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

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Abstract

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 unmanned 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. 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.

Summary

An automatic adult mosquito release device operated from a drone released sterile males without reducing their quality.

MAIN TEXT

Introduction

According to the World Health Organization (WHO), vector-borne diseases account for 17% of infectious diseases leading to more than one 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 for alternative mosquito control methods, particularly against *Aedes* vectors (*1*).

New 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 (1) 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*), (2) genome editing (*13*) and, (3) 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 programmes against fruit flies, moths and tsetse flies (*21-24*). However, few systems exists for the efficient aerial release of mosquitoes or drone release of any insect. In this Article, a fully automatic release system was developed for the release of adult sterile male *Aedes* mosquitoes that can be operated from an unmanned 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 (Suppl. Online Information).

Simulated release under laboratory conditions

Prior to the field trial, laboratory experiments were carried out for one year to develop and refine this release system and assess its potential impact on the released sterile males. We first investigated the effect of the different treatments (compaction, chilling, 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 ((*25*), Suppl. Online Information). The percentage of flyers was significantly correlated with the proportion of mosquitoes with damaged wings and legs ($r = -0.98$, $p = 0.02$, Suppl. Online Information). Male *Aedes aegypti* mosquitoes proved to be very sensitive to compaction up to 1.2 g/cm2 and therefore, a release cassette was developed with a maximal depth of 5 cm that contained no more than 50,000 *Ae. aegypti* males (Suppl. Online Information). 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 - 4 hours, became active again after 40 - 60 s when transferred to ambient temperatures. In view that *Ae. aegypti* males have an estimated average free fall speed of 2.5 m / sec, 50 and 100 m were selected as potentially appropriate release altitudes (Suppl. Online Information). Finally, 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 (*26*) (Fig. 1). The tsetse fly cylinder system, which is smaller and lighter than the fruit fly conveyer system, resulted in better quality mosquitoes (higher flight rate, $p=0.02$, Suppl. Online Information). 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 meters / s (25-68 km/h) did not reduce the quality of the males released with this system ($p>0.09$, Table S2). Finally, 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 x 60 x 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) (Fig. 2, $t = -0.036467$, $df = 3.9977$, $p = 0.97$).

Field trial

Following these positive results of the laboratory experiments, the UAV platform was tested on March 2018 under field conditions in Carnaìba do Sertão, Juazeiro, Brazil, within the Moscamed programme (Fig. 3 & Movie S1). Two series of trials were carried out simultaneously using differently color-marked sterile males: (1) point releases to compare ground and drone release from a fix point; (2) 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 sq. km). 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, Suppl. Online Information) but their average dispersal increased with release altitude ($p=0.011$), i.e. from 83 m (SD 21 m) to 133 m (SD 22m) and 153 m (SD 7m) for mosquitoes released from the ground, from 50 and 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 a 20ha monitored with 37 adult traps. To determine whether the males were

mating wild females, 37 ovitraps were deployed to monitor egg fertility, which was compared with an untreated control area. To implement this trial, the flight speed of the drone was set at 10 m/s and the cylinder speed at 2 revolutions per minute which allowed a release rate of ~5000 sterile males per ha. Approximately 12 minutes were 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 minutes (Fig. 4). Marked mosquitoes were recaptured in 24 out 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) *Ae. aegypti* males in Brazil (0.04%) (*14*) or that of *Ae. 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, X2 = 8.25, p-value = 0.7792 for 3 by 3 grid of tiles, X2 = 21.83, p-value = 0.8711 for 5 by 5 grid of tiles and X2 = 84.33, p-value = 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-value < 2.2e-16) and to a lesser extend to those of wild females (cor = 0.38, t = 2.3343, df = 33, p-value = 0.02582). These data indicate that the sterile males aggregated in the same sites than 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 an 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%CI [0.05-0.72] was estimated. However, this increase was surprisingly high and confounding factors might have inflated it. During a longitudinal monitoring effort conducted from 27th March 2017 to 14th 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.

Discussion

Prior to 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 SI 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 surprisingly high considering the number of sterile males released (~5000/ha/w) and the low sterile to wild ratio (<1), indicating the high competitiveness (~0.3) of the 35Gy irradiated sterile males. This compared favorably with an index of < 0.06 that was observed for ground-released RIDL *Ae. 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 impacting their biological quality (*29, 30*). For example, a competitiveness of 0.7–1.0 was observed for irradiated male *Aedes albopictus* under semi-field conditions in Italy (*31*) and of 0.4-0.8 in Reunion island (*32*). Moreover, flight ability of *Ae. albopictus*, *Ae. aegypti* and *Anopheles arabiensis* was not impaired with radiation

doses of up to 40 Gy, 90 Gy and 50Gy, respectively (*25*, *33*). Finally, triple *Wolbachia* infected male *Ae. albopictus* irradiated with 40 Gy showed a competitiveness close to 1 in walk-in field cage studies and of 0.5-0.7 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 as 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 2 field staff, a vehicle and two 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 20-25 minutes given the autonomy of the drone at the speed of 10m/sec used in this study), or by using several UAV's 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. whilst 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).

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 *Ae. albopictus* in China, the cost of releasing from the ground was estimated at 20 USD/ha/week, which could be reduced to an estimated 1 USD/ha/week using a drone (*9*). Irrespective of the size of the target areas, UAV's 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 to release sites within 48 h (*36*). This would make the technology even more cost effective, as 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 programme 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 non-profit organization, based in Juazeiro city (Bahia, Brazil), and operating since 2005. This facility has been working on the implementation of a pilot trial for the control of Aedes 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 sterile insect technique (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 as migration of *Ae. 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 are 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. Also, a TV 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 *Ae. aegypti* used in all laboratory experiments was sourced from Juazeiro, Brazil and transferred by Biofabrica Moscamed, Brazil to the Food and Agricultural Organisation/ International Atomic Energy Agency (FAO/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 (L:D) 12:12, with two one-hour twilight periods simulating dawn and dusk) as described in (*38*). Standardised guidelines developed at the IPCL were used to produce and hatch the eggs for all experiments (*39*). Larvae were reared in plastic trays (40 x 29 x 8 cm) containing 1 – 1.5 litres of deionized water at a density of roughly 1,500 – 2,000 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*)), prior to further examination under a stereomicroscope for increased accuracy. Adults were maintained in plastic Bugdorm cages (30 x

30 x 30 cm, Taiwan) unless otherwise stated with continuous access to a 10% sucrose solution. All experiments were carried out on 3 – 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 *Ae. 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 ± 1ºC, RH 80 ± 10% and a photoperiod of L10:D14h). Larvae were reared in plastic trays (51 x 30.3 x 9.7 cm), at a density of 1 larvae/ ml in 3 liters (L) of mineral water. Larvae were fed daily with a solution of the IAEA 2 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 by (*44*). Pupae were kept in trays containing mineral water in a climate controlled insectary until irradiation (T 27 ± 1ºC, RH 70 ± 10%, photoperiod of L10:D14h).

Irradiation protocols

At the IPCL in Seibersdorf, 36 to 48 hours old *Ae. aegypti* pupae were irradiated with 90 ± 5 Gy in a 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 a RS 2400 X-ray machine (RadSource, Suwanee, GA, USA) with a 125kV voltage, an 18mA current, and with a dose-energy ratio of 0.0207 Gy kW-1 s-1. Male pupae (30-36 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/well, area 3.66 cm2/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 x 30 x 30 cm) and kept in a climate controlled insectary until adult emergence (T 27 ± 1ºC, RH 70 ± 10%, photoperiod of 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 *Ae. aegypti*. Therefore, an actual aerial and ground field release was simulated prior to calculating the competitiveness index (CI). *Ae. 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 hours-old male pupae were irradiated with 95 ± 5 Gy as previously described. The remaining 1000 male pupae were not irradiated and 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, 2 batches of 100 served as back up adults. Sterile male pupae were caged in batches of approximately 3000 (volumetric estimation). On day 3 post-emergence, the number of females and fertile males were adjusted back to batches of 100 from the back up cages to compensate for failed emergence and mortality. All cages of sterile males, in addition to the 3 cages of 300 sterile males (ground release), were transferred to a cold room (4 ± 1 °C) for a period of 10 minutes until immobilization occurred. All cages were emptied into a plastic larval rearing tray (30 × 40 × 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, as 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 (3 repetitions per minute). Once all sterile males had passed through the release system, 3 batches of 300 were counted out and transferred to a small plastic container (100 ml) and closed. The 3 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 Bugdorms (60 x 60 x 60 cm) as follows: 2 sterile controls (100 sterile males), 2 fertile controls (100 fertile males), 3 ground release cages with sterile males which did not pass via the release mechanism (100 fertile males and 300 sterile males) and 3 aerial release cages, with males which 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 Bugdorms (30 x 30 x 30 cm). Bloodmeals were offered daily for the next 2 days and an egg cup placed in each Bugdorm 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 – 48 hours, the number of larvae in each tray was counted. Additionally, the egg paper was viewed under a stereomicroscope and the number of hatched and unhatched eggs 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 (*27*). Fried (1971) defined a competitiveness index, called Fried's index that can be calculated with the following formulae:

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

where Ha is the natural fertility of wild females and Ee the observed fertility rate under a given ratio of sterile over wild males, R. This formulae 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

Prior 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 approximately 25 000 sterile male *Ae. aegypti* were immobilized at 4°C in a cold room for 10 minutes prior to 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. A climate chamber was pre-programed to reflect likely environmental conditions in the field (35°C and 80% relative humidity). The release mechanism was connected to the software which controls the release when connected to the drone during an actual aerial release. The Bugdorm was placed inside the climate chamber and the door to the release mechanism removed. The Bugdorm was gently shaken to simulate the drone commencing flight. As 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 2 samples of approximately 100 mosquitoes from the Bugdorm and with 2 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/ 100 adult males (*47*). For the MRR, we marked in batches of 2400 requiring a L container and 24 mg of dust. To ensure dust adhered to the walls of the dusting container, the inside surfaces were rubbed 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 acclimatize. Cages of 2400 adult *Ae. aegypti* were then transferred to the cold room for immobilization for 20 minutes. Each cage was then emptied into a pre-dusted container and the lid closed. The container was then rotated for 30 seconds (equating to approximately 25 full rotations) to coat the sterile males uniformly. Dusting took place after 6pm 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 colour 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: (1) a storage unit consisting of a canister that keeps mosquitoes at low temperatures surrounded by insulation, (2) an ejection mechanism featuring a rotating cylinder that brings mosquitoes from the storing canister to the outside, (3) a release area where mosquitoes fall onto and then slowly ejected outside, (4) 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. In order to keep the insects at the target temperatures, we put phase change materials (PCM) 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 ERC REVOLINC project and patented under the reference PCT/EP2017/059832. The cylinder has 6 discrete holes that each can take up around 800 mosquitoes. Hence a full cylinder turn should release around 5,000 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 1-3 RPM are optimal, leading to release rates of 5,000-15,000 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 onto 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. Also, 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 a Raspberry Pi 3 (RPi, low-cost mini computer), interfacing the drone (and ground station) with the release mechanism. A 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.

In order 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. Further, we mounted two cameras to monitor and live-stream 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 DJI technologies, including a robust flight controller and a strong transmission system (up to 5km long range transmission). It enables a flight time of 30-35min when equipped with a payload of 1-2 kg. Also, 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

In order 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, monitoring drone state, mechanism state, sensor values and camera live-stream. 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 GIS tools.

System Calibration

In order to calibrate our system for a target mission, we mainly needed to set a flight route, the release rate of the mechanism as well as 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 swaths 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:

$$Release\ rate\ per\ flight\ line\ (Mosquito/m) = 5,000 * \frac{Cylinder\ speed\ (RPM)}{Flight\ speed\ (m/s)}$$

Mark-release-recapture protocol

Our final study aimed to estimate the dispersal, mortality and mating capacity of sterile male *Ae. aegypti* mosquitoes through mark – release – recapture (MRR) experiments after being released from either 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 hectares (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 irradiation section above for detailed protocol), in an open field setting. 3 releases were conducted within a 7 day period (Table 1). Aerial releases involved our prototype release mechanism attached to a DJi Matrice pro 600 drone (Fig. 1). Aerial releases occurred in 2 ways. Firstly, sterile males were released in the centre 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 were conducted as controls. Secondly, 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).

Prior to 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 ha. In each of the trapping stations, one ovitrap was set in the vicinity of the BG trap (<50m). Five ovitraps were also deployed in a neighbouring control area (at 0.9km from the release area) to measure the natural fertility of *Ae. aegypti* during the same period. In the early morning of March 21st 2018, sterile males were released either by air or by ground as described above. The following day (March 22nd) beginning early afternoon (12:00 pm to 14:00 pm), traps were inspected and the collected samples brought to the laboratory. All mosquitoes caught were given an identification code referring to the relevant station in order 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 (March 23rd) and after each collection day thereafter, field collected samples were analysed, classified and data stored. Samples were screened for colour under a UV-light stereomicroscope. Collections were made by 2 teams of 4 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 April 10th for the third and final release). Eggs collected were dried for 7 days and then hatched. Non-hatched eggs were bleached to check for the presence of an embryo. Release and recapture data were geo-referenced using a Global Positioning System device. All coordinates were entered into a Geographical Information System to calculate the distances between release and each recapture site.

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

Data analysis

Re-capture rates were compared using proportion comparison z-test and difference 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 comparison.

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 as random effects. The significance of fixed effects was tested using the likelihood ratio test (*48, 49*).

In order to obtain a confidence interval for the estimate of the Fried Index, we used a non parametric 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 (1,000 simulations). Assuming a symmetric distribution, we used the basic percentile method to get 95% confidence interval.

Tests of Complete Spatial Randomness (CRS) based on Monte Carlo simulations were carried out on positive traps. Pearson Chi2 tests were based on quadrat counts for sets of 3x3, 5x5 and 10x10 quadrats (Fig. S9).

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Author contributions: JB, NJC, AK, JG, RAH & FB designed all experiments; JB, NJC, MGP, MCP, LG, ATMP, AK, JG, TW, GSH, RAH, HY & FB performed all experiments; JB & AHD analyzed the data. JB, JV, AK & MJBV provided funding and supervised the experiments. JB, NJC, AHD, MGP, JG, FB, MJB wrote the first draft of the paper and all authors contributed to the submitted version.
Competing interests: The authors declare no competing interests.
Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials.

Figures and Tables

Fig. 1. The adult mosquito release system operated from an unmanned aerial vehicle (UAV). A: front right view of the release mechanism (technical drawing), B: half-section of the release mechanism (technical drawing), C: a canister filled with 50,000 marked mosquitoes, and D: a fully-assembled aerial mosquito release system attached to a DJI M600 UAV in flight.

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

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

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

Fig. 5. Induced sterility and sexual competitiveness of sterile male *Aedes 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 non-treated areas. (B) Estimation of the Fried index from 1000 bootstraps in the distributions of sterile to wild male ratios in traps and viable eggs rates in ovitraps in the release and non-treated areas (see SI for details). The Density corresponds to the percentage of the simulations for a given value.

Table 1. Main characteristics of the sterile male *Aedes aegypti* released in Carnaìba do Sertão, Brazil. Each row represents a series released separately with a different colour. Year 2018.

Release pattern	Date of Release	Colour	Number released	Recapture rate (%)	Number recaptured	Repeat	Survival rate	Median distance
Ground	March 21	B	9600	1.30	125	1	0.20 (0.96)	97
Ground	March 24	BY	7200	1.90	137	2	0.63 (0.59)	68
Drone_50m_stationary	March 21	O	9600	0.27	26	1	NA	117
Drone_50m_stationary	March 24	OY	7200	0.28	20	2	0.82 (0.48)	148
Drone_100m_stationary	March 21	G	9600	0.05	5	1	NA	158
Drone_100m_stationary	March 24	GY	7200	0.08	6	2	NA	148
Drone_100m_path	March 21	P	50700	0.27	138	1	0.45 (0.80)	NA
Drone_100m_path	March 24	PY	49000	0.42	207	2	0.64 (0.74)	NA
Drone_100m_path	March 27	Y	65700	0.27	175	3	0.70 (0.39)	NA

SUPPLEMENTARY MATERIALS

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

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

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

Fig. S4. Flight ability of male *Aedes aegypti* after passing through the cylinder release mechanism at different speeds (1 or 3 revolutions per minute (RPM)).

Fig. S5. Flight ability of male *Aedes 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. A (blue) first ground release. B (green) first stationary drone release (100m). C (orange) first stationary drone release (50m). D (pink) first drone release (100m flight path). E (blue-yellow) second ground release. F (green-yellow) second stationary drone release (100m). G (orange-yellow) second stationary drone release (50m). H (pink-yellow) second drone release (100m flight path. I (yellow) third drone release (100m flight path).

Fig. S8. Temporal dynamics of the fertility rate measured with ovitraps in a control site close to the release area from 27th March 2017 to 14th 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 (doted line).

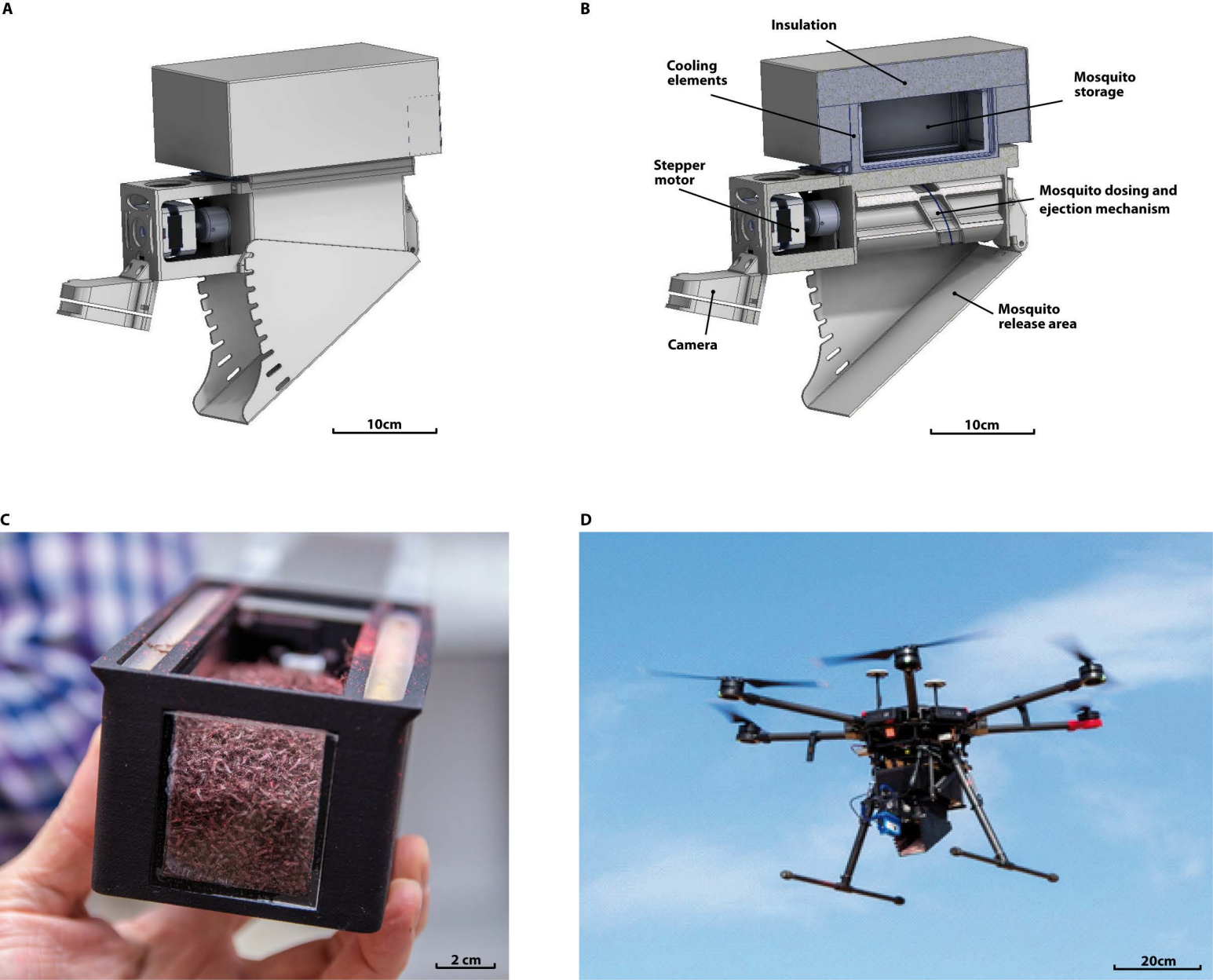
Table S1. Fixed-effects coefficients of a Gaussian model of the impact of temperature and chilling duration on the wake up time of *Aedes aegypti*. The reference temperature is 10°C.

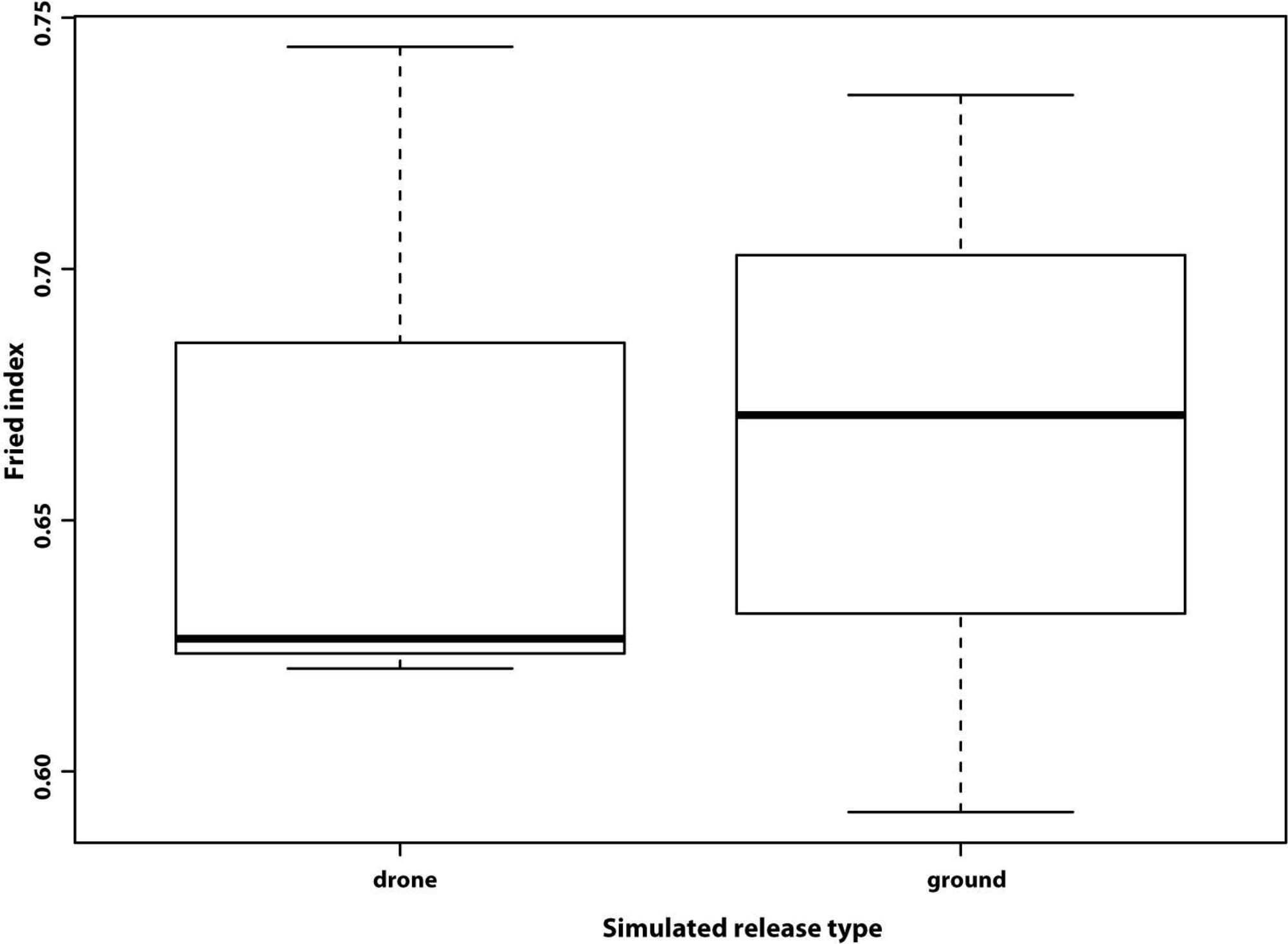
Table S2. Fixed-effects coefficients of a mixed-effect binomial model of the impact of wind speed in the wind tunnel on the escape rate of *Aedes aegypti* measured in the IAEA reference flight test.

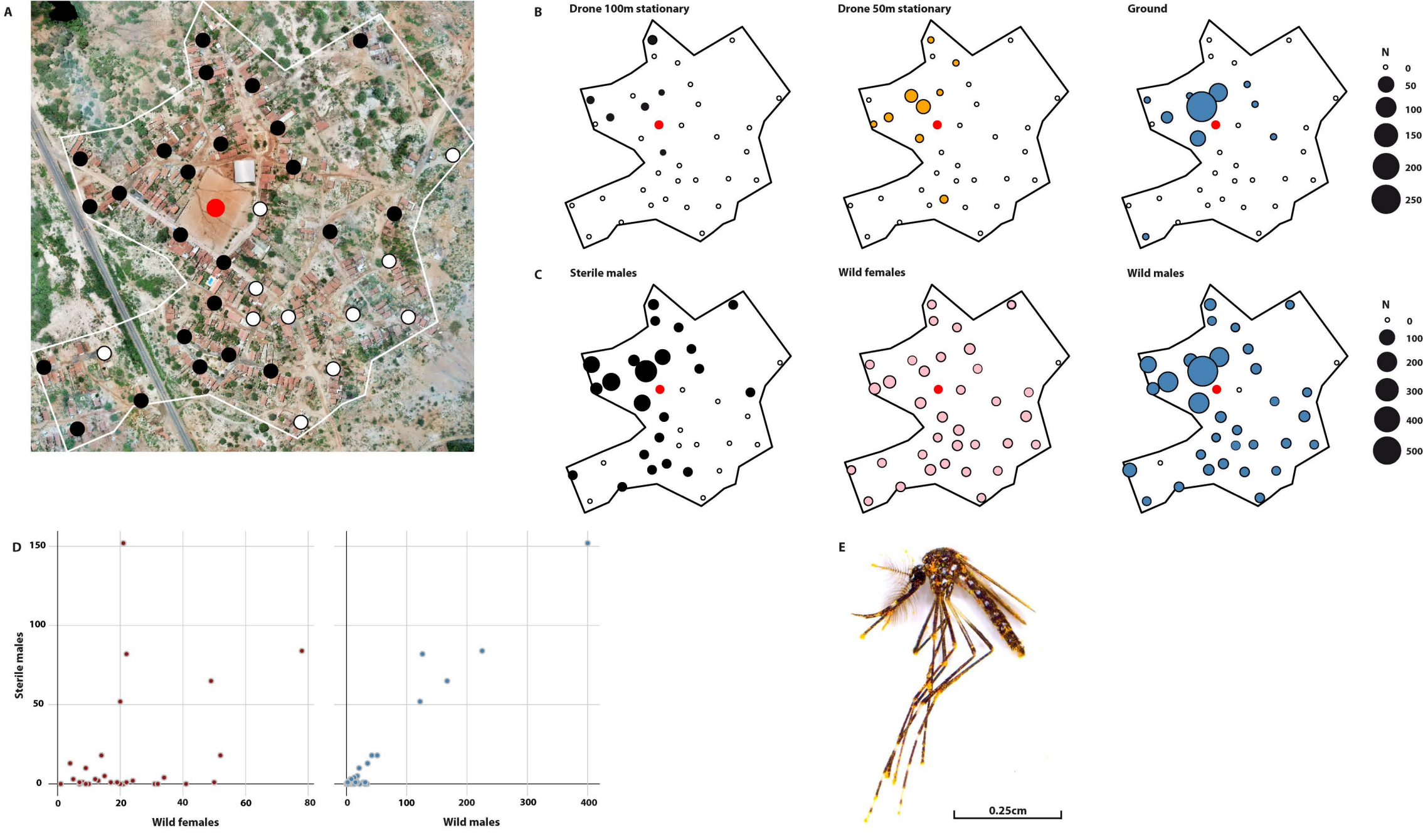
Table S3. Comparison of the mortality rates of the different series in the field.

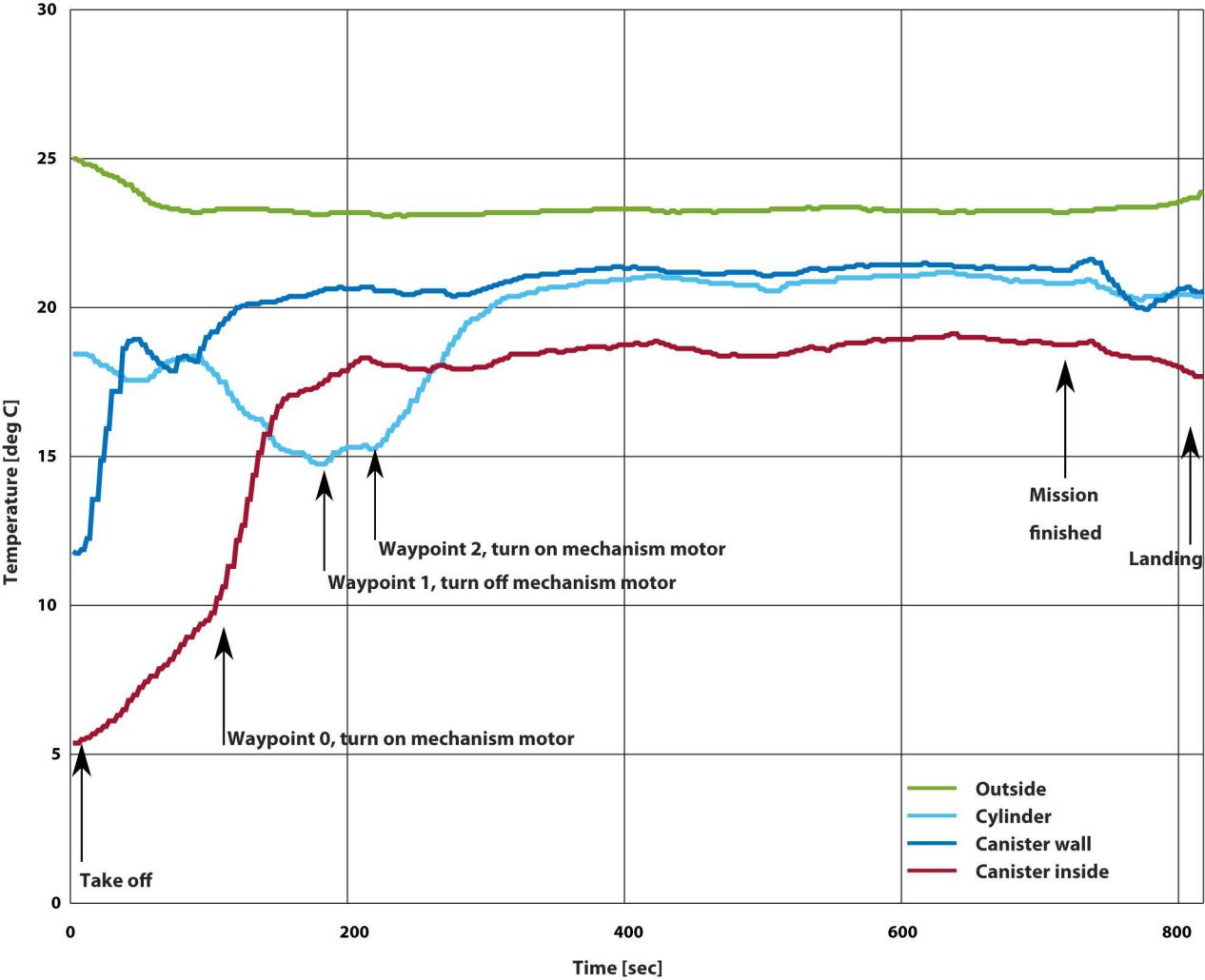
Data file S1. Raw dataset.

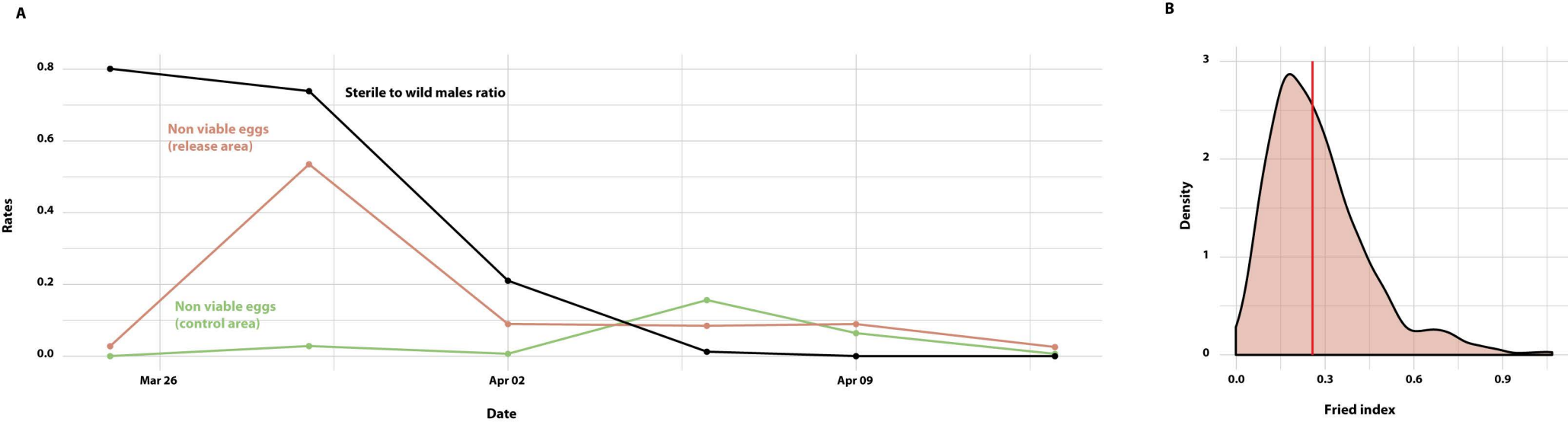
Movie S1. Presentation of the drone trial run in Brazil, March 2018.


A
10cm
B
Insulation
Cooling elements
Mosquito storage
Stepper motor
Mosquito dosing and ejection mechanism
Mosquito release area
Camera
10cm
C
2 cm
D
20cm


0.75
0.70
0.65
0.60
Fried index
drone
ground
Simulated release type


A
B
Drone 100m stationary
Drone 50m stationary
Ground
N
0
50
100
150
200
250
C
Sterile males
Wild females
Wild males
N
0
100
200
300
400
500
D
150
100
50
0
Sterile males
0
20
40
60
80
Wild females
0
100
200
300
400
Wild males
E
0.25cm


30
25
20
15
10
5
0
Temperature [deg C]
0
200
400
600
800
Time [sec]
Take off
Waypoint 0, turn on mechanism motor
Waypoint 1, turn off mechanism motor
Waypoint 2, turn on mechanism motor
Mission
finished
Landing
Outside
Cylinder
Canister wall
Canister inside


A
0.8
0.6
0.4
0.2
0.0
Rates
Sterile to wild males ratio
Non viable eggs
(release area)
Non viable eggs
(control area)
Mar 26
Apr 02
Apr 09
Date
B
3
2
1
0
Density
0.0
0.3
0.6
0.9
Fried index

Supplementary Materials for
Field performance of sterile male mosquitoes released from a drone

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Supplementary Materials and Methods

Quality control – flight test device

A flight test device (FTD), which aims to evaluate the flight ability of an adult mosquito, was created during the project (25). The FTD consists of a series of 40 transparent acrylic plastic (Polymethyl methacrylate - PMAA) flight tubes, surrounded by a larger PMAA tube. The first two series of tubes are housed within a third PMAA tube of greater size which serves as a containment box after mosquitoes escape the flight tubes. Mosquitoes are blown into the FTD via a mouth aspirator and are given a period of 2 hours to escape. Afterwards, the FTD is chilled at 4 °C and the number of adults that remain at the base of the flight tubes and those that have escaped are counted. Flight ability is calculated by dividing the number of adults which escaped by the total number put into the flight tube. An average is then calculated across 2 repetitions. A series of verification experiments confirmed that flight ability was an accurate predictor of male mosquito quality by comparing it to a series of reference predictors including survival and mating propensity (25). Thus, we used flight ability as an indicator of quality when conducting our laboratory tests related to the design and development of our prototype release mechanism. Further information regarding the FTD including schematic drawings, full dimensions and verification experiments results can be found in (25).

Defining the maximum tolerable height of compaction

The aim of this experiment was to measure the damage caused by the compaction of the mosquitoes and to ascertain the maximum pressure tolerable. This parameter was critical when designing the storage container for the release mechanism. We used a cylindrical tube (height 10 cm, diameter 4 cm) with holes (1 cm diameter) cut vertically at 2 cm intervals. 15 Bugdorm cages of male *Aedes aegypti* (approximately 50,000) were transferred to the cold room (4°C) and left for 10 minutes until immobile. The cylinder was placed on a scale and tared. All cages (except one which served as a control cage) were emptied into a larval rearing tray and mosquitoes transferred to the cylinder via a plastic funnel and a timer set for 2 hours. Following this 2 hour period of immobilization and compaction, a mouth aspirator was used to remove small samples (approximately 200) of mosquitoes via the pre-cut holes at different heights of 3, 5, 7 and 9 cm (equivalent to 0.76, 1.27, 1.78 and 2.29 g/ cm2 respectively). All samples, in addition to a sample from the control cage, were taken to the laboratory and split into 2, thus generating 10 batches of adults and flight ability tests were conducted in the flight test device (FTD).

Active compaction

In the event that the phase change material (PCM) was to fail during transportation to the field site and or during the actual release and the temperature were to rise above that necessary to maintain immobilisation, we were keen to know the impact this would have upon the compacted sterile males whilst held in the storage container. A Bugdorm containing approximately 3,000 *Ae. aegypti* males were immobilized for 10 mins inside a cold room (4°C). A cylindrical tube of 8 ml (height = 5.5 cm, diameter = 1 cm) was filled with immobile males, closed with a small square of mesh and secured with an elastic band. The volume of

the tube allows approximately 1,000 adult males to be contained. This was repeated with the remaining males, generating 3 tubes of immobilized males. The tubes were transferred to ambient laboratory conditions (described above) and a timer begun. After 10 minutes, one tube was emptied into a standard Bugdorm cage and again after 20 and 30 minutes.

Chilling and recovery times

In order to determine the altitude at which the drone will conduct an aerial release, it was important to know how long the sterile mosquitoes would need to recover following immobilisation for x amount of time at y temperature (in addition to the speed at which they fall when immobile, to be detailed later). Thus, we conducted an experiment at 3 temperatures across the range that we anticipated to store the mosquitoes during transport and release (6, 8 and 10°C) and for different lengths of time (1 to 4 hours) in order to ascertain how long it takes them to recover and regain the muscle activity necessary to fly. Batches of 1,000 male *Ae. aegypti* were immobilised and transferred to a small plastic tube (height = 5.5 cm, diameter = 1 cm) and transferred to a climate chamber pre-set to each of the temperatures to be tested. After each time interval, 2 tubes of immobile males were removed and emptied into separate Bugdorm cages. A timer was begun and when approximately 75% of the males had regained flight the timer was stopped. An average was calculated across the 2 repetitions for each temperature and duration.

Flow rates & homogeneity of the release in various prototypes of release machines

The final cylindrical design of the release mechanism was chosen after comparative tests with a second system based on a conveyor belt method and used in previous aerial releases of sterile insects (24). Male *Ae. aegypti* (reared as previously described) were immobilized in a cold room (4°C) and transferred to the storage containers of each release system (approximately 20,000 per container and thus the containers were at 40 -50% capacity). One Bugdorm was left aside to serve as a control sample. A timer was set for 1 hour after which each batch of males passed through one of the two mechanisms. Samples were taken from the top and bottom layers after passing via the release mechanisms. All samples were taken back to the laboratory along with a sample from the control cage. Two batches of approximately 100 males were taken from each of the top and bottom samples, in addition to from the control cage and placed within the flight test device (FTD) (25) to measure flight ability. Furthermore, to improve the homogeneity of the release, 4 different cylinder designs were tested to assess the approximate number of mosquitoes that were ejected with each turn of the cylinder. We 3D printed 4 designs and tested each one with a batch of approximately 20,000 male *Ae. aegypti* in a cold room at 4 °C whilst video recording each test.

Release mechanism speed

In order to further fine-tune the homogeneity of release, according to roughly how many mosquitoes we intended to release per hectare (ha), we investigated 2 different rotation speeds of the cylinder of 1 and 3 revolutions per minute (rpm). Cages totalling approximately 50,000 male *Ae. aegypti* were immobilized in the cold room (4°C) with half loaded into the storage container above the cylinder. We tested both 1 rpm and 3 rpm (half of the batch of 50,000 mosquitoes in each test) and video recorded each test to assess the homogeneity of each release speed. One cage of mosquitoes was set aside to serve as control. Samples were taken from each speed for flight ability tests, from the top, middle and bottom layers of mosquitoes within the storage container, in addition to a sample from the control cage.

Drop speed of mosquitoes

Another factor in determining the altitude at which mosquitoes are to be released was the speed at which they typically fall. Thus, we implemented a video capture set, which comprised a high performance industrial video camera (IDS camera), a metric ribbon resting on the wall (2 meters in length), and a white led light high power focus (40W). The optics used for this test had a maximum focal length of approximately 55cm and a 35° field of view and thus only allowed for focusing on a 35cm section of the metric ribbon. Therefore, it was decided to only focus on the last 35cm of the metric ribbon (closest to the floor). For the analysis, we used different male mosquito samples (*Ae. aegypti*) which had previously been frozen to kill them. We selected three different individuals that showed small differences in size. All samples were dropped repeated from four different heights: 50, 80, 110 and 140cm.

Wind resistance test

The aim of this experiment was to investigate the effect of exposure to various wind speeds on the flight ability of sterile male mosquitoes. Wind is a natural phenomenon and when releasing sterile male mosquitoes by air, it may be amplified by the movement of the drone in flight. Thus, it was crucial to ascertain if there was a wind speed above which significant damage occurred to the sterile males. It also allowed us to determine what speed we should conduct the aerial releases at. The wind tunnel was composed of a Plexiglas tube (diameter 150mm) with a powerful fan at one end, adapted from a basic garden leaf blower. The dropping tube, the point at which sterile males are introduced into the wind tunnel, was placed vertically at a distance of 10 cm on the laminar setup with the end of the tube being at the center of the wind tunnel. At the opposite end, a Bugdorm cage (30 x 30 x 30 cm) was used to catch the mosquitoes after they are blown through the wind tunnel. An anemometer was inserted through a small slot on the top of the Plexiglas tube, close to the dropping tube to enable the speed of the leaf blower to be adjusted until the correct speed was reached. Four speeds were tested in total, 7, 11, 15 and 19 meters/ second (m/s) with control samples simply dropped into the wind tunnel when the leaf blower was switched off (0 m/s). Several Bugdorms of three day old sterile male *Ae. aegypti* were taken to a cold room (4°C) and left for a period of 10 minutes until immobilization had occurred. Adults were gently tipped out of cages into a plastic tray and then further transferred to 10 Falcon-type tubes (15 ml) via a funnel, until a volume of 7.5 ml was reached in each tube. This volume equates to approximately 1,000 adult male *Ae. aegypti*, when no compaction is used. One tube of adults was introduced into the wind tunnel for each of the 4 tested wind speeds with 2 repetitions allocated to each speed and 2 control samples. A sub-sample of approximately 100 adults were taken from the Bugdorm following each wind speed test and quickly transferred to the flight test device (FTD) to calculate flight ability (as described previously).

Supplementary Results

Defining the maximum tolerable height of compaction

Compaction significantly reduced the flight ability of male *Ae. aegypti* from a height of 7 cm (0.178 g/cm^2) onwards, with no significant difference in flight ability observed between the control samples and those exposed to 3 or 5 cm or compaction (0.76 or 1.27 g/cm^2) (Figure S1). This finding confirmed an earlier observation using *Ae. aegypti* that 5cm of compaction is the maximum tolerable level that can be imposed upon immobile *Ae. aegypti* (25).

Active compaction

We observed a 100% recovery of all mosquitoes after 1, 2 and 3 hours of active compaction and thus no further experiments were carried out on active compaction.

Immobilization and storage temperatures
The suitable temperature range to conduct immobilization and store sterile male *Ae. aegypti* during transportation to the field and prior to release was identified previously as the range 8-10°C after testing temperatures between 0 and 10 °C for 2 hours (25).

Recovery times
The chilling temperature and duration, as well as their first order interaction, significantly impacted the recovery time (Table S1 & Figure S2). The longer and lower the chilling temperature, the higher the wake up time. At 10°C for one hour, the wake-up time of 75% of chilled adult males was around 40 seconds.

Release mechanism
The flight ability was higher for the cylinder than the conveyor belt ($p = 0.03$, Figure S3). Homogeneity of the mosquitoes passing via each mechanism (based upon video footage) was also better for the cylindrical release system which was selected as the final design. After testing four different variations of the cylinder shape and reviewing the video footage, we selected the one which gave the most homogenous release of male *Ae. aegypti* (Figure 1). Flight ability did not differ significantly between controls and males which passed via the release mechanism at either 1 or 3 RPM ($p > 0.714$, Figure S4). Furthermore, the position of the males within the storage container prior to passing via the release mechanism did not significantly decrease their flight ability in comparison to control males ($p > 0.488$, Figure S5). For the actual field releases in Brazil, we adjusted the flight speed of the drone to 10 m/s and the cylinder speed to 2 RPM to release approximately 5,000 sterile males per ha.

Drop speed of mosquitoes
Based on this experiment, we found that the maximum free fall speed of *Ae. aegypti*, in a closed space, would have a near upper limit of about 2.5m/s, which ensures a minimum falling time of 40s at a 100m dropping height. Due to the speed of the drone and climatic conditions, this falling time should be higher in a real scenario. Assuming a wake-up time of the mosquitoes around 40 seconds, flight altitudes of 50 and 100m were thus selected for testing in the field.

Wind resistance test
We did not observe any significant impact of wind speed on the quality of sterile males as measured with their flight ability ($p > 0.09$, Table S2), as measured in our wind speed test chamber (Figure S6).

Competitiveness analysis in large cage
Results of a Welch Two Sample t-test ($t = -0.036$, $df = 3.998$, p-value $= 0.973$) indicated that males which had passed via our prototype release mechanism in a simulated aerial release were of equal competitiveness as males exposed to a simulated ground release (Figure 2). The competitiveness index of males which underwent a simulated aerial release was 0.66 (SD 0.07) in comparison to those exposed to a simulated ground release (0.67, SD 0.07). Hatch rates from cages with only sterile males from simulated ground or aerial release were on average 1.12 and 1.10% respectively, corresponding to their residual fertility.

Simulated release within laboratory conditions
In laboratory conditions, sterile males that passed via the release mechanism in a simulated aerial release did not differ significantly in flight ability (76.01 ± 1.01 %) in comparison to controls males that did not, thus simulating a ground release (78.04 ± 0.54 %, $p = 0.837$).

Mark-Release-Recapture

Although not easy, we successfully discriminated the various series of released males using high magnification in the visible spectrum (Figure S7). The mortality rates of the different series released in the field were similar between treatments ($p>0.46$, Table S3), ie we did not find any differences between point and path releases or between release altitudes.

All other results are presented in the main text.

Supplementary Figures

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

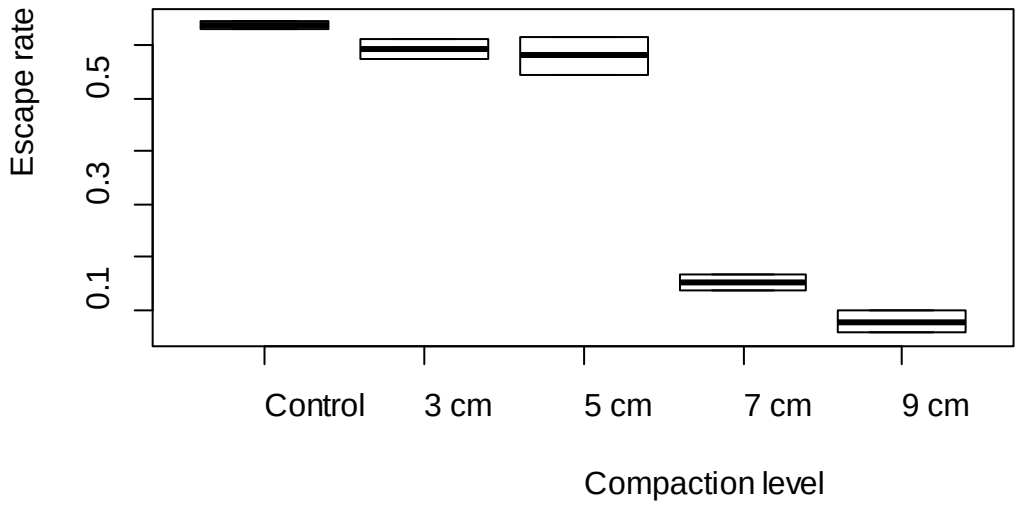


Fig. S2. The average time taken (secs) for 75% of adult male *Aedes aegypti* to regain flight ability following immobilization at 6, 8 and 10°C for 1 – 4 hours.

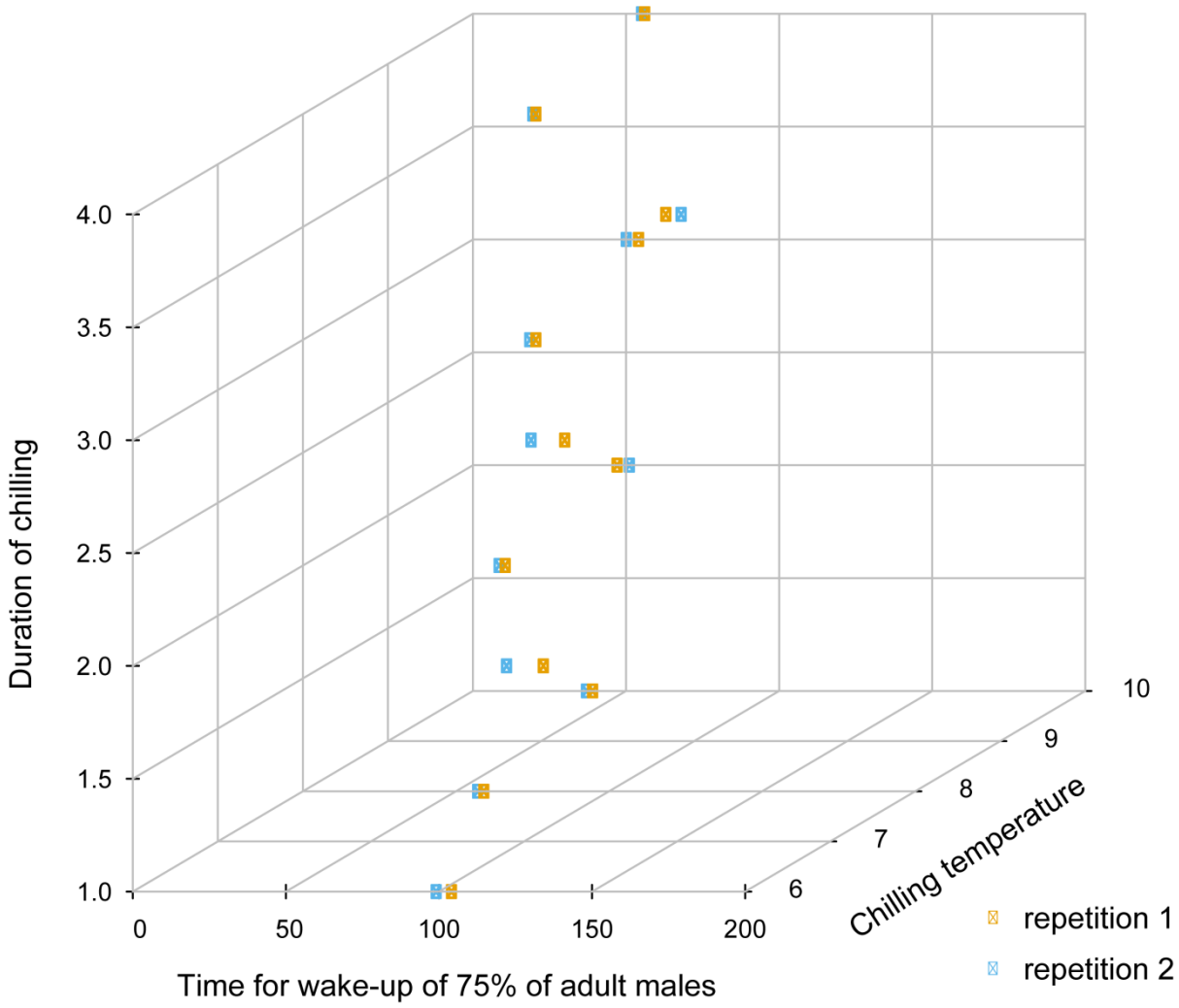


Fig. S3. Flight ability results of male *Aedes aegypti* after passing through two prototype release mechanisms versus a control sample.

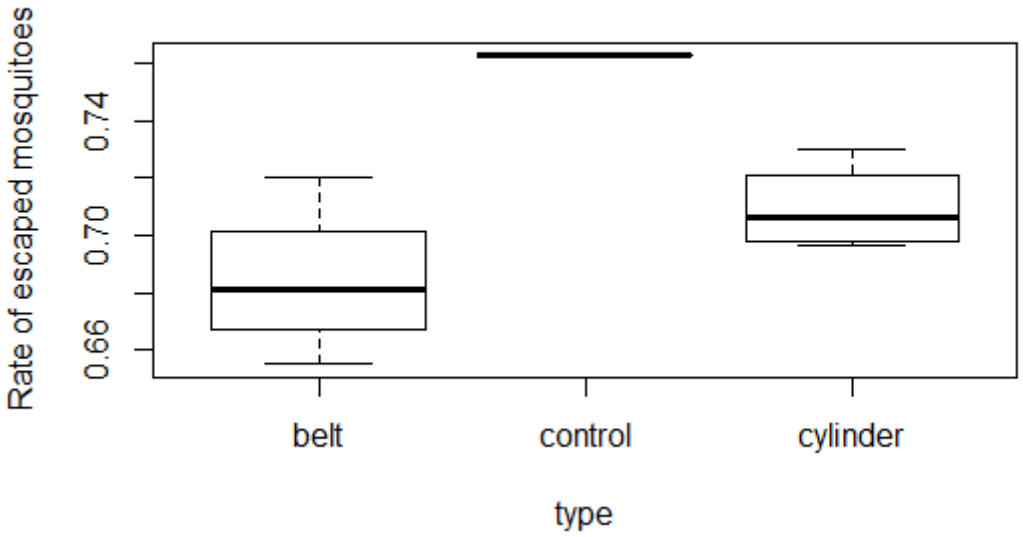


Fig. S4. Flight ability of male *Aedes aegypti* after passing through the cylinder release mechanism at different speeds (1 or 3 revolutions per minute (RPM)).

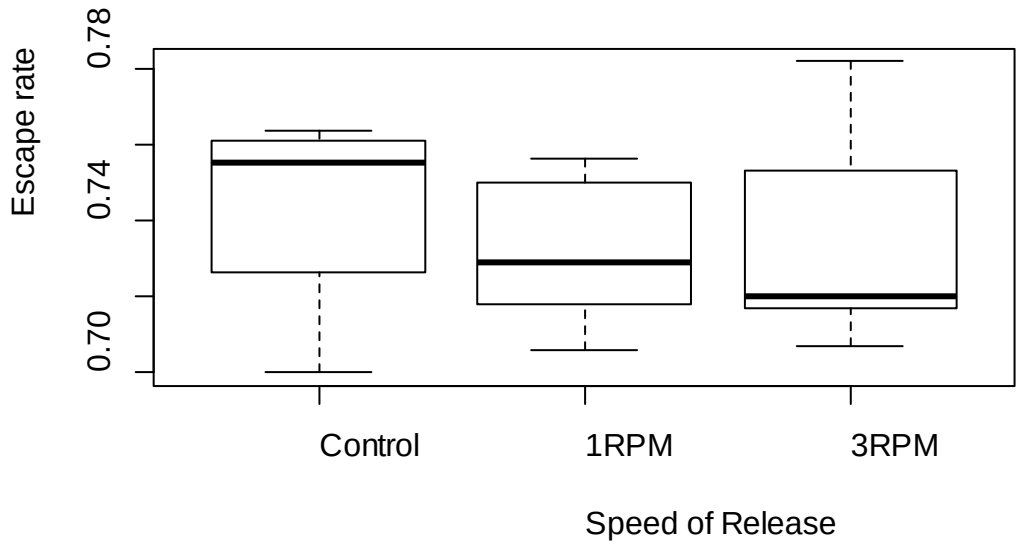


Fig. S5. Flight ability of male *Aedes aegypti* after passing through the cylinder release mechanism depending on their position in the canister.

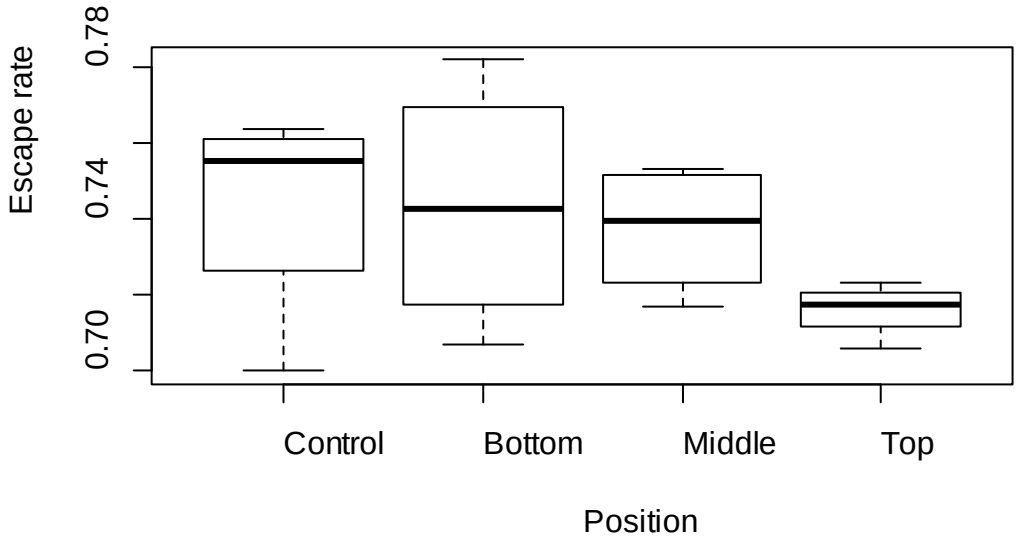


Fig. S6. Wind speed test chamber.

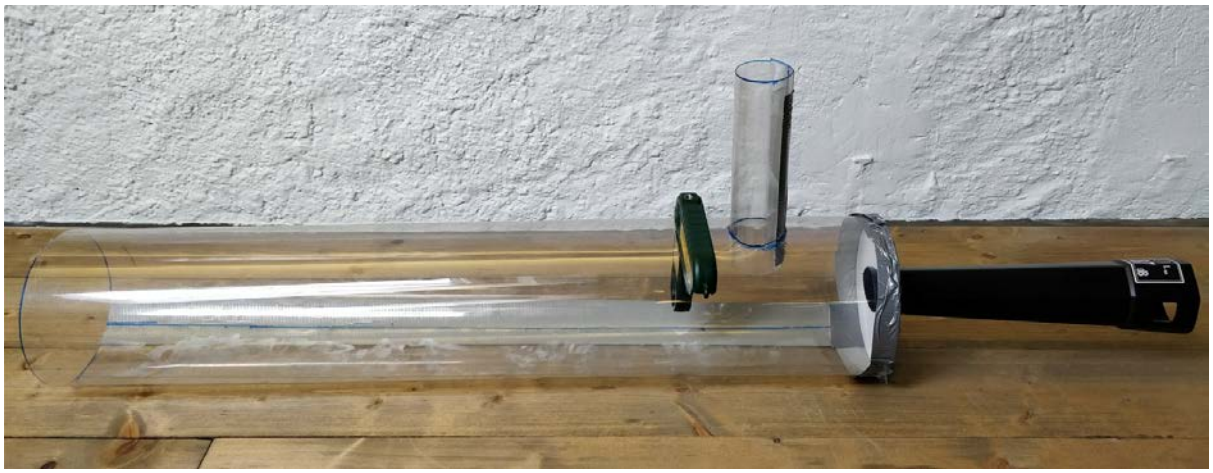


Fig. S7. Differentiation of sterile males from wild flies using fluorescent dust. A (blue) first ground release. B (green) first stationary drone release (100m). C (orange) first stationary drone release (50m). D (pink) first drone release (100m flight path). E (blue-yellow) second ground release. F (green-yellow) second stationary drone release (100m). G (orange-yellow) second stationary drone release (50m). H (pink-yellow) second drone release (100m flight path. I (yellow) third drone release (100m flight path).

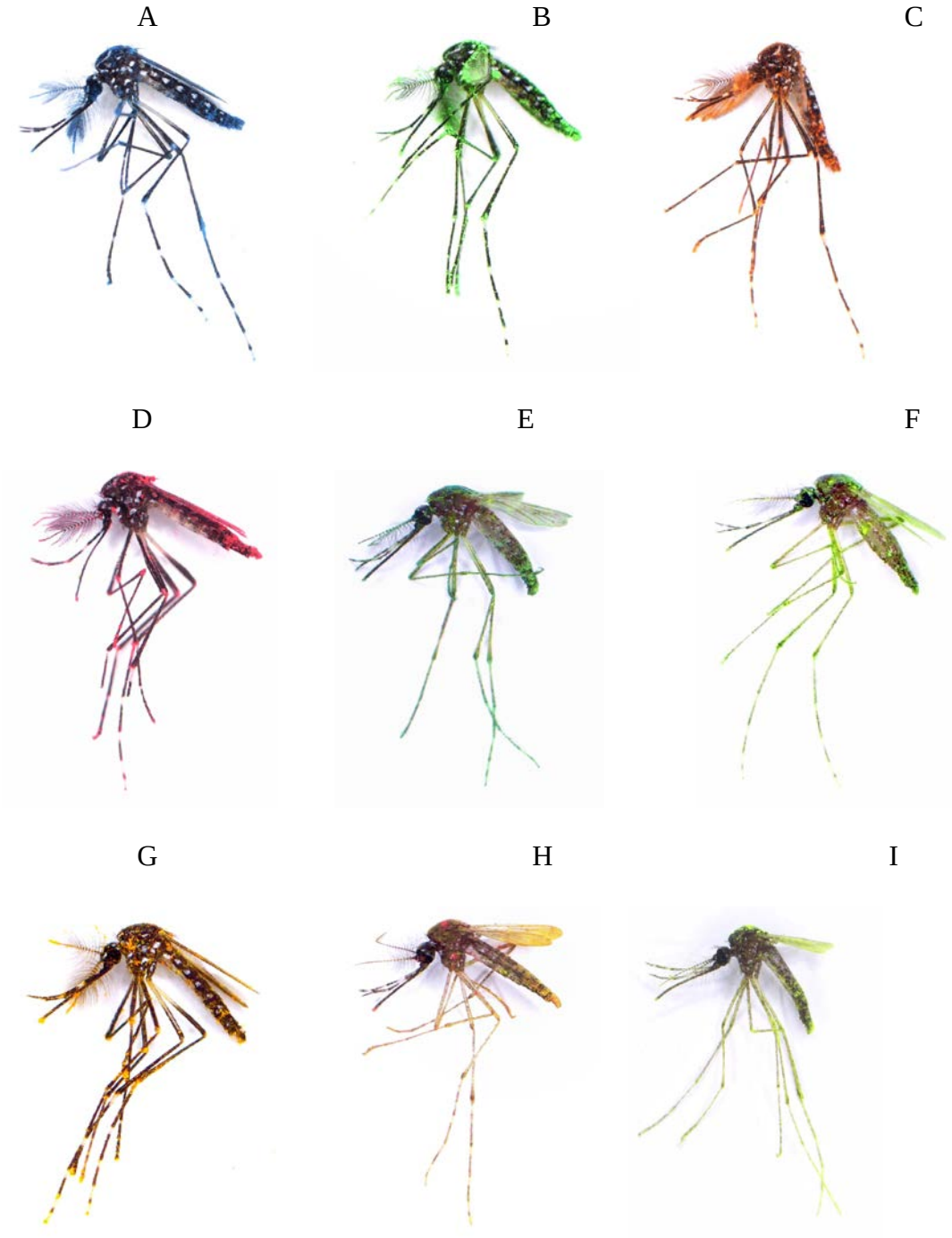


Fig. S8. Temporal dynamics of the fertility rate measured with ovitraps in a control site close to the release area from 27th March 2017 to 14th May 2018.

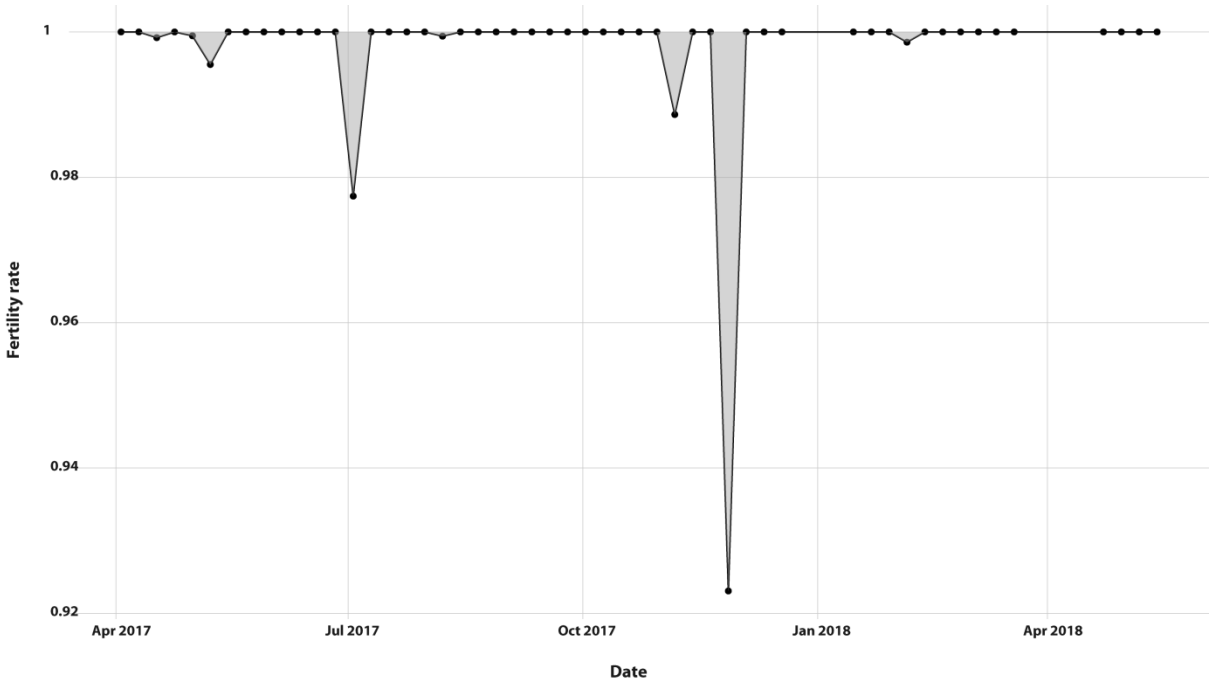
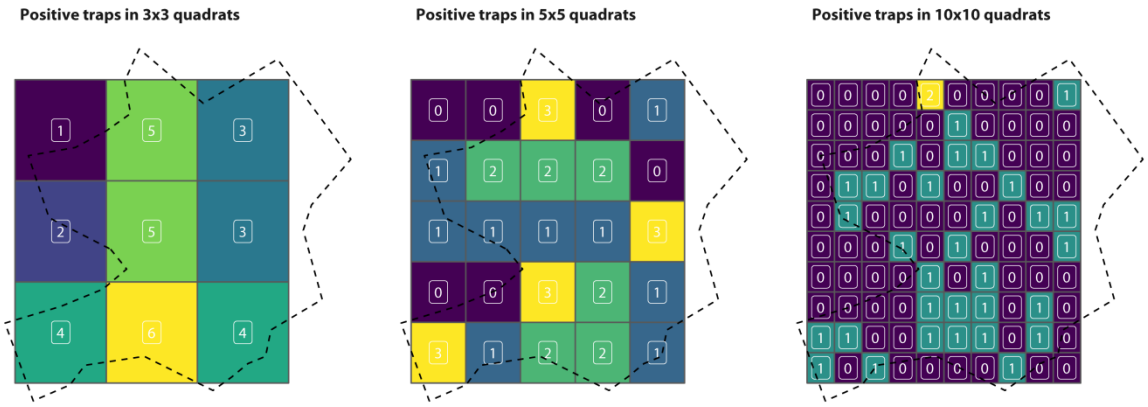


Fig. S9. Number of positive traps with at least one sterile male captured in quadrats of 3*3, 5*5 and 10*10 over the study area (doted line).



Supplementary Tables

Table S1. Fixed-effects coefficients of a Gaussian model of the impact of temperature and chilling duration on the wake up time of *Aedes aegypti*. The reference temperature is 10°C.

Fixed effects	Value	Std. Error	DF	t-value	p-value
Intercept	35.75	5.1452	17	6.9482	0
Temp 8 °C	18.00	7.2764	17	2.4738	0.0242
Temp 6 °C	42.50	7.2764	17	5.8408	0
Chilling duration	5.55	1.8788	17	2.9541	0.0089
Temp 8°C : Chil. dur.	0.70	2.6570	17	0.2635	0.7954
Temp 6°C : Chil. dur.	17.70	2.6570	17	6.6617	0

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 *Aedes aegypti* measured in the IAEA reference flight test.

Fixed effects	Value	Std. Error	z-value	p-value
Intercept	0.8553	0.1417	6.037	1.57e-09
7 m per sec	-0.1622	0.1965	-0.826	0.4091
11 m per sec	-0.0573	0.1880	-0.305	0.7604
15 m per sec	-0.3171	0.1891	-1.677	0.0935
19 m per sec	0.0845	0.1914	0.442	0.6588

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

Comparison	Estimate	SE	df	t.ratio	p.value
Drone_100m_stationary vs Air 100m path	0.084	0.146	23	0.574	0.939
Drone_100m_stationary vs Drone_50m_stationary	0.270	0.364	23	0.741	0.880
Drone_100m_stationary – Ground	0.251	0.169	23	1.488	0.460
Drone_100m_path - Drone_50m_stationary	0.186	0.349	23	0.532	0.951
Drone_100m_path – Ground	0.168	0.135	23	1.242	0.607
Drone_50m_stationary – Ground	-0.018	0.359	23	-0.050	1.000