INDUSTRIAL ROBOTS

Upscaling the production of sterile male mosquitoes with an automated pupa sex sorter

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Effective mosquito population suppression has been repeatedly demonstrated in field trials through the release of male mosquitoes to induce sterile mating with wild females using the incompatible insect technique (IIT), the sterile insect technique (SIT), or their combination. However, upscaling these techniques requires a highly efficient and scalable approach for the sex separation of mass-reared mosquitoes to minimize the unintentional release of females, which can lead to either population replacement or biting nuisance, a major bottleneck up to now. Here, we report the successful development of an automated mosquito pupa sex sorter that can effectively separate large numbers of males from females for population suppression of *Aedes aegypti*, *A. albopictus*, and *Culex quinquefasciatus*. The male production capacity of the automated sex sorter was increased by ~17-fold compared with manual sex separation with the Fay-Morlan sorter and enabled one person to separate 16 million males per week. With ~0.5% female contamination, the produced males exhibited high flight ability and mating performance. The field trial demonstrates that the quality of *A. albopictus* males produced using the automated sex sorter is suitable for inducing population suppression. These results indicate that the automated sex sorter offers the potential to upscale IIT and SIT against mosquito vectors for disease control.

INTRODUCTION

Accounting for more than 17% of all infectious diseases and causing more than 700,000 deaths annually, vector-borne diseases are unique in that the pathogens undergo an extrinsic incubation period in the vector before being transmitted to humans (*1*). This period offers an opportunity to prevent and control disease transmission by targeting vectors or pathogens transmitted through them (*2*). During the past decades, substantial efforts have been made to develop novel strategies to control mosquito-borne diseases, including dengue, Zika, and malaria, through naturally occurring symbiotic bacteria such as *Wolbachia* (*3*, *4*), the sterile insect technique (SIT) (*5*), release of insects carrying a dominant lethal (*6*), genetic modification of mosquitoes or their associated microbes (*7*), and gene drive technology (*8*). Although population replacement focuses on reducing a mosquito's ability to transmit a pathogen, the aim of population suppression is to eradicate or reduce and maintain a population at a density below a threshold required for sustaining pathogen transmission. Successful population suppression has been demonstrated in field trials in multiple countries by releasing sterile males into populations to mate with wild females and inhibit the production of fertile eggs (*3*, *4*, *9*, *10*). Regardless of whether sterility is induced by *Wolbachia*, radiation, or genetic modification, female mosquitoes need to be removed before release because they can transmit diseases, cause nuisance biting, compromise the efficiency of sterile mating, or even prevent the suppression in terms of the *Wolbachia*-based incompatible insect technique (IIT).

Current mosquito sex-separation methods are based on the natural and artificial differences in biology between females and males, including size, development rate, morphology, and feeding behavior. For *Aedes*, *Culex*, and some *Anopheles* mosquito species, female pupae are larger than male pupae, whereas males develop faster than females in the immature stage. Females can also be distinguished from males on the basis of morphological differences at both the pupa and adult stages. The fact that only female mosquitoes take a bloodmeal provides an opportunity to eliminate them by spiking blood meals with insecticides or other insect toxins (*11*). In addition to using the above natural differences between the two sexes for separation, efforts have been made to develop genetic sexing strains by linking a selectable marker to one sex through either classical mutagenesis or the CRISPR-Cas system (*12*). Genetically modifying mosquitoes to conditionally express female-specific lethality would be optimal for operational simplicity and cost-effectiveness (*13*). Unfortunately, public opposition to the use and release of transgenic organisms still exists in most countries or regions where new technologies for mosquito-borne disease control are urgently needed. There is thus currently no proposed method enabling the efficient upscaling of genetic control programs, which represents a major bottleneck (*13*, *14*).

Among all the field trials, the most common approach for mosquito sex separation is to use a mechanical separator, either the Fay-Morlan glass sorter (also named the Hock sorter) or pupal sieves, based on differences in pupa size between females and males. The changing width of the wedge-shaped opening between two glasses thus allows separation of sexes and stages in horizontal bands (Fig. 1), which is the principle used in our study for both manual and automated sorting. This method requires a lot of manual work and leads

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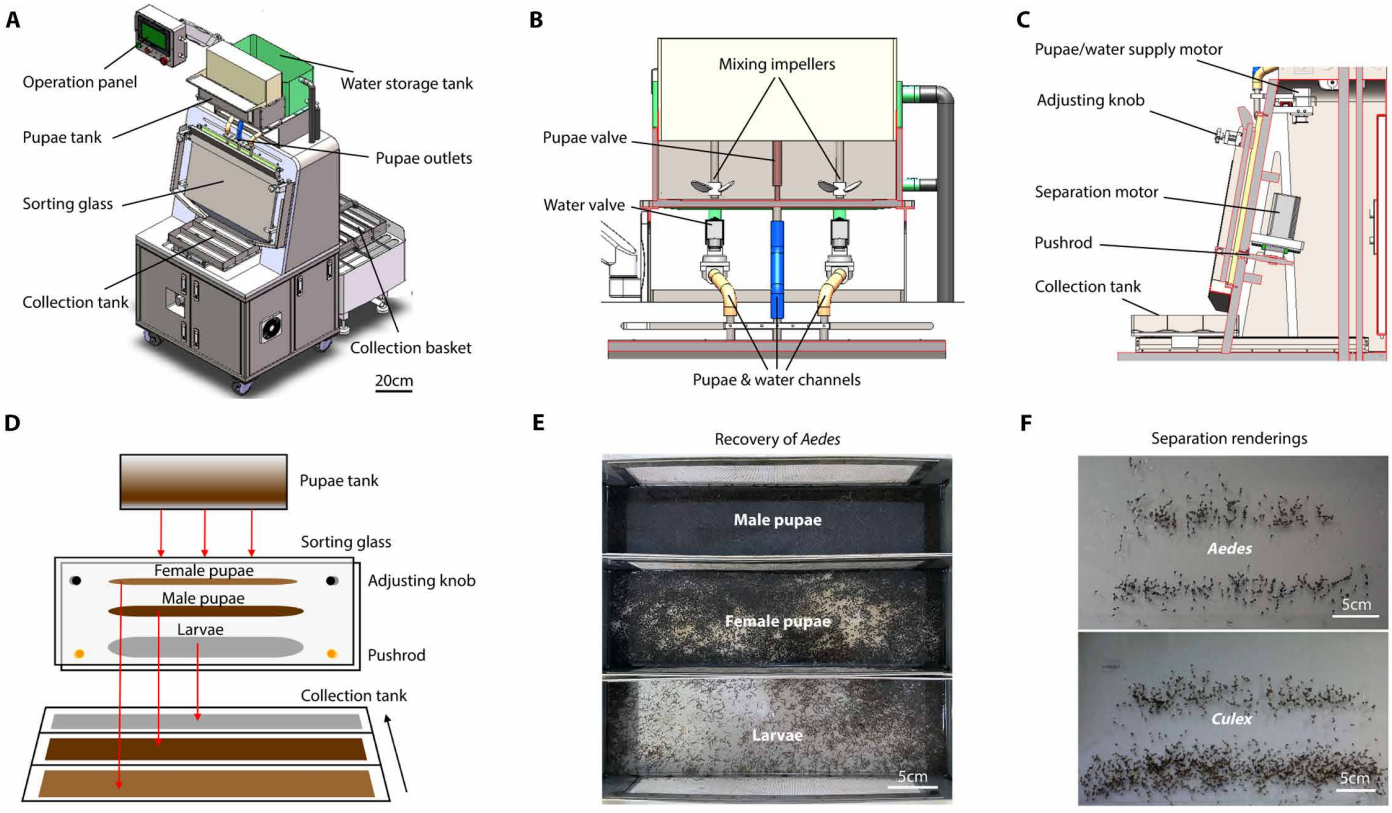

Fig. 1. The automated mosquito pupa sex-sorting system. Technical drawing of the automated sorting (**A**), pupa supply (**B**), and pupa sorting and collection (**C**) mechanisms. (**D**) Schematic diagram of automated mosquito pupa separation. Red arrows show the process of the mixture from the pupa tank separated into three different colonies. The black arrow shows the direction in which the collection tanks are moved into place. (**E**) Recovery of the male pupae, female pupae, and remaining larvae of *A. albopictus* in different collection baskets. (**F**) Separation renderings of the *A. albopictus* and *C. quinquefasciatus* pupae.

to female contamination rates sometimes higher than 1%, a threshold considered the maximum acceptable for release (*15*). Consequently, it has recently been supplemented with either radiation to sterilize accidentally released fertile females (*3*) or artificial intelligence to remove most residual fertile females, although the production capacity of the latter option has not been reported (*4*, *16*, *17*). This system has resulted in strong population suppression or even elimination of *Aedes aegypti* and *A. albopictus*, demonstrating proof of principle of the IIT/SIT being implemented for area-wide mosquito control. Nonetheless, further enhancements are necessary because these approaches are not scalable for mass-release programs and are constrained by limitations in human resources. Over the past decade, extensive technological developments have improved sex-separation methods; however, the current techniques remain time consuming and energy intensive and exhibit suboptimal sorting efficiency, posing challenges for their widespread implementation in population suppression strategies for vector control. To fit into mosquito industrialization processes, a highly effective sex-separation system must automate the loading, sorting, and collection of millions of male pupae each day, replacing the current manual labor with mechanization. The main challenge of such a system is the rapid separation of mosquito pupae in water, which requires distinguishing between the minor differences in size between the sexes (<0.2 mm) amid substantial size overlap (*18*) while maintaining a low tolerance for female contamination and minimizing the negative effects on male fitness.

Here, we report the development of an automated mosquito pupa sex sorter capable of efficiently loading, sorting, and collecting millions of male pupae daily (Movie 1). We experimentally compared the efficiency of this automated mosquito pupa sex sorter with that of the manual Fay-Morlan glass sorter for three mosquito species: *A. aegypti*, *A. albopictus*, and *Culex quinquefasciatus*. With ~0.5% female pupa contamination and an ~30% male loss rate from hatching to preemergence of pupae, the production capacity of the automated sex sorter increased by ~17-fold as compared with that of the manual sorter. We further evaluated its use and performance via a field trial and demonstrated the suppression of wild populations of *A. albopictus* in a target area by releasing incompatible *A. albopictus* with a triple-strain *Wolbachia* infection of *w*Pip (a *Wolbachia* strain from its native mosquito host *C. pipiens*), *w*AlbA, and *w*AlbB (*Wolbachia* strains from their native mosquito host *A. albopictus*) (*3*). These results support the potential of the automated sex sorter in the mass production of mosquito males and the deployment of IIT or SIT for area-wide management of mosquito vectors for disease control.

RESULTS

Mechanism of the automated sex sorter for mosquito pupae

The sex sorter developed in this study included three mechanisms with coherent and synchronized functions: a pupa tank for mosquito supply, a sorting glass for sex separation, and collection baskets for male

Movie 1. Summary video.

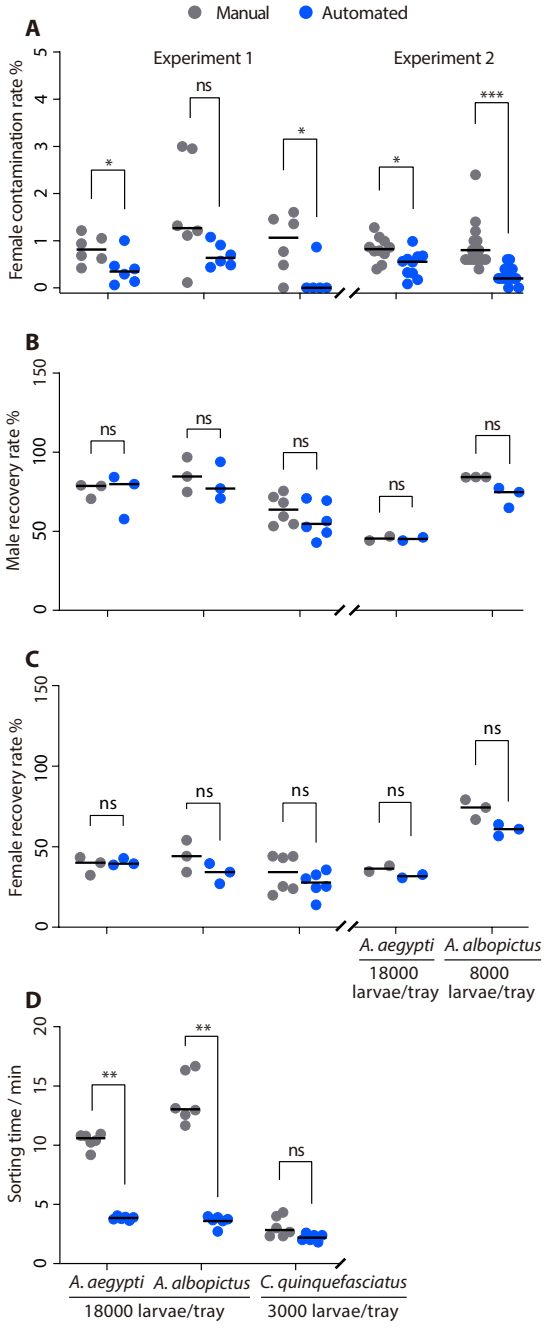

Fig. 2. Sex-separation test of three mosquito species, *A. albopictus*, *A. aegypti*, and *C. quinquefasciatus*, at laboratory scale. (**A**) Comparison of the female contamination rate per separation between the manual and automated mosquito pupa sex sorters. (**B**) Comparison of the average male recovery rate per batch between the manual and automated mosquito pupa sex sorters. (**C**) Comparison of the average female recovery rate per batch between the manual and automated mosquito pupa sex sorters. (**D**) Comparison of the average sorting time per tray between the manual and automated mosquito pupa sex sorters. Each dot in (A) and (D) represents one replicated sorting experiment, and each dot in (B) and (C) represents one replicated sorting batch. The two columns on the right represent a repetition of the experiment with larger batches (experiment 2). In experiments 1 and 2, each sample included 100 to ~300 ($N = 3$) and 500 to ~1000 ($N = 2$ for *A. aegypti* and $N = 3$ for *A. albopictus*) pupa per sex, respectively. Mann-Whitney *U* test, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Horizontal bars indicate medians.

and female pupae and larvae, all of which were incorporated with a water cycle between the tank and basket (Fig. 1 and figs. S1 and S2). To integrate and automate the operation, sensors, water pumps, stepper and servo motors, a customized transmission, and electromagnetic valves were controlled by a programmable logic controller from Mitsubishi (Fig. 1, B and C). Given the potential variation in mosquito immature development across different batches, the sorter was calibrated for each

batch of pupae at the beginning of sex separation to optimize the accuracy by adjusting the technical parameters, including the slope of the outer sorting glass, through the touch screen on the control panel. Once calibrated, sex separation operated automatically until all pupae were sorted (movie S1).

Testing the automated sex sorter in the laboratory

We tested the performance of the automated sex sorter in two independent experiments using different sample sizes. In experiment 1, three mosquito species, *A. aegypti*, *A. albopictus*, and *C. quinquefasciatus*, were assayed, because efforts to mass-produce their males for developing either SIT or IIT for population suppression are currently ongoing. Mosquito larvae were reared at densities corresponding to mass-rearing conditions (*A. aegypti* and *A. albopictus*, 18,000 larvae per tray; *C. quinquefasciatus*, 3000 larvae per tray), and three samples of approximately 100 to ~500 pupa per sex were randomly taken for analysis (see Materials and Methods, the section "Comparison of automated and manual sex-sorting methods in laboratory conditions"). The automated sorter significantly reduced the female contamination rates in male pupae by 56.79 and 100% for *A. aegypti* (median = 0.35%) and *C. quinquefasciatus* (median = 0.00%), respectively, as compared with manual sorting (median = 0.81 and 1.07% for *A. aegypti* and *C. quinquefasciatus*, respectively), although there was no statistical difference in *A. albopictus* (automated, median = 0.64%; and manual, median = 1.27%) (Fig. 2A). Regardless of the species, there was no significant difference in average male recovery rates relative to first-instar larvae between automated (*A. aegypti*, median = 79.79%; *A. albopictus*, median = 77.02%; and *C. quinquefasciatus*, median = 54.63%) and manual sorting (median = 78.73, 84.66, and 63.74%, for *A. aegypti*, *A. albopictus*, and *C. quinquefasciatus*, respectively) (Fig. 2B). Neither was a difference observed for female recovery rates from first-instar larvae to the pupa stage for the three mosquito species between automated (*A. aegypti*, median = 39.45%; *A. albopictus*, median = 34.24%; and *C. quinquefasciatus*, median = 27.74%) and manual sorting (median = 40.06, 44.18, and 34.24% for *A. aegypti*, *A. albopictus*, and *C. quinquefasciatus*, respectively) (Fig. 2C). The automated sorter significantly reduced the time spent on sex separation of *A. aegypti* (median = 3.85 min) and *A. albopictus* (median = 3.71 min) as compared with the manual sorter (median = 10.58 and 13.04 min, respectively), although no difference was observed for *C. quinquefasciatus* between automated (median = 2.20 min) and manual (median = 2.83 min) sorting, likely because of the limited number of pupae used (Fig. 2D). These results indicated that the automated sorter performed better in reducing both the female contamination rate and sorting time while maintaining similar male and female total recovery rates from first-instar larvae to preemergence pupae.

In experiment 2, we repeated this assay with larger samples for both *A. aegypti* and *A. albopictus* (1000 and 500 pupae, respectively; see Materials and Methods, the section "Comparison of automated and manual sex-sorting methods in laboratory conditions"). The automated sorter significantly reduced female contamination rates in male pupae in both species (Fig. 2A) without affecting the recovery rate of both males and females relative to first-instar larvae (Fig. 2, B and C). In addition, a sample with extreme deviation from the median level of female contamination (>2%) occurred in the manual sorting of *A. albopictus*, which did not happen with the automated sorter. These results demonstrated the improved accuracy in sex separation by the automated sorter relative to the manual sorter.

Evaluation of the automated sex sorter for mass production

We measured the female contamination rate, male pupa yield, and production capacity under mass-rearing conditions at the scale of millions of male mosquitoes per week. On the basis of historical analysis of 2-year data from our *A. albopictus* mass-rearing facility, the automated sorter had a female contamination rate of 0.38 to 0.56% (95% interval) (fig. S3A). The yield of male pupae derived from 1 g of eggs, which hatched into approximately 10^5 larvae under our mass-rearing conditions, was 3.02×10^4 to 3.58×10^4 (95% interval) (fig. S3B). Given that one person operated four automated sorters simultaneously, the production capacity, measured as the maximum number of male pupae produced per hour per person, was 9.93×10^4 to 1.42×10^5 (95% interval), accounting for the setup, maintenance, and cleaning of the sorter (fig. S3C). Because one experienced person had the capacity to operate eight automated sorters simultaneously (movie S2), the automated sorter allowed one person to produce up to 3.21×10^6 male pupae per day when working for 8 hours daily or 1.61×10^7 male pupae per week when working for 5 days.

Similar flight ability between males produced by the automated and manual sorters

To examine any difference in the quality of *A. aegypti* and *A. albopictus* males produced by the automated and manual sorters, we performed flight ability tests to measure the capacity of mosquitoes to escape flight tubes, serving as an indirect indicator of male competitiveness (*19*). There was no significant difference in the escape rates between males produced by the automated (*A. aegypti*, 78.62 ± 1.83%; and *A. albopictus*, 71.51 ± 1.74%) and manual sorters (*A. aegypti*, 79.57 ± 2.03%, $P = 0.730$; and *A. albopictus*, 69.09 ± 1.76%, $P = 0.337$) (Fig. 3), indicating that males produced using the two sorters had a similar ability to escape flight tubes.

Field evaluation of the quality of incompatible males sexed by the automated sorter

With the support of the above test results, we performed a field trial to examine the mating performance of *A. albopictus* incompatible males produced by the automated sorter in the field. The goal of this trial was not to compare manual and automated sorting but to assess the field performance of the males sexed by the automated sorter in

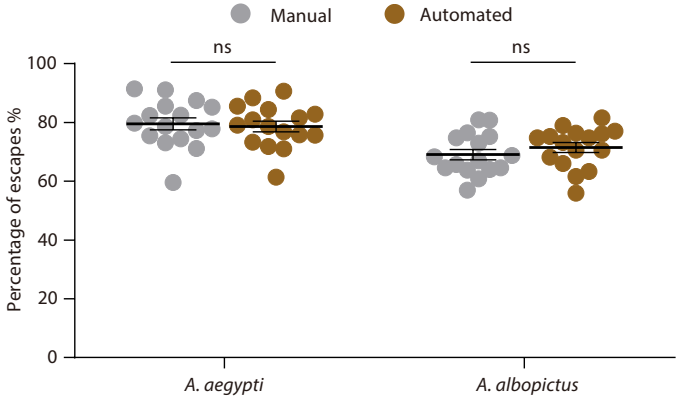

Fig. 3. Flight ability assessment of different *Aedes* males from the automated mosquito pupa sex sorter. Student's *t* test was used to determine statistical significance; ns, not significant. Horizontal bars indicate means based on $n = 16$ per treatment. Error bars indicate SEM.

operational conditions. Releases were conducted in Guangzhou from 27 July 2020 to 26 October 2020 (Fig. 4A). Before release, baseline data were collected weekly in both release and control sites to monitor the densities of mosquito adults and larvae using ovitraps and Biogents Sentinel (BG) traps, respectively. Overall, there was no difference in both female adults ($P = 0.90$) and hatched larvae ($P = 0.70$) between the release and control sites during the 3 weeks of monitoring before the first release. During the intervention period, 79,000 to 340,000 incompatible males were released weekly, resulting in ratios of incompatible versus wild-type males ranging between 3.56 and 158.60 (median = 40.95 and mean = 50.99), which were higher than the target overflood ratio of 5:1 (Fig. 4B). The relative mating performance of incompatible to wild-type males in the field was estimated from egg hatch rates and the observed release ratios. The observed egg hatch rates were significantly higher than expected for a mating competitiveness of 1 ($P < 0.0001$; Fig. 4C). The Fried index of incompatible males was 0.31 ± 0.36, consistent with the results of another field release site using males produced by the automated

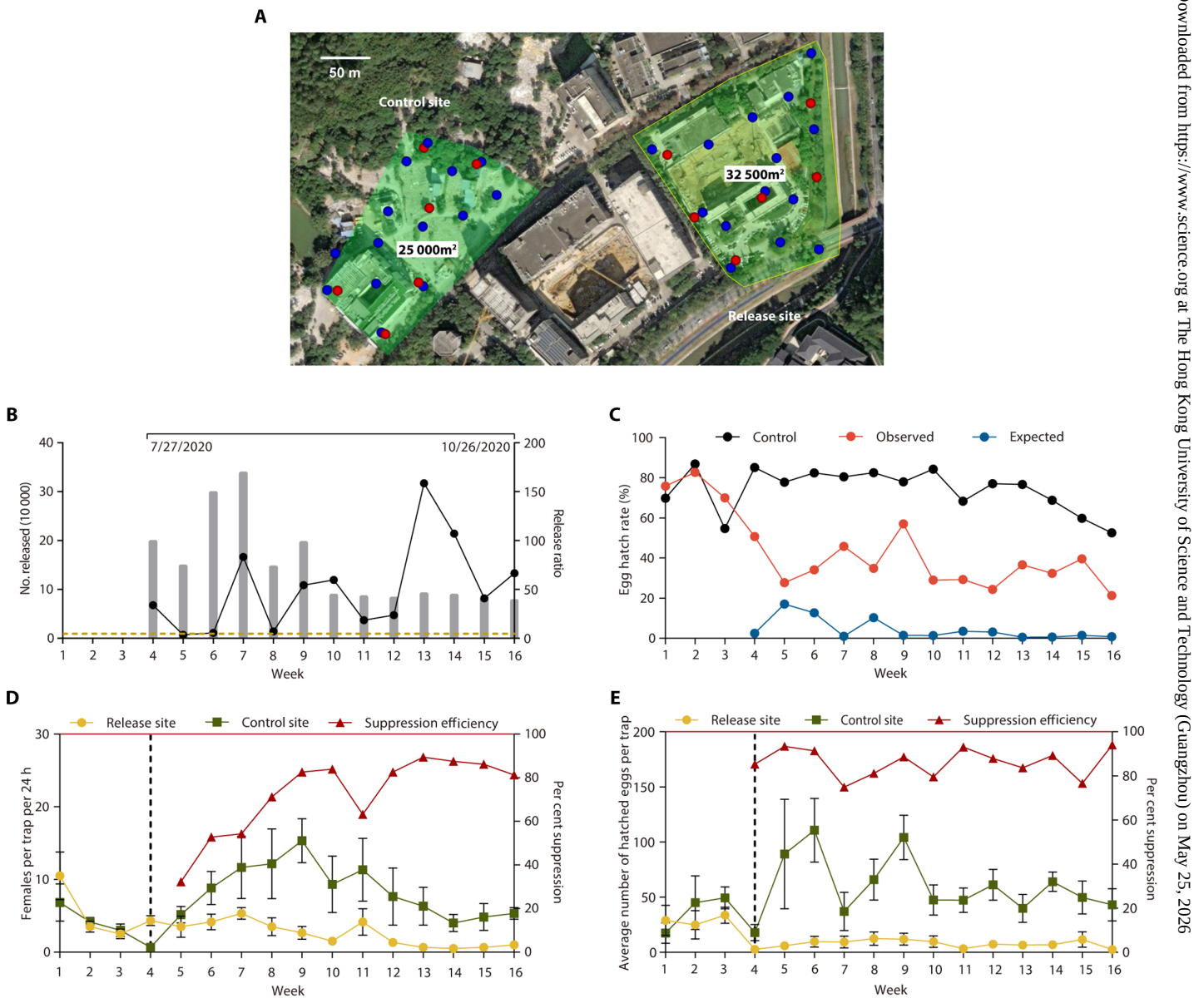

Fig. 4. Field trial of population suppression by releasing incompatible *A. albopictus* males produced by the automated sex sorter. (**A**) Satellite images of the release site (32,500 m^2) and control site (25,000 m^2) in Guangzhou. Red spots indicate six BG traps in each site. Blue spots indicate 14 ovitraps in each site. (**B**) Number of sterile males released weekly (vertical bars) and calculated ratios of released males to wild-type males in the field at the release site (black line). The brown dashed line indicates a target overflooding ratio of 5:1. (**C**) The observed and expected weekly egg hatch rates (total number of hatched eggs divided by total number of eggs) at the release site as compared with the egg hatch rates at the control site. (**D**) Population suppression at the adult stage, indicated by the densities of adult females collected weekly at the control and release sites (Mann-Whitney *U* test, $n = 12$, $P < 0.0001$). (**E**) Population suppression at the immature stage, indicated by the numbers of hatched eggs observed weekly at the control and release sites (Mann-Whitney *U* test, $n = 13$, $P < 0.0001$). Red solid lines in (D) and (E) indicate population suppression efficiency. Error bars indicate SEM.

sorter (*20*). Both egg hatch rates ($P < 0.0001$; Fig. 4C) and the proportion of egg-positive ovitraps ($P < 0.0001$; fig. S4) were significantly decreased in the release site as compared with the control site. Accordingly, we observed a marked decline in the number of female adults and hatched larvae in the release site as compared with the control site. Although the average number of female adults per BG trap dropped by 80% 7 weeks after the first release, the hatched eggs per ovitrap dropped by 85.9 ± 6.47% across all the release periods (Fig. 4, D and E), supporting a strong suppression of the mosquito population after the release of the incompatible males produced by the automated sorters.

DISCUSSION

The low efficacy of mosquito sex separation has long restricted the area-wide application of IIT and SIT for disease control. In this work, we have developed the first automated mosquito pupa sex sorter on the basis of the manual Fay-Morlan sorter and have demonstrated that the automated sorter exhibits lower female contamination rates and higher efficacy than the manual sorter in laboratory conditions, thus solving this bottleneck. Also, there was no difference in male recovery rates and flight ability between the automated and manual sorters. During a previous trial (*3*) based on sex separation using the manual method over a 27-week period, from 1 May 2017 to 5 November 2017, eight individuals participated in the separation work using eight Fay-Morlan glass sorters, each person spending 6 hours per day on the task. The female contamination rate of the manual sorter was 0.47 to 0.67% (95% interval), the yield of pupae was 3.04×10^4 to 3.79×10^4, and the personal production capacity was 1.99×10^4 to 2.29×10^4 (fig. S3). Because the time lag between these two trials may introduce various biases, we should be cautious when comparing the two trials even though we standardized the mosquito strain, person in charge, feeding procedures, mass-rearing conditions, and rearing period (27 weeks) in the year. However, this comparison is still meaningful because manual sex sorting was the main obstacle to upscaling production in 2017: The automated sorter performed similarly or better than the manual sorter in these operational conditions. Moreover, the automated sorter has the potential to increase personal production capacity up to ~17-fold as compared with the manual sorter, assuming that there are sufficient pupae to be separated, supporting its use to produce sterile males for upscaling releases to control mosquito vectors and their transmitted diseases.

The Fay-Morlan sorter has been successfully used in a number of IIT (*3*) and SIT (*9*) field trials for mosquito control, providing proof of concept that these technologies can lead to population suppression or elimination if sufficient numbers of high-quality males can be produced and released to exceed the critical overflooding ratio in the field. However, this manual sorter suffers from high demand for human labor, thus reduced cost-effectiveness, and poor consistency in quality across different individuals because of human errors (*21*). To address these issues, we have developed this automated sorter to replace human labor with mechanization.

With one person capable of operating up to eight machines and substantial reduction in time for sex separation, the automated sorter markedly increases personal production capacity and enables one person to produce 16 million male pupae per week. Accordingly, this automated sorter is already currently in use in multiple countries, including Austria, Italy, Brazil, China, and the US. Similar to the weekly release of a billion sterile males in screwworm and medfly SIT programs, the ability to develop IIT and SIT for mosquito population suppression through area-wide implementation would depend on the capacity to scale up sterile male production, which has long been restricted by sex separation as a bottleneck. The automated sorter not only facilitates the production scale-up but also produces males with consistently low female contamination such that population replacement may be prevented even without the irradiation step to sterilize the residual females post sex separation in specific weather conditions with extended cold temperatures because it is difficult for our triply *Wolbachia*-infected mosquitoes to survive winter (*20*). Thus, successful development of this automated sorter is a breakthrough in the effort to scale up the production by removing the current bottleneck and reducing human labor, moving one step closer to area-wide application of IIT and SIT for mosquito vector control. In the mass-production pipeline, the current scale of production would be determined by the capacity to mass-rear larvae and package and release the sterile males in the field (*22*) because a method to upscale irradiation of adult mosquitoes has recently been described (*23*).

The sex separation of the automated sorter is based on the size difference between male and female pupae, which is minor and subject to the mosquito's intrinsic biology and environmental conditions. Thus, standardizing the larvae-rearing protocol with quality assurance of larvae development is essential to protect the performance of this automated sorter. On the other hand, new strategies that can enhance sex dimorphism by expanding the difference in either size or development time between males and females will facilitate the use of the automated sorter for male mass production (*24*).

We demonstrated the high performance of males produced by the automated sorter through both a flight ability test in the laboratory and a field trial. The male mating competitiveness measured in the field was much higher than 0.2, the lower threshold for cost-effectiveness of mosquito SIT (*15*, *25*), which leads to successful suppression. It is worth noting that reduced mating competitiveness may be caused by the overall process of mass rearing, handling, irradiation, and release procedures, rather than sex separation alone (*25*).

Overall, our results show the successful development of an automated sorter for mass production of sterile males for mosquito suppression. The automated sorter is a promising instrument that can separate male and female pupae of three mosquito species, *A. aegypti*, *A. albopictus*, and *C. quinquefasciatus*, more efficiently than the manual sorter. Strong suppression by release of sterile males produced from the sorter highlights its feasibility in area-wide implementation for mosquito vector control. Future improvement in the design of the automated sorter includes integration of artificial intelligence to enhance its automation (for example, replacing manual calibration) and data collection (for example, pupa count and quality analysis and female contamination record).

MATERIALS AND METHODS

Study design

In all laboratory experiments, sample sizes were selected following reference protocols used in the Food and Agriculture Organization of the United Nations (FAO)/International Atomic Energy Agency (IAEA) Insect Pest Control Laboratory (IPCL) (*19*, *21*). The comparison

of automated and manual sex-sorting methods in laboratory conditions was repeated with harmonized and increased sample sizes following a reviewer request. No specific permit was needed for the laboratory experiments. The field trial in Guangzhou was authorized by the Ministry of Agriculture and Rural Affairs of the People's Republic of China (SY2019092) and was mainly based on a study design previously reported in 2019 (*3*) except no x-ray radiation treatment was performed. No data were excluded from the analysis.

Description and operating principles of the automated mosquito pupa sex sorter

We developed an automated mosquito pupa sex sorter (model WBK-P0001-V2) incorporating mechanical, electronic, and human-machine interface components (Fig. 1A and fig. S1A). The sorting system comprises two primary working modules: The pupa supply mechanism (Fig. 1B) serves as the first working module, consisting of two mixing impellers and a pupa valve within the pupa tank. The pupae are gently agitated at a controlled speed, as set by the speed regulation knob, to ensure a homogeneous concentration and prevent mechanical injury or hypoxia-induced mortality in the immature mosquitoes. The pupa valve is controlled by an electromagnet. Two water channels connected with water valves are combined with the pupae channel for the supply of pupae and water. The channels move together from start point to stop point repeatedly to ensure that pupae and larva are evenly distributed along their respective horizontal lines. For the separation, the start and stop points keep a certain distance from the edge of the sorting glasses to avoid the overflow of pupae. About 2000 to 3000 individuals are processed in one sorting action. The pupa tank was designed to accommodate approximately 200,000 mosquito pupae from the same feeding batch. To control the total number of pupae introduced in the tank, the number of mixed pupae/larvae collected after mass rearing must be estimated volumetrically. This allows for calculation of the total volume of water for each sorting action. Additional water is added at the end of each sorting action to ensure that the remaining pupae/larvae can be smoothly stirred by the mixing impellers and released into the space between the sorting glasses. There is no pupa/larva replenishment during a single sorting action.

The second working module is the pupa sorting and collection mechanism (Fig. 1C), which was designed after the first working module. A pupa/water supply motor controls the pupae/water channels to perform reciprocating motion on the basis of set parameters, which determine the amount of pupae/water supply and the separation range between the sorting glasses. The thickness and angle of the wedge-shaped space between the glasses can be regulated by the adjusting knobs (manual set for each batch) and pushrods (automatic set) (Fig. 1D). The eccentric wheels are driven by the separation motor to rotate, and the rotating motion is transformed into linear motion, so that the pushrod pushes the upper sorting glass to open with various angles. The repeated, synchronized actions of sorting and collection are supported by the connected stepper motors (Fig. 1, C and D). The lower opening is adjusted so that the larger organisms are retained in the tapering space between the panes of glass. Meanwhile, larvae and male and female pupae are sorted by the glasses before being washed into the corresponding collection tank at different angles, respectively, which ensures a very low friction, similar to the Hock sorter. The collection tanks also have reciprocating motion through a collection motor (fig. S1B), and then larvae, male pupae, and female pupae are collected and transported to the collection baskets through a pipeline under the tanks (Fig. 1E). Both *Aedes* and *Culex* could be efficiently separated by this machine (Fig. 1F).

The design of the collection tank not only ensures the continuous automatic operation of the system but also prevents overflows, which could lead to the remixing of the separated larvae, male pupae, and female pupae. The system notably reduces the potential for manual errors during the same work process, thereby minimizing the risk of female contamination in recovered male pupae. Excess water is pumped back to the water tank for the next cycle after seeping out of the collection baskets. Moreover, all the motors mentioned above are synchronized with each other and form an automatic working system in series, which makes the complex separation process fully automated. Different components cooperate and repeat the sorting motion until the pupa tank runs out of the mixture. The entire system needs to be cleaned and dried after each use. Moreover, the water needs to be replaced every 2 days.

Source of mosquitoes

For the experimental comparison against manual sorting, we used colonies of *A. aegypti* (Juazero, Brazil strain) and *A. albopictus* (Rimini, Italy strain). *A. aegypti* has been provided by Biofabrica Moscamed, IAEA Collaborative Center, since 2012, and *A. albopictus* by Centro Agricoltura Ambiente, IAEA Collaborative Center, since 2018. They were established and maintained at the IPCL (Seibersdorf, Austria) under controlled environmental conditions: The larval rearing room was maintained at 28° ± 2°C, 80 ± 10% relative humidity (RH), and the adult rearing room at 26° ± 2°C, 60 ± 10% RH, with a 14:10-hour light:dark (L:D) cycle with 1-hour periods of simulated dusk and dawn in both rooms. *C. quinquefasciatus* (Guangzhou, China) used here has been maintained at the Wolbaki factory since 2016. The colonies were established and mass-reared in a climate-controlled room at 27° ± 1°C, 70 ± 10% RH, and a photoperiod of 12:12 hours (L:D). The transinfected *A. albopictus* line, carrying three *Wolbachia* strains, *w*AlbA, *w*AlbB, and *w*Pip, was established and maintained in the Wolbaki facility using the standard conditions reported previously (*3*, *26*).

Comparison of automated and manual sex-sorting methods in laboratory conditions

We used a comparative approach against the manual sorting method using the Fay-Morlan glass separator. In experiment 1, *A. aegypti* and *A. albopictus* were mass-reared using the FAO/IAEA mass-rearing rack and procedures (*21*, *27*–*29*). First-instar larvae (18,000 per tray) hatched in tap water were fed with 6% IAEA slurry diet (tuna meal 50%, black soldier fly powder 35%, and brewer's yeast 15%) after the mass-rearing procedure with the following feeding regimen: 50 ml on day 1; 100 ml, day 2; 200 ml, day 3; 200 ml, day 4; 150 ml, day 5; and 200 ml, day 6. Trays were tilted to drain the water and pupae 20 to 24 hours after the first pupation and then 24 hours after the first sorting, and their contents were sorted for 2 consecutive days. Briefly, the contents of 10 trays were mixed together with reverse osmosis purified water for a total volume of 3 liters and sorted with the automated machine. For the manual sorting, the content of each tray (six trays) was sorted separately. For each day, the time spent on pupa mixing, calibration, sorting, and pupa collection was recorded to estimate time efficiency. Male and female pupae were estimated volumetrically using a modified tube following standard operating procedures developed at the IPCL (*27*). Three samples of approximately 500 and 100 pupa per sex for the automated and the manual

sorting, respectively, were randomly taken and placed into emergence cages, representing a global sampling effort of approximatively 5 and 8% for the two sorting strategies. All larvae in the samples were removed. After emergence and when all adults had died, they were separated by sex with the naked eye (or when in doubt verified under a stereomicroscope) to calculate female/male contamination percentages. The whole experiment was carried out three times. The male pupa recovery rate and female contamination rate were calculated as previously described (*30*). Specifically, the male pupa recovery rate was defined as the percentage of collected male pupae relative to the initial number of first-instar male larvae in the rearing tray, assuming an equal proportion of males and females. The same methodology was applied to calculate the female pupa recovery rate. The female contamination rate was calculated as the percentage of females relative to the total sorted male pupae. Any contaminating females were excluded when calculating the male pupa recovery rate using the following equation:

$$\text{Male recovery rate (\%)} = (\text{number of collected male pupae}/\text{total number of first-instar larvae}) \times 100 \times 2 \times (1 - \text{female contamination rate}/100)$$

The triply *Wolbachia*-infected *A. albopictus* used was mass-reared in the Wolbaki facility at Guangzhou, China, according to the methods previously reported (*3*), except that the irradiation procedure was removed, and the manual sex separation was replaced with the automated sex separation. Given the different maximum feeding density and biological characteristics between *Aedes* and *Culex* and different feeding equipment and protocol between IPCL and Wolbaki, there were some adjustments in *Culex*. First-instar larvae (3000 per tray) were fed with 33.3% Wolbaki slurry diet (beef liver powder 50% and shrimp meal 50%) according to the following feeding regimen: 5 ml, day 1; 0 ml, day 2; 5 to ~8 ml, day 3; 10 to ~15 ml, day 4; 10 to ~20 ml, day 5; 10 to ~15 ml, day 6; and 10 to 12 ml, day 7. Trays were tilted 40 to 48 hours after first pupation. The contents of five trays were mixed with tap water for a total volume of three to five times the pupae and were then sorted with the automated machine. For the manual sorting, the contents of three trays were mixed and sorted together. The whole-sorting experiment was carried out two times, each time with three replicates. Three samples of approximately 100 to ~300 pupa per sex for both the automated and manual sorting methods were randomly taken and separated by sex with microscopic examination. The rest of the larvae were not included in the statistics because in operational programs, the timing of male recovery allows for getting rid of them, because male pupae complete emergence in a shorter time than larvae.

Experiment 2 was conducted on *A. aegypti* and *A. albopictus* with harmonized and increased sample sizes. *A. aegypti* (Brazil strain) first instars (18,000 per tray) were reared as described above with a slight modification of the feeding regimen, implemented as follows: 300 ml on day 1 (20 hours after egg hatching), 300 ml on day 4, 200 ml on day 5, and 300 ml on day 6. For *A. albopictus* (*Wolbachia*-transinfected strain), 8000 first instars per tray were fed with the Wolbaki liquid diet as described above (*3*). On day 7, the contents of five mass-rearing trays were combined and tilted for sorting separately, for each sorting method. After pupa estimation, five random aliquots (about 1000 in *A. aegypti* and 500 in *A. albopictus*) per sex and sorting method were selected and sexed. In *A. aegypti*, pupae were placed in separate emergence cages for a 4-day period and sexed as adults. In *A. albopictus*, male and female pupae were identified by microscopy on the basis of morphological differences. Last, contamination and pupa recovery rates were assessed for both sexes. The experiment was repeated two and three times for *A. aegypti* and for *A. albopictus*, respectively.

Evaluation of the automated sex sorter at a mass-production scale

Data on automated sex separation were collected from 29 April 2019 to 3 November 2019 for a 27-week period. Three individuals participated in the separation using 12 automated sex sorters. The average working time for each person was 5 hours.

Flight ability test

One hundred and five male pupae (*A. aegypti* and *A. albopictus*, four replicates for each sex-sorting method) were manually counted and transferred into 15-cm^3 emergence cages (BugDorm, Taichung, Taiwan) containing 10% sugar solution. After complete emergence, a male flight ability test was performed on 2- to-3-day-old adult males according to the method reported previously (*19*).

Field trial of population suppression by releasing *A. albopictus* incompatible males produced by the automated sex sorter

To verify the field effectiveness of incompatible male mosquitoes (*A. albopictus*) obtained by the automated separator, we conducted a field release trial in Guangzhou to evaluate their suppression effect on female mosquitoes and larvae from 6 July 2020 to 26 October 2020. To reduce the cost, the release ratio (incompatible male to wild-type male) was not determined by polymerase chain reaction but was calculated by the male to female ratio in the BG traps, with a normalization by the male to female ratio at the control site. First, we calculated the male-to-female ratio (r) according to the BG trap data by counting the number of male and female *A. albopictus* at the control site. Then, we counted the number of male and female *A. albopictus* at the release site and assumed that the number of males and females was n and m, respectively. The release ratio (R) was calculated as follows: $R = [n/(r \times m)] - 1$.

Statistical analysis

We used Mann-Whitney U tests to compare the differences between manual and automated sorting methods and the mosquito density between release and control sites, including the female contamination rate, the sorting efficiency and capacity, the recovery of different sexes, the proportion of egg-positive ovitraps, the proportion of eggs hatching per ovitrap, and the total number of female adults per trap per 24 hours. Student's t test was used to compare the flight ability of males sorted by different methods. All analyses were done using either GraphPad Prism version 6.00 or SPSS version 20.

Supplementary Materials

The PDF file includes:
Figs. S1 to S4

Other Supplementary Material for this manuscript includes the following:
Movies S1 and S2
MDAR Reproducibility Checklist

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Upscaling the production of sterile male mosquitoes with an automated pupa sex sorter

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Editor's summary

Several techniques have been developed to fight mosquito-borne diseases, including the release of sterile or incompatible male mosquitoes into the wild to reduce population growth. However, sorting mosquitoes by sex is a laborious process. Gong *et al.* have now developed an automated mosquito pupa sex sorter, which was tested on three mosquito species and shown to increase the production of males 17-fold when compared with a manual separation process. A field trial in Guangzhou, China showed that the process was capable of producing sufficient incompatible males without reducing their quality, which led to the suppression of wild populations of mosquitoes. — Amos Matsiko

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