ROBOTS AND SOCIETY

Field performance of sterile male mosquitoes released from an uncrewed aerial vehicle

J. Bouyer[1]*†‡, N. J. Culbert[2,3‡], A. H. Dicko[4,5], M. Gomez Pacheco[6], J. Virginio[6], M. C. Pedrosa[6], L. Garziera[6], A. T. Macedo Pinto[6], A. Klaptocz[4], J. Germann[4], T. Wallner[2,4], G. Salvador-Herranz[4,7], R. Argiles Herrero[2], H. Yamada[2], F. Balestrino[2,8], M. J. B. Vreysen[2]

Genetic control methods of mosquito vectors of malaria, dengue, yellow fever, and Zika are becoming increasingly popular due to the limitations of other techniques such as the use of insecticides. The sterile insect technique is an effective genetic control method to manage insect populations. However, it is crucial to release sterile mosquitoes by air to ensure homogeneous coverage, especially in large areas. Here, we report a fully automated adult mosquito release system operated from an uncrewed aerial vehicle or drone. Our system, developed and tested in Brazil, enabled a homogeneous dispersal of sterile male *Aedes aegypti* while maintaining their quality, leading to a homogeneous sterile-to-wild male ratio due to their aggregation in the same sites. Our results indicate that the released sterile males were able to compete with the wild males in mating with the wild females; thus, the sterile males were able to induce sterility in the native female population. The use of drones to implement the sterile insect technique will lead to improvements in areal coverage and savings in operational costs due to the requirement of fewer release sites and field staff.

INTRODUCTION

According to the World Health Organization (WHO), vector-borne diseases account for 17% of infectious diseases, leading to more than 1 million human casualties each year. This includes (in order of importance) malaria; lymphatic filariasis; and arboviruses like dengue, yellow fever, and Zika (*1*). In a growing number of countries, awareness of the toxicity of insecticides to living organisms and ecosystems is leading governments to increasingly ban these chemicals. Moreover, resistance to pyrethroids, the most commonly used group of insecticides against insects, is increasing. Therefore, WHO's global vector control response 2017–2030 urgently demands alternative mosquito control methods, particularly against *Aedes* vectors (*1*).

Innovative mosquito control methods have become available (*2*), and some genetic control methods show great promise (*3*). The sterile insect technique (SIT) is the original genetic control method and has been used with great success against insect pests of agriculture and livestock, i.e., the New World screwworm (*4*), fruit flies (*5*), moths (*6*), and tsetse flies (*7*, *8*). Very recent genetic control methods include (i) the use of symbionts like *Wolbachia* for the incompatible insect technique (IIT) that was successfully combined with the SIT for the suppression of *Aedes albopictus* (*9*) or for population transformation using virus-blocking strains (*10*–*12*), (ii) genome editing (*13*), and (iii) the use of transgenic insect strains (*10*, *14*). Gene drive is the latest developed technology (*15*, *16*) and is associated with male-determining factors (*17*), now described in both *Anopheles* (*18*) and *Aedes* (*19*) families. It is expected to become a powerful mosquito control tool, but it is not ready to be released in the field (*20*).

Aerial release approaches will be required to ensure cost-effective releases of the sterile male mosquitoes, especially when large areas need to be covered. Such release systems have been developed for SIT programs against fruit flies, moths, and tsetse flies (*21*–*24*). However, few systems exist for the efficient aerial release of mosquitoes or drone release of any insect. Here, a fully automatic release system was developed for the release of adult sterile male *Aedes* mosquitoes that can be operated from an uncrewed aerial vehicle (UAV), commonly known as a drone.

RESULTS

Mosquito release mechanism design

Mosquitoes have long fragile legs and delicate wings, which makes the design of a release system that does not cause injuries, and hence reduce their quality, very challenging. From an entomological perspective, the main challenges to address were compaction, chilling, and the development of a conveyor system to permit stacking an adequate number of mosquitoes per flight, ensuring their complete immobilization and controlling the release flow rate without causing injuries (*25*). From a mechanical engineering perspective, the release platform developed in this study included the release mechanism that consisted of an insulated storage unit, a mechanism that ejects the mosquitoes onto a release area ramp, and onboard electronics featuring sensors and cameras to control and monitor the state of the mechanism and mosquitoes (Fig. 1). A custom-made Android-based software application was also developed to operate mosquito release flights autonomously, which does make the planning and implementation of the releases more effective (Supplementary Materials).

Simulated release under laboratory conditions

Before the field trial, laboratory experiments were carried out for 1 year to develop and refine this release system and assess its potential

[1]Unité Mixte de Recherche ASTRE, CIRAD, INRA, Univ Montpellier, Montpellier, France. [2]Insect Pest Control Laboratory, Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture, A-1400 Vienna, Austria. [3]Institute of Integrative Biology and the Centre for Genomic Research, University of Liverpool, Liverpool, Merseyside, UK. [4]WeRobotics, Rue d'Italie 11, 1204 Geneva, Switzerland. [5]Statistics for Development–STATS4D, Sacre Coeur III, 1 bis, P.O 11000, Dakar, Senegal. [6]Biofábrica Moscamed Brasil, Av. C1, 992 - Quadra D 13, Lote 15, Distrito Industrial do São Francisco, Bahia, Brazil. [7]Instituto de Investigación e Innovación en Bioingeniería (I3B), Universitat Politècnica de València, Camino de Vera, s/n, 46022 Valencia, Spain. [8]Centro Agricoltura Ambiente "Giorgio Nicoli" S.r.l. (CAA), Via Sant'Agata, 835, 40014 Crevalcore BO, Italy.
*Corresponding author. Email: j.bouyer@iaea.org, bouyer@cirad.fr
†Present address: Insect Pest Control Laboratory, Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture, A-1400 Vienna, Austria.
‡These authors contributed equally to this work.

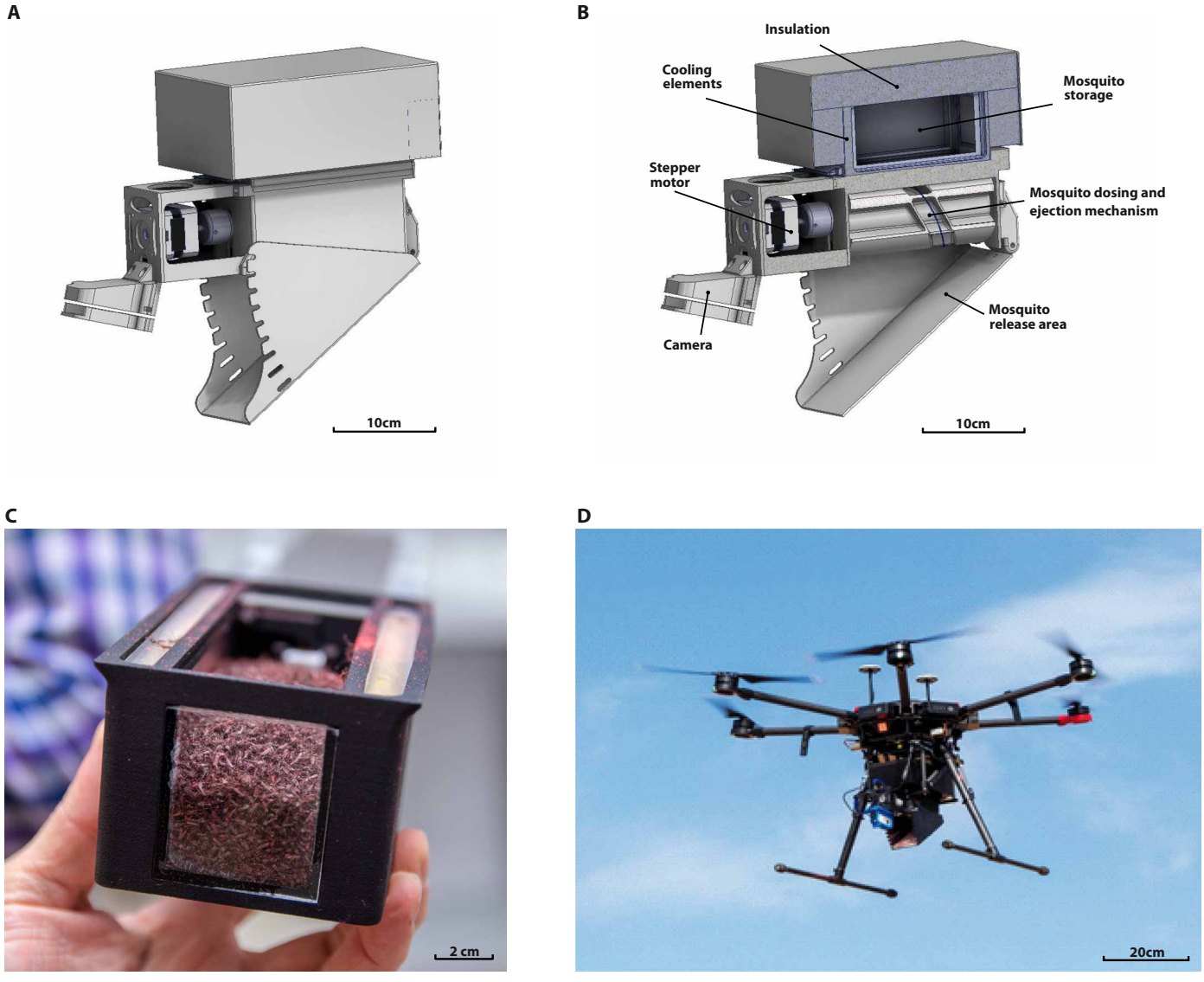

Fig. 1. The adult mosquito release system operated from an UAV. (**A**) Front right view of the release mechanism (technical drawing). (**B**) Half-section of the release mechanism (technical drawing). (**C**) Canister filled with 50,000 marked mosquitoes. (**D**) Fully assembled aerial mosquito release system attached to a DJI M600 UAV in flight.

impact on the released sterile males. We first investigated the effect of the different treatments (compaction, chilling, and release) on the quality of the released mosquitoes using a standardized flight ability test that determines the proportion of adult mosquitoes escaping from a 25-cm-tall vertical tube (Supplementary Materials) (*25*). The percentage of flyers was significantly correlated with the proportion of mosquitoes with damaged wings and legs ($r = -0.98$, $P = 0.02$; Supplementary Materials). Male *Aedes aegypti* mosquitoes proved to be very sensitive to compaction up to 1.2 g/cm^2, and therefore, a release cassette was developed with a maximal depth of 5 cm that contained no more than 50,000 *A. aegypti* males (Supplementary Materials). Moreover, at temperatures below 8°C, the mosquitoes were behaving like inert particles and their quality was reduced (*25*), whereas at 11°C, some mobility was restored. Therefore, an insulated container was developed that could hold phase-change material packs (S8, PureTemp, MN) to maintain the temperature between 8° and 10°C throughout the flight. Mosquitoes exposed to these temperatures for 1 to 4 hours became active again after 40 to 60 s when transferred to ambient temperatures. In view that *A. aegypti* males have an estimated average free fall speed of 2.5 m/s, 50 and 100 m were selected as potentially appropriate release altitudes (Supplementary Materials). Last, two conveyor systems were compared, i.e., a conveyor system commonly used to release fruit flies (*24*) and a cylinder system initially developed to release tsetse flies (Fig. 1) (*26*). The tsetse fly cylinder system, which is smaller and lighter than the fruit fly conveyer system, resulted in better quality mosquitoes (higher flight rate, $P = 0.02$; Supplementary Materials). To simulate the forces experienced by the mosquitoes when released from the drone, a wind tunnel experiment confirmed that a wind speed of 7 to 19 m/s (25 to 68 km/hour) did not reduce the quality of the males released with this system ($P > 0.09$; table S2). Last, a laboratory study was carried out to assess the impact of a simulated release process on the competitiveness of the sterile male mosquitoes. The competitiveness of irradiated sterile males that had been exposed to the final design of

the release system was assessed in laboratory cages (60 cm by 60 cm by 60 cm) and proved to be similar to that of untreated control mosquitoes, i.e., a Fried index (*27*) of 0.66 (SD 0.06) ($t = -0.036467$, df = 3.9977, $P = 0.97$; Fig. 2).

Field trial

Following these positive results of the laboratory experiments, the UAV platform was tested on March 2018 under field conditions in Carnaíba do Sertão, Juazeiro, Brazil, within the Moscamed program (Fig. 3 and movie S1). Two series of trials were carried out simultaneously using differently color-marked sterile males: (i) point releases to compare ground and drone release from a fix point and (ii) drone releases covering the entire area (see Materials and Methods for details). In the first part of the experiment, we compared the drone hovering over the ground at a fixed location that was the same location used for the ground releases. A total of 50,400 sterile irradiated males were either released from a central point on the ground or released from an UAV in stationary flight at an altitude of 50 or 100 m (two repeats, see Table 1 for details). The mosquitoes were recaptured with 35 baited Biogents' Professional Mosquito Traps (BG-Sentinel, Biogents, Germany) in the 20-ha trial area (0.2 km^2). More ground-released mosquitoes (1.60%; SD, 0.42%) were recaptured than UAV-released mosquitoes ($P < 10^{-3}$), and recapture rate of mosquitoes released from an altitude of 50 m (0.27%; SD, 0.01%) was significantly better than those released from an altitude of 100 m (0.07%; SD, 0.02%; $P < 10^{-3}$). Survival of the three groups was similar ($P > 0.46$, stats; Supplementary Materials), but their average dispersal increased with release altitude ($P = 0.011$), i.e., from 83 m (SD, 21 m) to 133 m (SD, 22 m) and 153 m (SD, 7 m) for mosquitoes released from the ground, from 50 to 100 m, respectively.

In the second part of the experiment, a total of 165,400 sterile males were released along release lines 80 m apart, in 20 ha monitored with 37 adult traps. To determine whether the males were mating wild females, we deployed 37 ovitraps to monitor egg fertility, which was compared with an untreated control area. To implement this trial, we set the flight speed of the drone at 10 m/s and the cylinder speed at 2 revolutions per minute (rpm), which allowed a release rate of ~5000 sterile males per hectare. About 12 min was needed to cover 20 ha. During the flight, the temperature exceeded 10°C but remained below 18°C, which did not cause a strong reduction of the quality given that the flight duration remained below 13 min (Fig. 4). Marked mosquitoes were recaptured in 24 of 35 active monitoring traps (69%; Fig. 1), which indicated a uniform release pattern. The recapture rate of 0.32% (SD 0.09%) in this study was better than that of RIDL (Release of Insects carrying a Dominant Lethal) *A. aegypti* males in Brazil (0.04%) (*14*) or that of *A. albopictus* males used in an IIT-SIT trial in China (0.09%; SD, 0.07%), both released from the ground (*9*). Catches of sterile males were homogeneous in space (conditional Monte Carlo test of complete spatial randomness using quadrat counts, $\chi^2 = 8.25$, $P = 0.7792$ for 3 by 3 grid of tiles; $\chi^2 = 21.83$, $P = 0.8711$ for 5 by 5 grid of tiles; and $\chi^2 = 84.33$, $P = 0.2997$; fig. S8). Catches of sterile males released along lines over the whole study area were also strongly correlated with those of wild males (cor = 0.98, $t = 25.73$, df = 33, $P < 2.2 \times 10^{-16}$) and, to a lesser extent, to those of wild females (cor = 0.38, $t = 2.3343$, df = 33, $P = 0.02582$). These data indicate that the sterile males aggregated in the same sites as the wild mosquitoes and/or that they responded in a similar way as wild males to the cues conditioning trap attractiveness (Fig. 3), which is a prerequisite for success in a SIT program (*28*). A maximum ratio of 0.8 sterile to 1 wild male was obtained in the release area (Fig. 5). Following the release of sterile males, the proportion of unviable eggs collected in the release area increased by more than 50% as compared with that of a neighboring control area where no mosquitoes were released ($P < 10^{-3}$). This indicates that the released males were able to compete with wild males, mate with wild females, and transfer their sterile sperm inducing sterility in the native female population. A Fried competitiveness index of 0.26 (95% confidence interval, 0.05 to 0.72) was estimated. However, this increase was unexpectedly high, and confounding factors might have inflated it. During a longitudinal monitoring effort conducted from 27 March 2017 to 14 May 2018 in this area, a peak of sterility of almost 8% was observed in November 2017 in the absence of sterile males (fig. S8). It may be possible that, in this dry area, with a low density of mosquitoes, the probability of encounter between wild males and females is sometime reduced, thus artificially increasing the proportion of virgin females in the population.

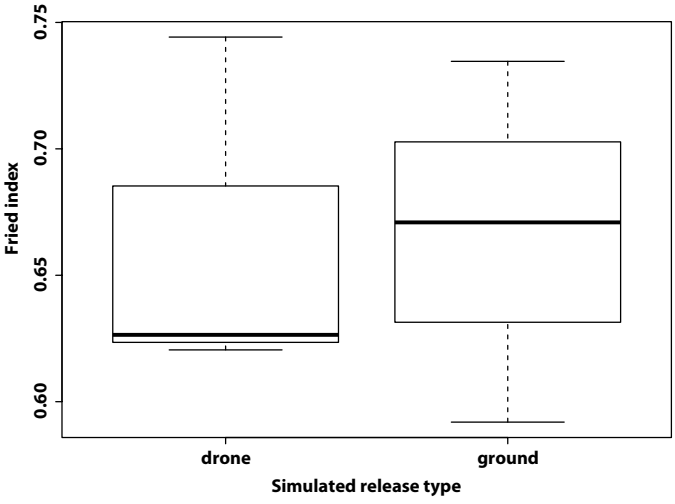

Fig. 2. Fried competitiveness index of sterile male *A. aegypti*. Sterile males were released using our prototype aerial release system or by ground in large cages at the laboratory.

DISCUSSION

Before the release experiments, the Moscamed team was engaged in several public relations activities in the release area, which resulted in an overall good acceptance of the drone releases by the general public (see the Supplementary Materials for details). The data of this trial indicate that releasing sterile *Aedes* mosquitoes from an UAV platform is feasible with a uniform dispersal of sterile males in the field and a homogeneous sterile-to-wild male ratio as a result.

The induced sterility observed in our trial was unexpectedly high considering the number of sterile males released (~5000 per hectare per week) and the low sterile-to-wild ratio (<1), indicating the high competitiveness (~0.3) of the 35-Gy irradiated sterile males. This compared favorably with an index of <0.06 that was observed for ground-released RIDL *A. aegypti* males (*14*). It is generally assumed that the competitiveness of irradiated sterile male mosquitoes is reduced because irradiation causes somatic damage. Obviously, excessive irradiation will impair competitiveness of any insect, but in general, it is possible to obtain a trade-off between a dose obtaining >99% sterility of the males without substantially affecting their biological

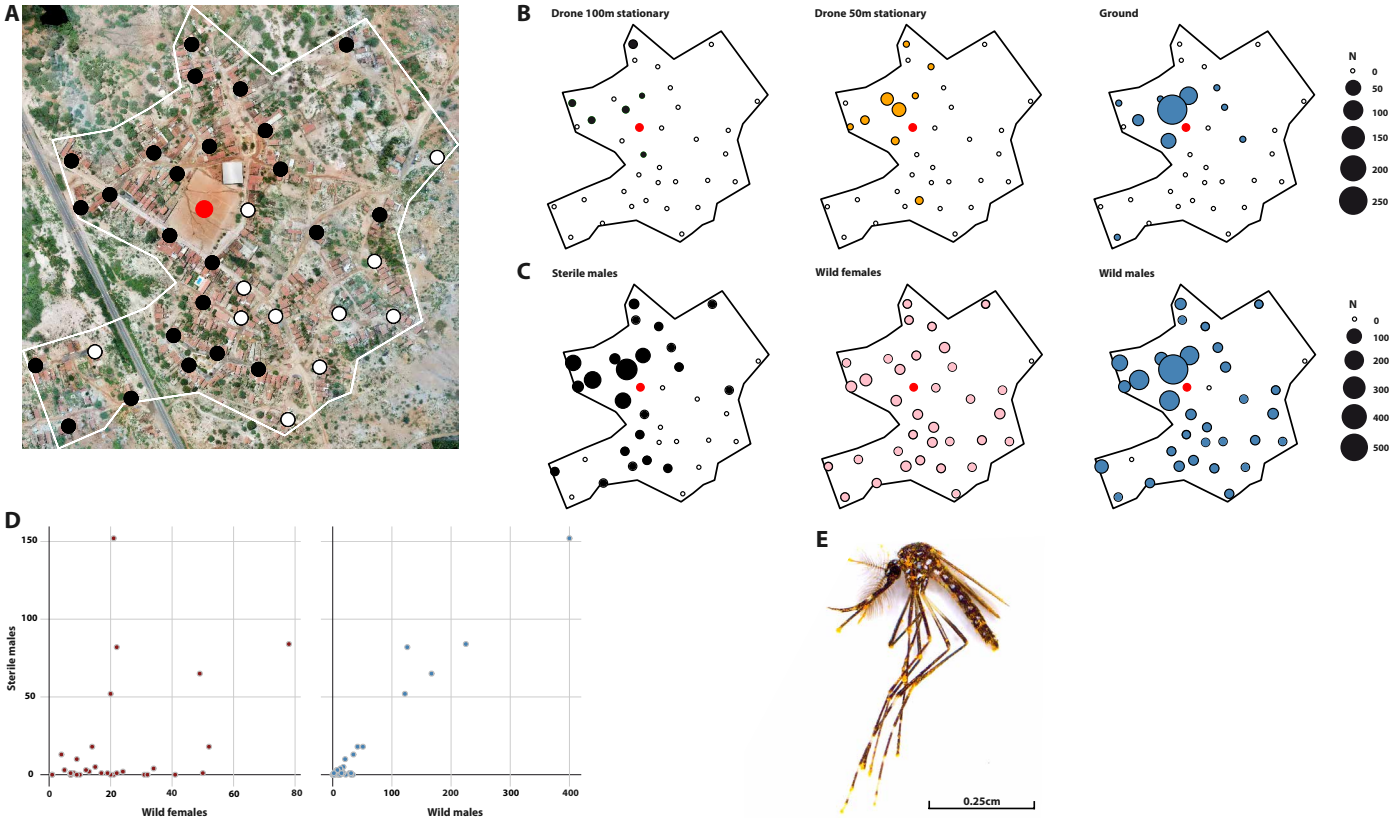

Fig. 3. Results of an MRR experiment in Carnaíba do Sertão, Brazil. (**A**) Map of the monitoring system using BG monitoring TM traps (Biogents, Germany) deployed from 20 March to 11 April 2018. Black points represent traps with catches of sterile *A. aegypti* during the line releases, whereas white points represent negative traps. The red point represents the location of ground releases and point releases in the middle of a football field. (**B**) Trap catches of sterile males after point releases by drone at 100 and 50 m and on the ground. Each data point represents the total catch of one trap during the experimental period. (**C**) Trap catches of sterile males after line releases by drone at 100 m, wild females and wild males. Each data point represents the total catch of one trap during the experimental period. (**D**) Relationship between sterile male catches and those of wild females and males. (**E**) Photography of a marked sterile male *A. aegypti*.

Table 1. Main characteristics of the sterile male *A. aegypti* released in Carnaíba do Sertão, Brazil, in 2018. Each row represents a series released separately with a different color: B, blue; O, orange; Y, yellow; G, green; P, pink. Colors can be combined (e.g., BY, blue + yellow). The numbers in parentheses in the column labeled "Survival rate" are the r^2 of the survival models fitted to the data, i.e., the percentage of inertia explained. NA, not applicable.

Release pattern	Date of release	Color	Number released	Recapture rate (%)	Number recaptured	Repeat	Survival rate	Median distance
Ground	21 March	B	9,600	1.30	125	1	0.20 (0.96)	97
Ground	24 March	BY	7,200	1.90	137	2	0.63 (0.59)	68
Drone_50m_stationary	21 March	O	9,600	0.27	26	1	NA	117
Drone_50m_stationary	24 March	OY	7,200	0.28	20	2	0.82 (0.48)	148
Drone_100m_stationary	21 March	G	9,600	0.05	5	1	NA	158
Drone_100m_stationary	24 March	GY	7,200	0.08	6	2	NA	148
Drone_100m_path	21 March	P	50,700	0.27	138	1	0.45 (0.80)	NA
Drone_100m_path	24 March	PY	49,000	0.42	207	2	0.64 (0.74)	NA
Drone_100m_path	27 March	Y	65,700	0.27	175	3	0.70 (0.39)	NA

quality (*29*, *30*). For example, a competitiveness of 0.7 to 1.0 was observed for irradiated male *A. albopictus* under semi-field conditions in Italy (*31*) and of 0.4 to 0.8 in Reunion island (*32*). Moreover, flight ability of *A. albopictus*, *A. Aegypti*, and *Anopheles arabiensis* was not impaired with radiation doses of up to 40, 90, and 50 Gy, respectively (*25*, *33*). Last, triple *Wolbachia*-infected male *A. albopictus* irradiated with 40 Gy showed a competitiveness close to 1 in walk-in field cage studies and of 0.5 to 0.7 in the field. This resulted in successful suppression of two isolated target populations in China (*9*).

In the present study, the high competitiveness was also possible because good irradiation practices were adhered to by irradiating low amounts of pupae under normoxic conditions. This is very relevant

because irradiating large numbers of pupae can lead to anoxia, which increases their radiation resistance, thus making it necessary to increase the dose, which consequently will increase the somatic damage, not to forget that anoxia is in itself damaging (*34*). It needs to be emphasized that most of the reduction in quality of the irradiated males needed for the SIT is not related to irradiation per se but mostly to the mass-rearing, handling, and release processes of the sterile males (*29*). This study shows that, when these components are mastered, competitiveness of the released male mosquitoes will be adequate to ensure success in the field.

Successful release of sterile males from a drone is an important outcome, especially in view of the low dispersal capacity of *Aedes* mosquitoes. To obtain the same coverage using ground releases would have required a release site every 80 m, taking into account the observed median dispersal distance. Releases from the ground in the required 63 release sites would have necessitated two field staff, a vehicle, and 2 hours of work. The UAV release system used in this trial could cover much larger areas by replacing the battery and release cassette more frequently (every 20 to 25 min, given the autonomy of the drone at the speed of 10 m/s used in this study) or by using several UAVs that would fly in an echelon formation. The release system might also be mounted on a motorcycle or a bicycle for ground releases in an urban setting. Further improvements to the system are currently under development; i.e., while ensuring the same autonomy, the mosquito load may be doubled (100,000), the total weight would remain below 2 kg, and a parachute could be added to the system to operate safely in urban areas (*35*). In addition, improvements will be needed with respect to insulation to ensure a stable temperature below 10°C throughout the flight (fig. S10).

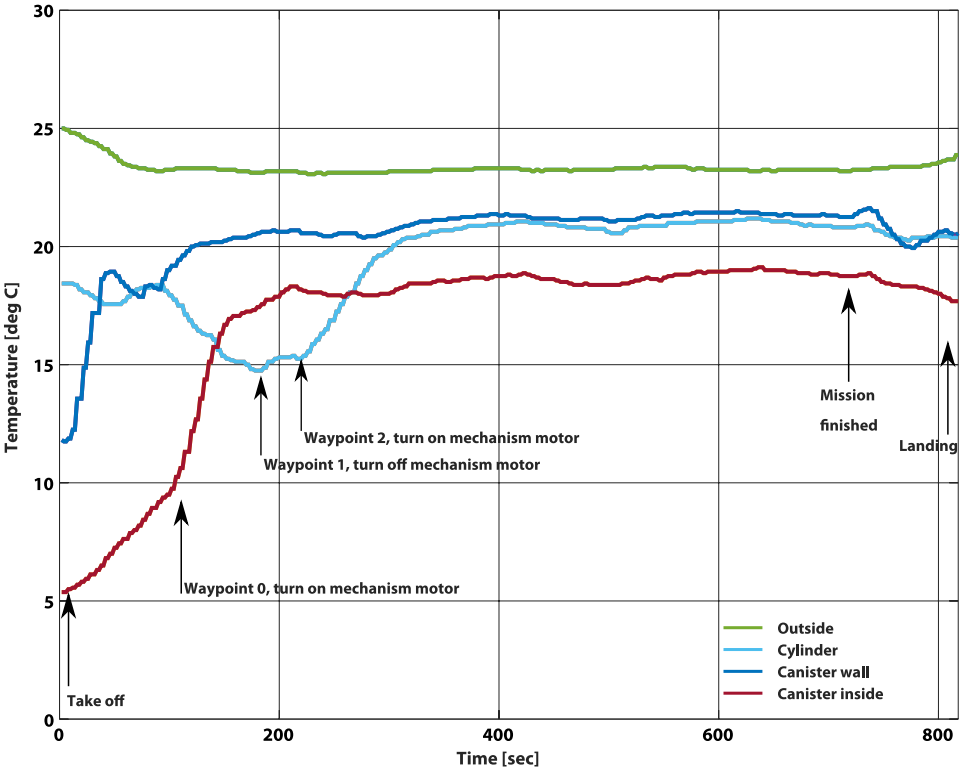

Fig. 4. Dynamics of the temperature inside the release system during a flight. The flight altitude was 100 m and corresponds to the line release of 21 March 2018 described in Table 1.

The use of a UAV-based system for the aerial release of mosquitoes will substantially reduce the operational release costs. For example, in an IIT-SIT trial against *A. albopictus* in China, the cost of releasing from the ground was estimated at 20 USD per hectare per week, which could be reduced to an estimated 1 USD per hectare per week using a drone (*9*). Irrespective of the size of the target areas, UAVs might be a good substitute for ground releases to mitigate some of the limitations of ground releases, i.e., no uniform distribution of the sterile males due to the point releases and accessibility of some sites.

In the future, it might even be envisioned that chilled adult mosquitoes are irradiated when already packed into the release cassettes that could then be shipped using courier services from production

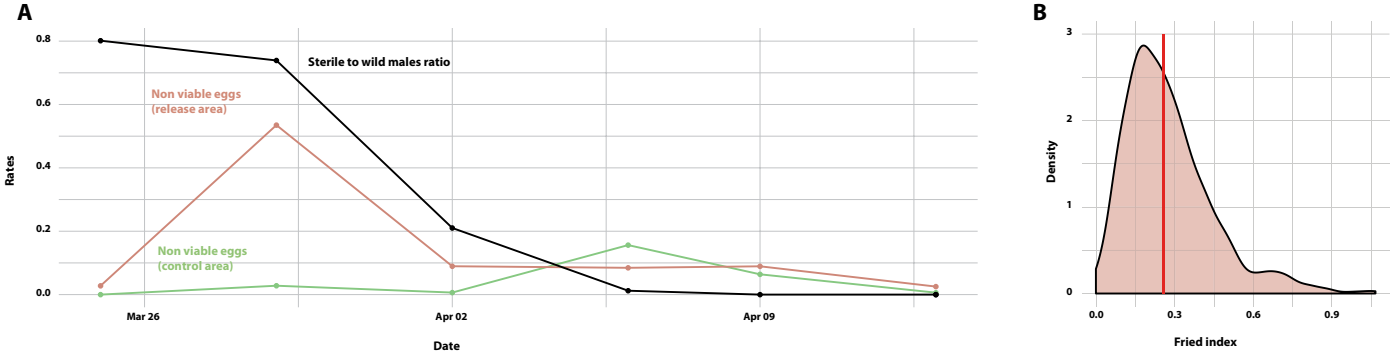

Fig. 5. Induced sterility and sexual competitiveness of sterile male *A. aegypti* released from an UAV-operated release system. (**A**) Temporal dynamics of the sterile-to-wild male ratio and rate of viable eggs in the release and nontreated areas. (**B**) Estimation of the Fried index from 1000 bootstraps in the distributions of sterile to wild male ratios in traps and viable egg rates in ovitraps in the release and nontreated areas (see the Supplementary Materials for details). The density corresponds to the percentage of the simulations for a given value.

to release sites within 48 hours (*36*). This would make the technology even more cost-effective, because it would abolish the need for costly emergence and release centers in the target areas. The International Atomic Energy Agency (IAEA) and the WHO recently published a guidance framework to assess the feasibility of using the SIT as a mosquito control tool and thus reducing or eliminating *Aedes*-borne diseases (*30*). This guidance covers all processes for decision support, including risk assessment, regulatory and technical aspects (e.g., insect mass rearing), entomological and epidemiological indicators, as well as community involvement, cost-effectiveness, and program monitoring and evaluation. These recommendations will be applied in the 34 currently implemented SIT pilot projects against *Aedes* species worldwide to maximize the chances of success (*37*).

MATERIALS AND METHODS

Study area and the Moscamed program

Moscamed Brasil is a nonprofit organization, based in Juazeiro city (Bahia, Brazil), and is operating since 2005. This facility has been working on the implementation of a pilot trial for the control of *A. aegypti* since 2011 in different rural and semi-urban areas in Bahia.

Carnaíba do Sertão village was selected as a target site to perform a new SIT pilot project. This project started in 2017, and it has support from the national and local authorities, including the local vector control authorities and local community leaders, who participated in previous project activities. Carnaíba is located in Juazeiro (Bahia: 9°35′37.48″S, 40°25′7.17″W), and its population is around 3100 residents and covers an area of ~51 ha. It is a typical rural area surrounded by native vegetation (Bioma Caatinga) and crops, which provides ecological isolation because migration of *A. aegypti* is reduced. The mean annual rainfall is around 400 mm, with a rainy season occurring between November and April. Sanitation and water supply systems are precarious with several open drains, cisterns, tanks, and other types of reservoirs of the community available as mosquito breeding sites. The essential criteria used for the selection of this area for the present study were a manageable size, presence of a vector population, adequate topographic surroundings, and consent from the local community and authorities. The vector surveillance activities using ovitraps have been ongoing in this area since 2017, which facilitated the interactions with the local community.

Community engagement

Before the trial, the Moscamed team informed the Bahia Municipality Health Public Secretary about the objective of these aerial releases, its support, and achievements. Two meetings were carried out with the Health Surveillance Superintendence to share the goals of the trial with supervisors and discuss entail points to access people's knowledge about the use of SIT for mosquito control. They contributed with crucial recommendations for the best approach to obtain local community agreement to perform the study. Besides the authorities, the vector control agents and local community leaders were trained in communication and stakeholder engagement so that they would be able to support and disseminate the trial objectives among the local community. Their role was critical to set up appropriate locations for monitoring traps used in this study. Most of the community engagement activities took place locally to clarify as much as possible the trial steps, such as visiting households for monitoring, and included the distribution of leaflets. In addition, a television interview by the local press took place, with Moscamed representative and researchers, to provide information about the study. All these activities allowed a high acceptance of the use of drone releases by the community.

Rearing of the laboratory mosquito strain in Seibersdorf

The strain of *A. aegypti* used in all laboratory experiments was sourced from Juazeiro, Brazil and transferred by Biofábrica Moscamed, Brazil to the Food and Agricultural Organization/IAEA Insect Pest Control Laboratory (IPCL) in Seibersdorf, Austria. The strain was maintained since 2010 without further colony regeneration. Adults are maintained in a climate-controlled insectary [temperature (*T*), 27 ± 1°C; relative humidity (RH), 70 ± 10%; photoperiod light:dark (L:D), 12:12; with two 1-hour twilight periods simulating dawn and dusk] as described in (*38*). Standardized guidelines developed at the IPCL were used to produce and hatch the eggs for all experiments (*39*). Larvae were reared in plastic trays (40 cm by 29 cm by 8 cm) containing 1 to 1.5 liters of deionized water at a density of roughly 1500 to 2000 first instar (L1) per tray and were fed daily with IAEA diet developed and described in (*38*, *40*, *41*). Pupae were sexed mechanically using a Fay-Morlan (*42*) glass plate separator as redesigned by Focks [John W. Hock Co., Gainesville, FL, USA (*43*)] before further examination under a stereomicroscope for increased accuracy. Adults were maintained in plastic BugDorm cages (30 cm by 30 cm by 30 cm, Taiwan), unless otherwise stated with continuous access to a 10% sucrose solution. All experiments were carried out on 3- to 4-day-old adults to reflect the likely age of release, unless otherwise stated.

Colony rearing of the mosquito strain used for the field trial

The strain of *A. aegypti* (MBR-001) used in the present study was obtained from field material (eggs) collected in the Carnaíba neighborhood (09°35′40″S, 40°24′58″W), Juazeiro city, Bahia State, northeast Brazil. Sterile males were reared in a climate-controlled insectary at the mass-rearing Unit of Moscamed Brasil (T = 28 ± 1°C; RH, 80 ± 10%; photoperiod, L10:D14h). Larvae were reared in plastic trays (51 cm by 30.3 cm by 9.7 cm) at a density of 1 larva/ml in 3 liters of mineral water. Larvae were fed daily with a solution of the IAEA two liquid diet (4%, w/v) until pupation (*40*). Pupal separation was carried out by size (female pupae > male pupae > larvae) using a glass plate separator (Moscamed Brasil model) (*41*–*43*), as described (*44*). Pupae were kept in trays containing mineral water in a climate-controlled insectary until irradiation (T = 27 ± 1°C; RH, 70 ± 10%; photoperiod, L10:D14h).

Irradiation protocols

At the IPCL in Seibersdorf, 36- to 48-hour-old *A. aegypti* pupae were irradiated with 90 ± 5 Gy in 60Co Gamma Cell 220, with all water removed. The actual dose received was measured with a dosimetry system using Gafchromic MD film (International Specialty Products, NJ, USA) (*44*).

In Juazeiro, male pupae were sterilized at the Moscamed Brasil using an RS 2400 x-ray machine (RadSource, Suwanee, GA, USA) with a 125-kV voltage, an 18-mA current, and a dose-energy ratio of 0.0207 Gy kW^{-1} s^{-1}. Male pupae (30 to 36 hours old) were irradiated with a dose of 35 Gy, resulting in >99% sterility. The pupae were placed in 12-well cell culture plates (diameter, 2.14 cm per well; area, 3.66 cm^2 per well) containing a small amount of water (1.5 ml and ~100 pupae in each well). The plates were placed in a horizontal position inside of polyfoam prototype (diameter, 16.7 cm; length, 11.7 cm) developed in the workshop of Moscamed Brasil to position

pupae in the most central part of the irradiation cylinder (diameter, 17.5 cm; length, 14 cm) to minimize dose variation. After irradiation, the pupae were transferred to laboratory cages (30 cm by 30 cm by 30 cm) and kept in a climate-controlled insectary until adult emergence (T = 27 ± 1°C; RH, 70 ± 10%; photoperiod, L10:D14h). Sterile males were provided with 10% sucrose solution ad libitum.

Competitiveness of the sterile male mosquitoes

It was critical that we investigated the impact of the release system on the competitiveness of sterile male *A. aegypti*. Therefore, an actual aerial and ground field release was simulated before calculating the competitiveness index. *A. aegypti* were reared as described above, separated into batches of males and females, and further screened under a stereomicroscope to ensure the accurate sex separation of 30,000 males and 1200 females. Twenty-nine thousand 40- ± 4-hour-old male pupae were irradiated with 95 ± 5 Gy, as previously described. The remaining 1000 male pupae were not irradiated, served as fertile males, and were caged in batches of 100. Female pupae were also caged in groups of 100. For both females and fertile males, two batches of 100 served as backup adults. Sterile male pupae were caged in batches of about 3000 (volumetric estimation). On day 3 post-emergence, the number of females and fertile males was adjusted back to batches of 100 from the backup cages to compensate for failed emergence and mortality. All cages of sterile males, in addition to the three cages of 300 sterile males (ground release), were transferred to a cold room (4 ± 1°C) for a period of 10 min until immobilization occurred. All cages were emptied into a plastic larval rearing tray (30 cm by 40 cm by 7 cm) and carefully transferred to the storage unit of the aerial release system. The storage unit is designed to hold 50,000 mosquitoes and thus was only at half capacity, because in total only 900 sterile males were used for this experiment. The rearing tray was placed underneath the ejection mechanism to collect the sterile males after they passed through the aerial release system, which was set to operate at the speed chosen for the actual aerial releases in Brazil (three repetitions per minute). Once all sterile males had passed through the release system, three batches of 300 were counted out and transferred to a small plastic container (100 ml) and closed. The three cages of ground released sterile males were also transferred to such containers. All containers were returned to the laboratory to be transferred to allocated large BugDorm cages (60 cm by 60 cm by 60 cm) as follows: two sterile controls (100 sterile males), two fertile controls (100 fertile males), three ground release cages with sterile males that did not pass via the release mechanism (100 fertile males and 300 sterile males), and three aerial release cages, with males that did pass through the release mechanism (100 fertile males and 300 sterile males). Once all males had been assigned to their cages, 100 females were added to each of the 10 cages. A period of 72 hours was given for mating to occur, after which females were recollected from each of the 10 cages and transferred to 10 new BugDorm cages (30 cm by 30 cm by 30 cm). Blood meals were offered daily for the next 2 days, and an egg cup was placed in each BugDorm cage of females. After 72 hours, the egg papers were collected and dried for a period of 2 weeks to allow the eggs to mature. The eggs on each paper were then hatched, and after 24 to 48 hours, the number of larvae in each tray was counted. In addition, the egg paper was viewed under a stereomicroscope, and the number of hatched and unhatched eggs was counted to calculate the hatch rate.

The SIT relies on the release of mass-produced male flies that are sterilized by ionizing irradiation. Consecutively, wild female flies produce no offspring after mating with sterile males. This is due to insemination with sperm that contains numerous dominant lethal mutations that will cause embryonic arrest. A good competitiveness of the released sterile males is crucial to warrant the success of this technique (*45*, *46*). The evaluation of this competitiveness was based on the assessment of the impact of sterile males on female fertility. Fried (*27*) defined a competitiveness index, called Fried's index, which can be calculated with the following formula

$$F = \frac{\frac{\mathrm{Ha} - \mathrm{Ee}}{\mathrm{Ee}}}{R}$$

where Ha is the natural fertility of wild females and Ee is the observed fertility rate under a given ratio of sterile over wild males, R. This formula can be applied when the residual fertility of males can be neglected, which was the case for a 90-Gy dose.

Simulated release under laboratory conditions

Before the mark-release-recapture (MRR) study in Brazil, we carried out one last laboratory test with the aim to simulate a release with all predefined parameters. Cages with about 25,000 sterile male *A. aegypti* were immobilized at 4°C in a cold room for 10 min before being transferred to the storage container, with one cage left aside to serve as controls. The container was fully surrounded by phase change material (PCM) to keep the temperature below 10°C during this experiment. The storage container was connected to the release mechanism and placed inside a BugDorm cage. A climate chamber was preprogrammed to reflect likely environmental conditions in the field (35°C and 80% RH). The release mechanism was connected to the software, which controlled the release when connected to the drone during an actual aerial release. The BugDorm cage was placed inside the climate chamber, and the door to the release mechanism was removed. The BugDorm cage was gently shaken to simulate the drone commencing flight. Because the storage container was only at half capacity, the speed of release was set at 1 rpm, taking 15 min for all mosquitoes to be ejected from the storage container (similar to the time it will take at 2 rpm to release a full container of sterile males). Flight ability tests were carried out with two samples of about 100 mosquitoes from the BugDorm cage and with two control samples.

Marking protocol

Sterile male mosquitoes were dusted with pigments from the DayGlo series in a 100-ml cylindrical container with the equivalent of 0.001 g or 1 mg per 100 adult males (*47*). For the MRR, we marked in batches of 2400 requiring a liter container and 24 mg of dust. To ensure that dust adhered to the walls of the dusting container, we rubbed the inside surfaces with sandpaper to create a rough surface. The dust for each container was weighed on an analytical balance and then transferred to the container and closed (dust colors and combinations can be found in table S2). The container was shaken vigorously to coat the inner surfaces evenly. All containers were taken to a cold room (4°C) and left to acclimate. Cages of 2400 adult *A. aegypti* were then transferred to the cold room for immobilization for 20 min. Each cage was then emptied into a predusted container, and the lid was closed. The container was then rotated for 30 s (equating to about 25 full rotations) to coat the sterile males uniformly. Dusting took place after 6:00 p.m., which was around 12 hours before each release the following day. Sterile males were left immobilized in the dust containers overnight with the cold room temperature raised to

8°C. The following morning, dusted mosquitoes were transferred to storage containers according to their dust color and packed into a cool box for transport to the field site.

Mosquito release mechanism design

We designed a release mechanism including mechanics, electronics, and software. The mechanism mounts on a drone and enables aerial release of mosquitoes. The main parts of the release mechanism are (i) a storage unit consisting of a canister that keeps mosquitoes at low temperatures surrounded by insulation, (ii) an ejection mechanism featuring a rotating cylinder that brings mosquitoes from the storing canister to the outside, (iii) a release area where mosquitoes fall onto and then slowly ejected outside, and (iv) onboard electronics featuring sensors and cameras to control and monitor the state of the mechanism and mosquitoes. A comparison of the final prototype to other systems is presented in the Supplementary Materials.

The storage unit or holding canister was designed to contain 50,000 mosquitoes. To keep the insects at the target temperatures, we put PCMs with a target temperature of 4°C in the double-sided canister walls. The canister was placed in an insulation box made of styrofoam to minimize heat exchange. The whole storage unit featuring the canister and insulation box could then be loaded into the ejection unit. This enabled us to load the release mechanism multiple times in the field without the need to remove any parts from the drone.

The ejection mechanism consists of a rotating cylinder connected to a stepper motor. This mechanism was developed for other fragile insects within the European Research Council REVOLINC project and patented under reference PCT/EP2017/059832. The cylinder has six discrete holes that each can take up around 800 mosquitoes. Hence, a full cylinder turn should release around 5000 mosquitoes. The stepper motor controls the rotation of the cylinder with high accuracy and high torque. The motor can be set to various speeds. We found that values between 1 and 3 rpm are optimal, leading to release rates of 5000 to 15,000 mosquitoes per minute. The structure around the cylinder is built to minimize airflow from the outside to the inside of the canister. In addition, the connection between cylinder and structure is designed in such a way that it is easy to remove the cylinder for cleaning.

The release area is simply an inclined surface where mosquitoes fall after transportation through the cylinder. While the cylinder ejects discrete amounts of mosquitoes, the airflow through the release area moves the mosquitoes more gently into the surrounding air, making the release more continuous. In addition, the white background on the inclined surface (and a camera pointing at it) allows the user to see and monitor the release using a real-time video stream.

The onboard electronics control is running on Raspberry Pi 3 (RPi; low-cost minicomputer), interfacing the drone (and ground station) with the release mechanism. A liquid crystal display (LCD) screen is mounted on the drone and gives visual feedback of the onboard control when in the field. The stepper motor is controlled using an STM32 microcontroller and a motor controller shield that receives motor commands from the RPi.

To monitor the mechanism during flight, we embedded several sensors into the mechanism. Four temperature sensors are mounted at locations outside the mechanism, at the cylinder, at the canister wall, and inside the canister. Two humidity sensors measure outside humidity and humidity in the canister. Furthermore, we mounted two cameras to monitor and livestream the release area as well as the canister load and drone flight. The cameras give direct visual feedback to the user about the release of the mosquitoes.

Drone integration

The whole mechanism is embedded on a DJI M600 Pro hexacopter drone using a custom-made holding structure that allows for simple mounting and unmounting. The M600 Pro is a professional six-rotor drone made for industrial applications that comes with a range of Dà-Jiāng Innovations technologies, including a robust flight controller and a strong transmission system (up to 5-km long-range transmission). It enables a flight time of 30 to 35 min when equipped with a payload of 1 to 2 kg. In addition, it features a dust-proof propulsion system with actively cooled motors, making it reliable and robust during extended missions. The M600 Pro can be extended with third-party hardware components and is fully compatible with the DJI Onboard SDK and Mobile SDK to build software adapted for our own purpose.

Ground station software

To implement mosquito-release missions autonomously, we developed a custom Android-based app that allowed for efficient planning and running of such missions. The main features of this ground-station app were planning of flight route, speed, and altitude; setting release points and rates; uploading a mission to drone; running a mission autonomously; and monitoring drone state, mechanism state, sensor values, and camera livestream. Missions could be saved and loaded for repeating the exact same missions. In addition, KML files featuring GPS positions could be imported, allowing to plan the flight route using standard geographic information system (GIS) tools.

System calibration

To calibrate our system for a target mission, we mainly needed to set a flight route, the release rate of the mechanism, and the flight altitude. The flight route is best set as a regular polygon pattern above the target area. The distance between release lines (distance between release lines or swaths) is mainly related to the dispersal of the mosquitoes. Assuming a dispersion of around 50 m a priori, we chose a swath of 80 m.

The release rate per area depends on the turning speed of the cylinder and the flight speed. Using the formula below, we could derive a cylinder speed and flight speed for a target release rate for a given flight route/line

$$\text{Release rate per flight line (Mosquito/m)} = 5000 \times \frac{\text{Cylinder speed (rpm)}}{\text{Flight speed (m/s)}}$$

MRR protocol

Our final study aimed to estimate the dispersal, mortality, and mating capacity of sterile male *A. aegypti* mosquitoes through MRR experiments after being released either from the ground or by air in a pilot site in Brazil. The MRR experiments were carried out in a pilot site situated in Carnaíba do Sertão, Juazeiro, Brazil. A pilot site of 20 ha was mapped, with 35 trap locations (Fig. 3). The average daytime temperature in this area was 32°C with a monthly precipitation of 101 mm (based on March averages). The MRR study involved releasing sterile males irradiated with 35 Gy by x-ray (see the "Irradiation protocols" section for detailed protocol) in an open-field setting. Three releases were conducted within a 7-day period (Table 1). Aerial releases involved our prototype release mechanism attached to a DJi Matrice 600 Pro drone (Fig. 1). Aerial releases occurred in two

ways. First, sterile males were released in the center of the pilot site at altitudes of 50 and 100 m with the drone hovering in a stationary position (Fig. 3). Ground releases entailed adults being released from a container in the same release site and were conducted as controls. Second, sterile males were released along selected paths at an altitude of 100 m with release lines spaced 80 m apart over all the area. Sterile males were marked according to their release type and release day (for detailed marking protocol, see marking method above).

Before the day of the first release (20 March), 35 baited BG-Sentinel traps were deployed in the MRR pilot site, being a rectangular area of 20 ha (Fig. 3), at a density of 1.75 traps per hectare. In each of the trapping stations, one ovitrap was set in the vicinity of the BG trap (<50 m). Five ovitraps were also deployed in a neighboring control area (0.9 km from the release area) to measure the natural fertility of *A. aegypti* during the same period. In the early morning of 21 March 2018, sterile males were released either by air or by ground as described above. The following day (22 March) beginning early afternoon (12:00 p.m. to 14:00 p.m.), traps were inspected, and the collected samples were brought to the laboratory. All mosquitoes caught were given an identification code referring to the relevant station to calculate dispersal capacity. Collected adults were immediately placed in an insulated storage container. In the laboratory, all samples were transferred to a freezer (−20°). The following day (23 March) and after each collection day thereafter, field collected samples were analyzed and classified and data were stored. Samples were screened for color under an ultraviolet light stereomicroscope. Collections were made by two teams of four people, with each team responsible for monitoring 17 or 18 traps. Traps were monitored daily for a period of 14 days after each release (thus, until 10 April for the third and final release). Eggs collected were dried for 7 days and then hatched. Nonhatched eggs were bleached to check for the presence of an embryo. Release and recapture data were georeferenced using a GPS device. All coordinates were entered into a GIS to calculate the distances between release and each recapture site.

The release area was also very close (1.3 km) from a part of Carnaíba that has been monitored weekly with ovitraps since March 2017, i.e., 1 year before the release trial (fig. S8). The hatch rate of the eggs was thus estimated with 113 ovitraps from 27 March 2017 to 20 November 2017, then 60 ovitraps until 19 March 2018, and 45 ovitraps until 14 May 2018 (fig. S8).

Data analysis

Recapture rates were compared using proportion comparison *z* test, and differences between release patterns were tested using pairwise proportion test between each mechanism. We used a Kruskall-Wallis rank test to compare the overall mortality and dispersal data, and we then used some pairwise tests to compare each release pattern correcting the *P* value to account for multiple comparisons.

Binomial linear mixed-effect models were used to analyze the impact of the various treatments on escape rates from the flight test device (response variables). The treatment regimens were then used as fixed effects, and the repetitions were used as random effects. The significance of fixed effects was tested using the likelihood ratio test (*48*, *49*).

To obtain a confidence interval for the estimate of the Fried index, we used a nonparametric bootstrap approach (*50*). The data on fertility and ratio of wild male over wild one were resampled without replacement, and for each set of resampled data, we computed the Fried index (1000 simulations). Assuming a symmetric distribution, we used the basic percentile method to get a 95% confidence interval.

Tests of complete spatial randomness based on Monte Carlo simulations were carried out on positive traps. Pearson χ^2 tests were based on quadrat counts for sets of 3-by-3, 5-by-5, and 10-by-10 quadrants (fig. S9).

SUPPLEMENTARY MATERIALS

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Materials and Methods

Results

Fig. S1. Flight ability results of male *A. aegypti* following 2 hours of immobilization at 4°C under various levels of compaction.

Fig. S2. The average time taken (seconds) for 75% of adult male *A. aegypti* to regain flight ability following immobilization at 6°, 8°, and 10°C for 1 to 4 hours.

Fig. S3. Flight ability results of male *A. aegypti* after passing through two prototype release mechanisms versus a control sample.

Fig. S4. Flight ability of male *A. aegypti* after passing through the cylinder release mechanism at different speeds (1 or 3 rpm).

Fig. S5. Flight ability of male *A. aegypti* after passing through the cylinder release mechanism depending on their position in the canister.

Fig. S6. Wind speed test chamber.

Fig. S7. Differentiation of sterile males from wild flies using fluorescent dust.

Fig. S8. Temporal dynamics of the fertility rate measured with ovitraps in a control site close to the release area from 27 March 2017 to 14 May 2018.

Fig. S9. Number of positive traps with at least one sterile male captured in quadrats of 3*3, 5*5, and 10*10 over the study area (dotted line).

Table S1. Fixed-effects coefficients of a Gaussian model of the impact of temperature and chilling duration on the wake-up time of *A. aegypti*.

Table S2. Fixed-effects coefficients of a mixed-effect binomial model of the impact of wind speed in the wind tunnel on the escape rate of *A. aegypti* measured in the IAEA reference flight test.

Table S3. Comparison of the mortality rates of the different series in the field.

Data file S1. Raw dataset.

Movie S1. Presentation of the drone trial run in Brazil, March 2018.

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