect the rate of re-infestation will depend on both the strength of suppression in the treated area as well as the abundance and proximity of nearby source populations. In addition, as recommended by the World Health Organization, a cluster-randomized control trial(s) would further validate the efficacy of our approach and, if conducted in an area with *Aedes*-borne disease, could be used to measure the impact of mosquito population suppression on arboviral transmission. When first responding to an epidemic outbreak of *Aedes*-borne disease, however, our study and others have shown that SIT-based interventions take multiple weeks to begin reducing mosquito numbers and thus should be combined with more fast-acting abatement techniques.

In this study, automation enabled unprecedented consistency in larval rearing, accuracy during sex separation, and precision in mosquito release, allowing us to avoid pitfalls that limited the success or scale of most previous mosquito SIT trials, such as uncompetitive males, insufficient production yields, and high female contamination rates[6,10,35]. Residual adult female removal after mechanical pupal sex-separation has been especially difficult to scale, but our highly accurate automated sex-sorting pipeline and female sterilization by low-dose pupal irradiation[29,30] both solve this problem, enabling SIT to be effective in large-scale suppression of wild populations. We expect that continued improvements in mosquito production, separation, and release technologies will increase performance and efficiency. The results described here support the prospect of removing invasive *A. aegypti* populations from large swaths of land without the use of chemical insecticides, aiding the ongoing public health battle against *A. aegypti*.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41587-020-0471-x.

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Methods

Mosquito strains. In 2018, we released male mosquitoes from two colonies of the WB1 strain of *A. aegypti* with a Fresno-Clovis genetic background. In each case, *Wolbachia*-infected females were crossed en masse with field-derived wild-type Fresno and Clovis males (collected and colonized in the Summer of 2017) for at least four generations (Supplementary Text). Each backcrossed strain was confirmed to have 100% incompatibility when mated with wild-type females sourced from Fresno and Clovis. We confirmed genetic similarity between backcross colonies and wild-type Fresno-Clovis colonies using genome sequencing (Supplementary Text and Supplementary Fig. 4). The first colony (denoted WB1-CL4-BC4) was released on 148 days of the trial, while the second colony (WB1-CL5-BC5) was released on 70 days (Supplementary Table 2).

Mass rearing. For egg production, approximately 4,500 adults at a 1:1 sex ratio were held in 60 cm^3 Bugdorm cages at 75% relative humidity and 28 °C, and given 10% sucrose ad libitum. Cages were fed organic bovine blood warmed to 37 °C in petri dishes covered with parafilm. Eggs were collected in soup cups lined with wet seed germination paper, allowed to embryonate, and then stored for up to 1 month prior to hatching. For larval production, eggs were scraped from germination papers, weighed, and 0.5 g of eggs were hatched in 0.15 optical density *E. coli* (DH5a) broth. L1 larvae were automatically counted into batches of 1,500, 2,000, or 3,000 and transferred into a thermoformed container containing 1 liter of double-distilled water, 40 ml of fermented bovine liver powder (fBLP) (MP Biomedicals), activated carbon pellets (Imagitarium), and 0.5 g of bovine liver powder (BLP). fBLP was made by allowing 4.5 g of BLP to ferment in a closed carboy containing approximately 20 liters of water for 7 days. After loading onto the incubated rearing frame, larvae were given three additional BLP feeds (Supplementary Text), and removed after 6 days at 28 °C.

Field releases. After sex-sorting, males were transported from our rearing facility at Verily in South San Francisco to Fresno and Clovis, in 6-inch-diameter release tubes with 10% sucrose ad libitum, where they were held overnight for release the next morning. Males ranging in age from 2 to 3 days old were released from the side of customized vans typically between 6:00 and 11:00, 7 days per week for 26 weeks. See Supplementary Text for more details.

Study sites. We chose three communities in Fresno County for male mosquito releases. They are almost exclusively residential neighborhoods within incorporated cities, except for T1, which includes a community center and elementary school. Treatment sites ranged in size, with T1 being the largest (1,563 households within 130 ha), followed by T3 (683 households within 89 ha), and T2 (665 households within 74 ha) (Supplementary Table 1). T2 is bordered on three sides by other neighborhoods known to have established *A. aegypti* populations, so although we treated and monitored 74 ha, we designated buffers on the northern, eastern, and southern borders, leaving a core area of 44 ha designated as the core treatment area (see Fig. 4c) for all subsequent analyses. Treatment site T3 was somewhat disconnected from other residential areas and bordered on most sides by either a road or open space and residential areas, so no buffer areas were treated around this site.

We also monitored three geographically matched control areas. Although smaller than the release areas in overall size (Supplementary Table 1), the control areas were almost exclusively residential and very similar to the release areas with respect to housing density and landscape. One control site, C1, is adjacent to the T1 treatment site. Although we monitored the entire C1 site, we excluded from downstream analysis traps in a buffer region (Fig. 4d) (size defined as three times the expected average flight range of this species, or approximately 300 m, of the edge of T1) to minimize any confounding effects of *Wolbachia*-males dispersing into this site in appreciable numbers (Fig. 4d).

Treatment areas were chosen based on several criteria: (1) the degree of isolation from untreated areas; (2) historical trapping data indicating establishment of *A. aegypti*; and (3) how well the area represented typical landscape in Fresno County. Control areas were chosen based on the same criteria, except that criterion 1 was relaxed given that the number of sites that fitted this criterion was small. Assignment of each site as control or treatment was not randomized, but we believe that any potential bias associated with site assignment would be negligible compared to the effect size observed in comparisons between treatment and control areas.

Field monitoring. All mosquito field monitoring was conducted by CMAD staff using protocols developed in collaboration with Verily and MosquitoMate. Following consent from residents, adult BG-Sentinel (v2, Biogents) and custom-made egg traps were placed in front yards at residences thought to be preferred by *A. aegypti* based on physical characteristics of the yard. Trap density was similar (Supplementary Table 1) between treatment and control areas. Treatment and control areas were paired such that pairs were always collected on the same day. Adult trap data from treatment and control sites can be found in Supplementary Table 9.

Statistics. We summarized trap counts on a weekly basis, but we only included weeks with valid collection data from greater than 75% of traps in a site to minimize fluctuations resulting from small sample sizes. Non-parametric 95% bootstrap CIs were calculated by taking 1,000 bootstrap samples with replacement of all valid trap collections for a week within a site for site-wise statistics, or samples of all valid trap collections across the merged site classes for the aggregate statistics. We calculated means from each bootstrap sample and found the 2.5% and 97.5% quantiles of the sorted distribution. Target sample sizes were $n_{T1}=44$, $n_{T2}=24$, $n_{T3}=35$, $n_{C1}=17$, $n_{C2}=28$, $n_{C3}=15$ independent trap samples per collection day, but trap problems led to slight reductions in sample size for some collection days. For aggregate statistics, sample sizes vary and are specified in the figure legends.

We calculated suppression as $1-(T_i/C_i)$ where T_i is the 2-week trailing average of all valid treatment site collections and C_i is the 2-week trailing average of all valid control site collections. This formula is numerically identical to Abbott's formula[43]. As adult traps were baited with dry ice and collected twice per week in 2017 and once per week in 2018, the 2-week windows include four collections on average in 2017, but only two collections in 2018. We calculated non-parametric 95% CIs as described above but within 2-week windows within each class separately for suppression analysis, re-calculated the mean for each bootstrapped sample and found the 2.5% and 97.5% quantiles of all bootstrap means for that window. We chose to use 2-week trailing averages for comparison to reduce emphasis on collection-to-collection fluctuations in the data.

To quantify the statistical power afforded by the 2018 trapping regime, we conducted a power analysis for the 2018 trial calibrated using data from the control areas during peak mosquito season. For this analysis, we assumed a three-level hierarchical model with three clusters (representing control areas) in the trial and an average of 21 traps per cluster. Each cluster is described by an *N*-mixture model[44], in which the underlying population is drawn from a Poisson distribution and then the trap counts are drawn from a binomial distribution where the parameter n is the Poisson draw and p is set to the trap efficiency. Previous work suggests that the BG-Sentinel trap efficiency is approximately 10% (ref. [45]). To parameterize the Poisson distributions, we back-calculated the mean trap counts from peak-season control area data assuming a trap efficiency of 10%. Specifically, we defined 'peak mosquito season' as the window of time when the mean number of females per trap in the aggregate control area exceeded 10, which corresponds to 17 July to 19 October (gray bar in Fig. 6c). After accounting for trap efficiency, the mean female trap count is 129 and the between-cluster variance was 1,695. To simulate each cluster, we drew lambda for the Poisson distribution from a normal distribution (mean = 129, s.d. = 1,695), and each cluster was assumed to be independent. We then simulated control area trap counts using Monte Carlo sampling of this hierarchical model and treatment area trap counts using the same approach but scaling lambda by the target suppression value. The suppression values were then calculated and compared using bootstrapping with replacement on the resulting simulated trap counts aggregated across clusters. This process was repeated 1,000 times to calculate the power and p values of the simulated experiment. The analysis shows that this suppression study has >80% power and has a p value of <0.05 when there is at least 40% suppression observed (that is 80% probability of recovering true positive result when suppression is at least 40%).

To explore differences between treatment and control sites, we made all pairwise comparisons between the aggregate control and treatment sites, and all treatment and control sites individually. The maximum suppression value within a 2-week window and 95% CIs are presented in Supplementary Table 5.

To determine whether the levels of suppression observed in our treatment areas are significantly different from the null hypothesis of no suppression (that is, no difference between treatment and controls), we applied a permutation test. We calculated the observed level of suppression across the entire 14-week peak mosquito season and compared this value to suppression calculated after randomly permuting traps among sites. We compared the aggregate treatment site and all individual treatment areas to the aggregate control sites as well as each control area individually using one million permutations per comparison in a one-sided test. In all cases, all permuted data sets produced levels of suppression less than the observed value, corresponding to a Bonferroni-adjusted p value of 1.6×10^{-5}.

After excluding collections with trap problems or traps in which the paper was dry at the time of collection, we calculated aggregate egg hatch rates as the number of larvae divided by the number of viable eggs. Hatch rate 95% CIs were calculated by bootstrap sampling with replacement of egg papers that were positive for eggs. We calculated the cumulative number of larvae per trap by cumulatively summing the total number of larvae that hatched from all egg collections. Target samples sizes were $n_{T1}=61$, $n_{T2}=35$, $n_{T3}=41$, $n_{C1}=30$, $n_{C2}=32$, $n_{C3}=15$ independent trap samples per collection day, but trap problems led to slight reductions in sample size for some collection days. We excluded collection days on which more than 25% of trap collections were missing owing to trap problems.

Analyses were conducted using custom R[46] and Python scripts.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Adult count data from field traps analyzed in this study are included as supplementary tables. Per-site male release numbers are also included as

supplementary tables. Genome sequencing data can be found under Bioproject PRJNA600991 at NCBI. Training image data and the trained neural-net model for male–female classification can be accessed by visiting https://github.com/verilylifesciences/classifaedes.

Code availability

Scripts for analysis of trap data are available upon request. Scripts for organizing machine learning training data and conducting model training can be found at https://github.com/verilylifesciences/classifaedes.

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Author contributions

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Competing interests

J.E.C., D.W.C, V.C., M.D., K.G., K.C.H., P. H., J.S.H., J.L., C.B., R.B., W.C., C.E., D.G., Y.H., B.H., E.K., J.K., A.K., E.L., T.L., J.L., M.L., W.M., M.M., S.N.M., D.M., J.R.O., K.P., A.P., C.R., M.S., R.S., P.S., J.S., J.S., B.W. A.M.W., M.W., J.W., A.Y., W.C.C., N.S., L.U., T.Z., P.M. and B.J.W. were paid employees of Verily Life Sciences, a for-profit company developing products for mosquito control, at the time they performed research for this study. K.L.D., J.W.M. and S.L.D. were paid employees of Mosquito Mate, a for-profit company developing products for mosquito control, at the time they performed research for this study.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41587-020-0471-x.

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☐	☒ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☐	☒ Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Custom website was built for data entry of trap collection and hatch rate data.
Data analysis	Custom R scripts were used to analyze mosquito counts. Python scripts were written to analyze male release data and sex-sorting performance.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Adult count data from field traps analyzed in this study are included as a supplementary tables. Per-site male release numbers are also included as supplementary tables. Genome sequencing data can be found under Bioproject PRJNA600991 at NCBI SRA. Training image data and the trained neural-net model for male-female classification can be accessed by visiting github.com/verilylifesciences/classifaedes.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	We chose three treatment sites for male releases as a balance between logistical/production constraints, need for replication, and physical size of the areas required to minimize migration.
Data exclusions	We excluded trap collections determined to be compromised by trap malfunctions.
Replication	We applied Wolbachia-infected male releases in three independent areas providing replicate tests of their effect on mosquito reproduction.
Randomization	Randomization was not appropriate for our experimental design.
Blinding	Blinding was not incorporated into the data entry or analysis for this study.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology
☐	☒ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals	A laboratory Wolbachia-infected strain of Aedes aegypti (WB1) was kept in the insectary and used as the source of Wolbachia for making new Wolbachia-infected colonies with local mosquito genetic background.
Wild animals	Adult Aedes aegypti mosquitoes were captured in field traps and immediately frozen on dry ice for preservation. Mosquito eggs were collected using egg traps, matured in the laboratory, and hatched to obtain hatch rate data. Resulting larvae were either boiled or used to establish new colonies in the laboratory.
Field-collected samples	Adult mosquitoes were frozen after removal from the trap, underwent several freeze-thaw cycles during transport and counting, and remained frozen at -20 until DNA extraction. Mosquito eggs were held at ambient temperatures for 48 hours followed by a 72 hour incubation at 28C.
Ethics oversight	General experimental design guidance and approval was provided by the US Environmental Protection Agency as part of the Experimental Use Permit approval process.

Note that full information on the approval of the study protocol must also be provided in the manuscript.