Joint FAO/IAEA Programme
Nuclear Techniques in Food and Agriculture

GUIDELINES FOR

MARK-RELEASE-RECAPTURE

PROCEDURES OF *AEDES* MOSQUITOES

Version 2

Food and Agriculture Organization of the United Nations
International Atomic Energy Agency
Vienna, 2023

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The proper citation for this document is:

FAO/IAEA. 2023. Guidelines for Mark-Release-Recapture Procedures of *Aedes* Mosquitoes, Jérémy Bouyer and Wadaka Mamai (eds.), Food and Agriculture Organization of the United Nations/International Atomic Energy Agency. Vienna, Austria. 26 pp.

GUIDELINES FOR

MARK-RELEASE-RECAPTURE PROCEDURES OF *AEDES* MOSQUITOES

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FOREWORD

The success of any Area-Wide Integrated Pest Management (AW-IPM) programme that includes a Sterile Insect Technique (SIT) component relies on the ability of the irradiated sterile males to survive, disperse and compete sexually with their wild counterparts to induce sterility in wild females.

In a phased conditional approach for such a programme, it is considered essential to estimate these quality control parameters (survival, dispersal, and competitiveness) under field conditions using mark-release-recapture (MRR) trials during the baseline data collection phase (phase I) before advancing to a pilot trial. MRR can also be conducted during the pilot and operational phases as a quality control tool but this is beyond the scope of this guideline. In case of low quality of the insects produced, it will then be necessary to determinate which step(s) of the of the sterile males' production process must be corrected. These can be related to colonizing of the strain to mass-rearing, irradiation or handling procedures, among others. Another important result from MRR will be the estimation of the real density of the target population of mosquitoes. Other results of MRR's will allow informed decision-making on release densities, release frequencies and swath widths (distance between release lines). If possible, it is thus recommended to conduct this MRR in the same sites targeted for the pilot trials in the next phase.

The protocols presented in these guidelines are the results of lessons from collaborations with Member States preparing SIT pilot trials against *Aedes* species. They were developed to expose the problems encountered in order to provide useful insights to develop MRR protocols that should be adapted to each situation and research questions.

We also did not consider areas where two *Aedes* species co-occur, which make egg analyses more complex. With the extension of the number of ongoing projects, more information on field trials will become available and this guideline will be updated regularly. This version is an update of version 1 (FAO/IAEA 2020).

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1. Objective

The objective of these guidelines is to provide guidance to implement Mark-Release-Recapture (MRR) experiments to evaluate sterile male dispersal capacity, survival rate and their field competitiveness in an open field setting, as well as the density of the wild population. The protocols presented below have been tested in the field for *Aedes albopictus* in Tirana, Albania (Velo et al. 2022) and northern Italy (Bellini et al. 2021). For *Aedes aegypti*, this methodology was tested in Juazeiro, Brazil (Bouyer et al. 2020c) and Havana City, Cuba (Gato et al. 2022, Gato et al. 2023). However, each ecological situation, each mosquito species and each set of particular environmental conditions require an adapted protocol. The major originality of the presented protocols is that they allow measuring competitiveness (Bouyer and Vreysen 2020), by estimating the ratio of sterile to wild males and the induced sterility of females in the same sites.

Estimating these entomological parameters is crucial for any SIT programme and should be carried out during the feasibility phase since they are mandatory to define project needs in terms of sterile males production, release frequency and density, and swath widths between release lines or distance between release points (Vreysen et al. 2013, Bouyer et al. 2020a, WHO and IAEA 2020).

2. Marking of adult mosquitoes with fluorescent pigments

2.1 Marking of sterile males at room temperature (not chilled)

This protocol applies only for *Aedes albopictus* (Petric, personal communication).

A plastic Petri dish that contains about 2.000 male pupae, is introduced in a cardboard box (length x width x height: 12 × 12 × 18 cm; volume 2.6 litres) for emergence. The top side of the box is covered with a white netting and one feeder with 10% sucrose solution is placed on the top of each box as an energy source for adult males.

A manual insufflator is used to dust the fluorescent pigment (RADGLO® JST, Radiant NV, Houthalen, Belgium or DayGlo® Color Corp., Cleveland, USA) through the netting at a dose of about 300 mg per 1000, 2-3 day-old males (Hygienic vaginal douche, Farlin® Industrial Co., Taiwan) *(Figure 1*). To accelerate male movement through the created fluorescent powder mist, the box should be gently shaken for two minutes.

Dusted males are released after a few hours of resting or after transport in the field. This delay allows male mosquitoes to groom, thus reducing the amount of dust on their body. Males not taking flight after 30 minutes are considered as non-fliers or dead and counted. A sample of about 300 males is randomly taken from several boxes and examined under a fluorescent stereomicroscope to check that they are all coloured.

The time to mark one box is one minute for one person, considering that two operators are working together, and that several boxes are shaken at the same time.

Figure 1. Procedure for marking mosquitoes with fluorescent dust. A. Weighing of the dust and introduction into a manual insuflator; B. spraying of the mosquitoes within a plastic bag; C. box containing of marked mosquito placed into a transport coolbox.

The survival of male *Ae. albopictus* marked with this protocol was compared with that of a control group in laboratory conditions and no difference was observed (Table 1).

Table 1. Mean Aedes albopictus male age at death (survival) in the treatment and control groups (Source: Petric, pers. Com).

*Replicate**	*Survival (days) Treatment**		*Survival (days) Control*	
	mean	*SD*	*mean*	*SD*
1	*37.9*	*±18.69*	*40.78*	*±16.98*
2	*41.06*	*±18.89*	*39.4*	*±20.54*
3	*39.64*	*±16.72*	*34.74*	*±19.58*
total	*39.53*	*±18.05*	*38.46*	*±19.10*

This protocol has been the reference in mosquito ecology for many years, but it has showed several drawbacks (i.e. the heterogeneous marking of the males and the loss of large amounts of fluorescent dye during the treatment). Also, it can take quite a lot of time and therefore it is recommended to use the chilled method below, especially if large numbers of males need to be marked (>10,000).

2.2 Marking procedure for chilled sterile males

This protocol applies to both *Aedes aegypti* and *Aedes albopictus*.

The dust for each container must be individually weighed on an analytical balance and then transferred to the container and closed. Sterile male mosquitoes are dusted in groups by applying 5 mg of fluorescent pigments (RADGLO® JST, Radiant NV, Houthalen, Belgium or DayGlo® Color Corp., Cleveland, USA) per 1,000 adult males in a 100mL cylindrical container (5.5cm of diameter and 7cm of height, see Figure 2). Batches of 2,400 sterile males can be marked together by increasing the size of the container and the quantity of dust (1L container, 11.5cm of diameter and 13.5cm of height, for 12mg of dust). To ensure that dust adheres to the walls of the dusting container, the internal surface must be rubbed with sandpaper to create a rough surface. The container must then be shaken vigorously to coat the inner surfaces evenly. After that, containers need to be transferred to a cold room (4°C) and left to acclimatize for at least 30 min.

Sterile male mosquitoes are then transferred to the cold room for immobilization for 10 minutes at 4-7°C. Mosquitoes are then introduced into a pre-dusted container and the lid closed. The container must be rotated for 10 seconds (equating to approximately 20 rotations) to coat the sterile males uniformly. It must be turned around and shaken after the ten first rotations.

Using this procedure, 70,600 sterile males were marked in 23 minutes by two persons during a MRR trial conducted in Brazil (24th March 2018).

Figure 2. 100 mL cylindrical containers containing 100 mosquitoes and increasing amounts of dust (source: N. Culbert 2019).

For aerial release, it is recommended to mark mosquitoes immediately before release, when mosquitoes are chilled for handling and transport to the release site. It is highly recommended to reduce the chilling time for this operation as much as possible (and keep it below 30 min by adjusting the number of mosquitoes to be marked at once). The dusted mosquitoes can be transferred to release containers taking into account their dust colour and then packed into a cool box for transportation to the field site.

The dose of dust applied with this technique is 60 times lower than in the procedure described in section 2.1. This is important because marking the mosquitoes with dust when they are chilled increases its toxicity, i.e. chilling opens the respiratory spiracles (Parker A., personal communication) allowing the dust to enter the trachea. Any amount above 5mg/ 1000 adults was found to decrease the mosquito survival significantly (Culbert et al. 2020).

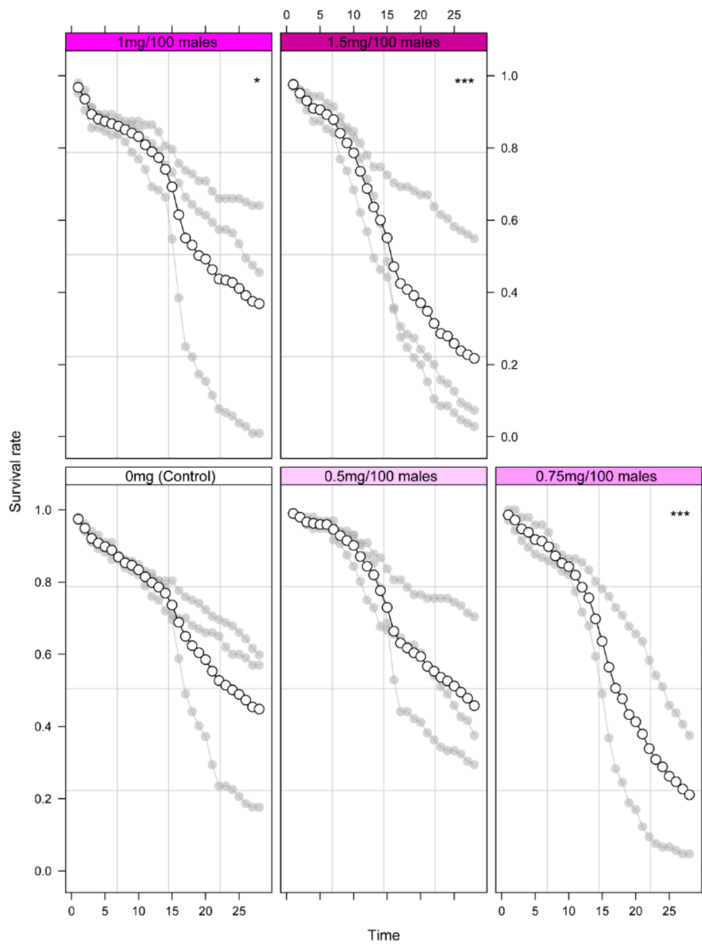

Figure 3. Survival rate of adult male Aedes aegypti *in relation to the amount of fluorescent dust (pink DayGlo) in mg per 100 adults. Source: modified from (Culbert et al. 2020).*

3. Mark-Release-Recapture protocol for ground releases

3.1 Study area

The study area should be representative of the area where the pilot SIT trial will be implemented and the target mosquito species should be present and well established. For this type of MRR trial, two areas should be selected: a target and a control area and they should be similar (in terms of mosquito population dynamic, environmental setting, etc). The target area should be isolated as much as possible and with only one *Aedes* species present. This should have been previously assessed by monitoring activities. The MRR area should be at least 20ha in large cities but can be smaller in an isolated village. The control area should be located at least 2km from the release area and no releases will be carried out in this area.

Field data collection on population dynamics and eggs fertility should be started with ovitraps, BG traps and/or Human Landing collection (HLC) at least two weeks before the release of sterile males in the study and control areas but the longer the better. Fertility of wild population eggs will be assessed by standardised hatching procedures (ANNEX 1: PROTOCOL FOR EGG EMBRYONATION & HATCHING) in both sites during the MRR trials.

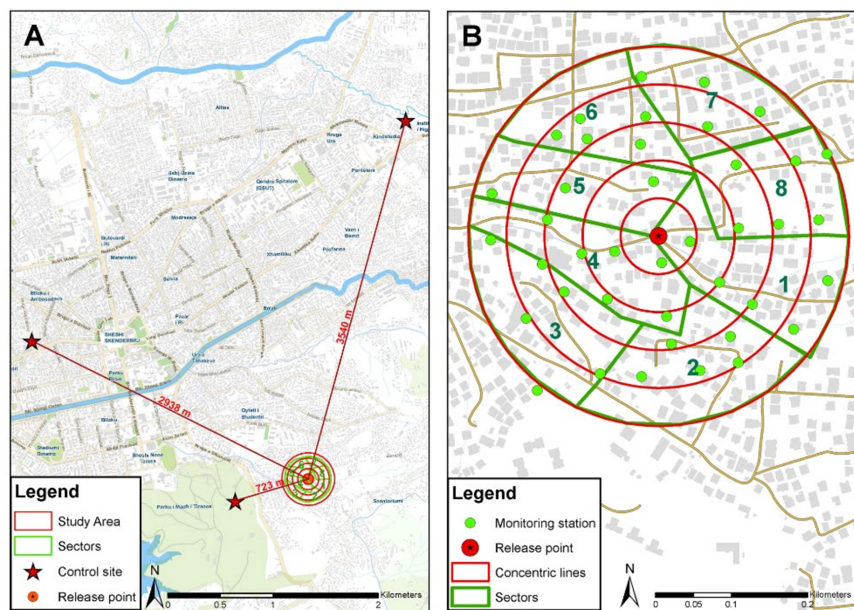

Figure 4. Example of an MRR study area in Tirana. A. Location of the release and control sites. The red stars represent the control sites, 723, 2938 and 3540 m from the release site. B. The study area was divided in sectors for simultaneous monitoring by different teams (green lines). The central point corresponds to the sterile males' release site. Concentric red lines represent five annuli at 50, 100, 150, 200 and 250 m from the release point (red point in the centre). Source: modified from (Velo et al. 2022).

3.2 Mosquito release

The dusted males are released as 2-4 day-old adults depending on what is planned for future operational SIT trials. After the marking procedures, the male mosquitoes are transferred to an isothermal box and transported to the study area for release. In the case of unchilled adults (marking protocol 2.1), the temperature and relative humidity in the box should be 21°C and 70% RH. The cardboard boxes or cages are left open for about 10 min allowing the males to fly out (Figure 5).

In the case of chilled adults (marking protocol 2.2), the temperature and relative humidity in the box during transport should be 8-12°C and 80% RH. Once in the study site, containers are exposed to the sun and gently shaken for about 10 min, to induce the males to exit (Figure 5). It is also advisable to put the container in a larger one with walls of at least 10 cm to prevent mosquitoes from escaping without flying.

The males that remain in the cages or in the containers after 10 min are considered in the non-fliers / dead counts. Different colours should be used to differentiate release sites and release times.

It is difficult to discriminate between yellow and green, between orange and red, and between red and violet, and even more for combinations of colours. It is thus recommended to reduce as much as possible the number of colours used simultaneously. If necessary, combinations of single colours like yellow, pink, and blue can be used.

It is suggested to release at least 10,000 males for each release point / time. Males should be released at least once a week for at least four consecutive weeks. The MRR should be repeated during the various seasons when SIT will be conducted.

Figure 5. Release of marked sterile male Aedes albopictus from the ground in Albania (unchilled males).

Figure 6. Release of marked sterile male Aedes aegypti *from the ground in (left) La Reunion island and (right) Brazil (chilled males).*

3.3 Monitoring protocol

The distribution of trapping stations should be as homogeneous as possible, and traps should be deployed at a density of at least 2 per ha (Figure 9). The sampling stations should be deployed the day before the first release, and they should be georeferenced. At least 5 ovitraps, and if possible 30% of the number of traps used in the release site, should be set in a neighbouring area serving as control (2 to 5km from the release site).

In each sampling site, one BG Sentinel 2™ (Biogents, Regensburg, Germany) baited with dry ice (1kg) or CO_2 (throughput of 0.2kg-0.5kg/24H) is deployed together with an ovitrap (500 mL black polypropylene cups; hydroculture pot; 11cm diameter × 10cm height; Luwasa Interhydro AG, Allmendingen, Switzerland) lined with heavy-weight seed germination paper (#76, Extra Heavy Weight, Anchor Paper Co., St Paul, MN, USA; 40 x 9cm) and spaced 10-20 m apart. The traps can also be used without CO_2 but it will result in a lower recapture rate.

In countries without endemic circulation of arbovirus and after agreement by the local authorities, Human Landing Collection (HLC) can replace adult traps, giving similar results for the various entomological parameters measured (Velo et al. 2022). HLC sessions should be carried out during the period of peak mosquito activity (such as late afternoon in temperate climates and early morning in tropical environments). Ovitraps and BG-traps or HLC samples obtained in the same sampling station should be coded with the same station number.

Adult BG traps should be checked daily at the same time starting the day after release and they should be sampled until no marked mosquitoes are captured for two weeks. Ovitraps should be checked once a week until two weeks after the last release. The water of each ovitrap should be filtered using a coffee filtered a 100µm sieve to collect floating eggs, which can represent up to 50% of the eggs.

The study area should be divided in sectors that can be monitored simultaneously by different teams. The exact position of each trap should be decided in the field according to the characteristics of the local environment. Each team will be in charge of the deployment and the daily inspection of 5 to 10 stations depending on local conditions.

For HLC, one member of the team will collect adult male and female mosquitoes that are flying around them in each sampling station with a manual battery aspirator for 15 min. Only one operator per team should perform the HLC and the collected adults are recorded with an identification code referring to the sampling station and the collection date. Teams should be rotated between sectors.

Air temperature and relative humidity should be recorded throughout the study using a weather data logger placed in the study area and/or collected from a station situated in or near the study area (rainfall, wind speed and direction).

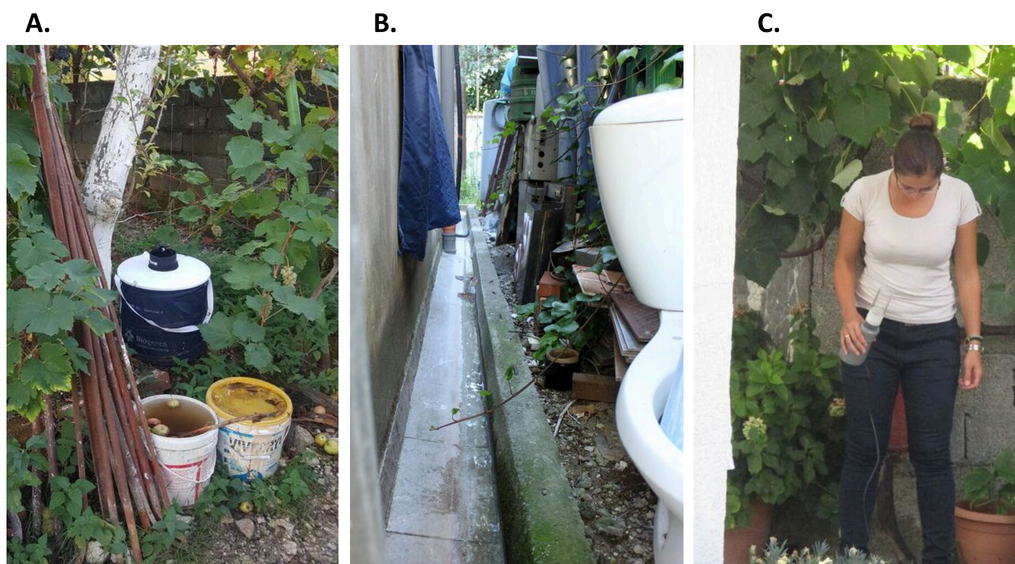

Figure7. BG-trap (A), ovitrap (B) and HLC session (C) as used in a trial conducted in Albania on Aedes albopictus*.*

3.4 Entomological analyses

Each team should be provided with an insulated thermal container that contains dry ice to store the adult mosquitoes sampled during the daily checks of the traps. If dry ice is not available, ice packs can be used. Collected adult mosquitoes can be analysed at once or stored overnight. Species and colour marks are identified under a stereomicroscope and using a 12 volts UV lamp or using a USB fluorescent microscope (Dinolite®) and a laptop.

The number of collected eggs on each paper should be counted under a stereomicroscope and the portions of paper with eggs cut out in stripes, stored, and kept for at least 7 days to allow the embryonic development and then, hatched according to the procedures described in Annex 1. Non-hatched eggs can be bleached or dissected to check for the presence of embryos.

Field data samples are analysed, classified, and stored into a database to calculate the distances between release and recapture sites.

3.5 General comments

In Albania, the data obtained with the BG-traps and the HLC were similar with respect to recapture rates, estimates of mortality rates, the sterile-to-wild male ratio, the wild population size and dispersal of the sterile males. These data indicate that both methods can be used to make these estimations. However, whereas BG traps are deployed for 24h periods and thus cover a full activity period for mosquitoes, the HLC is implemented for only 15 mins and should therefore be done during the peak activity periods of *Aedes*. This to minimize inaccuracies in the statistical analyses. Whenever possible, it is advised to use BG-traps baited with CO_2 in the case of *Ae. albopictus*. HLC will however be useful in areas of low transmission risk, with no electrical supply, or where traps can be stolen or damaged.

The competitiveness of the sterile males, the induced sterility and the ratio of sterile to wild males must be estimated in the same area. The sterile to wild ratio generally drops quickly within 200m from the release point (Figure 8), whereas the spatial trend in the induced sterility is much

smoother because inseminated females disperse more. This higher dispersal potential of females in comparison to males was demonstrated before with *Ae. aegypti* in Singapore (Liew and Curtis 2004).

This entails that traps should be deployed far enough from the release points (up to 500m) to enable an assessment of the decrease in egg fertility with distance. To obtain a good estimation of the competitiveness in the release area, it is thus necessary to release sterile males homogeneously during the MRR (every 50-80m depending on the dispersal distance measured) over the monitored area, but also to release additional sterile males at the same density in a buffer of at least 250m around the monitored section. Finally, the release frequency should be adapted to the estimated survival: if the mean survival is lower than seven days, two releases per week will be needed to maintain a more homogeneous ratio of sterile to wild males. Otherwise, the competitiveness will be underestimated because part of the wild female population will not be exposed to sterile males between the release events. Wild females immigrating from outside into the study area will also result in an underestimation of the field competitiveness. If the study area is not isolated, a ratio of sterile to wild males below 4 is recommended to reduce the impact of fertile female immigration on the underestimation of the competitiveness (Bouyer and Vreysen 2020). This is a complex issue and we recommend to read this paper (particularly box 1 and Table S2 for a demonstration).

Therefore, it is necessary to mark with different colours the males released in the centre of the study area and those released over the rest of the study area (like in the other example presented below) to assess dispersal and competitiveness simultaneously. A single release in the center of the monitored area will lead to a heterogeneous ratio of sterile to wild males which is sub-optimal to estimate competitiveness. A single release point is also sub-optimal to estimate dispersal as well, since directional attraction or heterogeneity of the landscape can influence the dispersal capacity. It is therefore recommended to release males in at least three release points using different colours.

It is also recommended to deploy ovitraps in the control and release area at least two weeks before the first release to make sure that the natural fertility is similar in both areas.

Ovitrap types may differ between regions and it is recommended to keep the same type during the MRR and pilot trial.

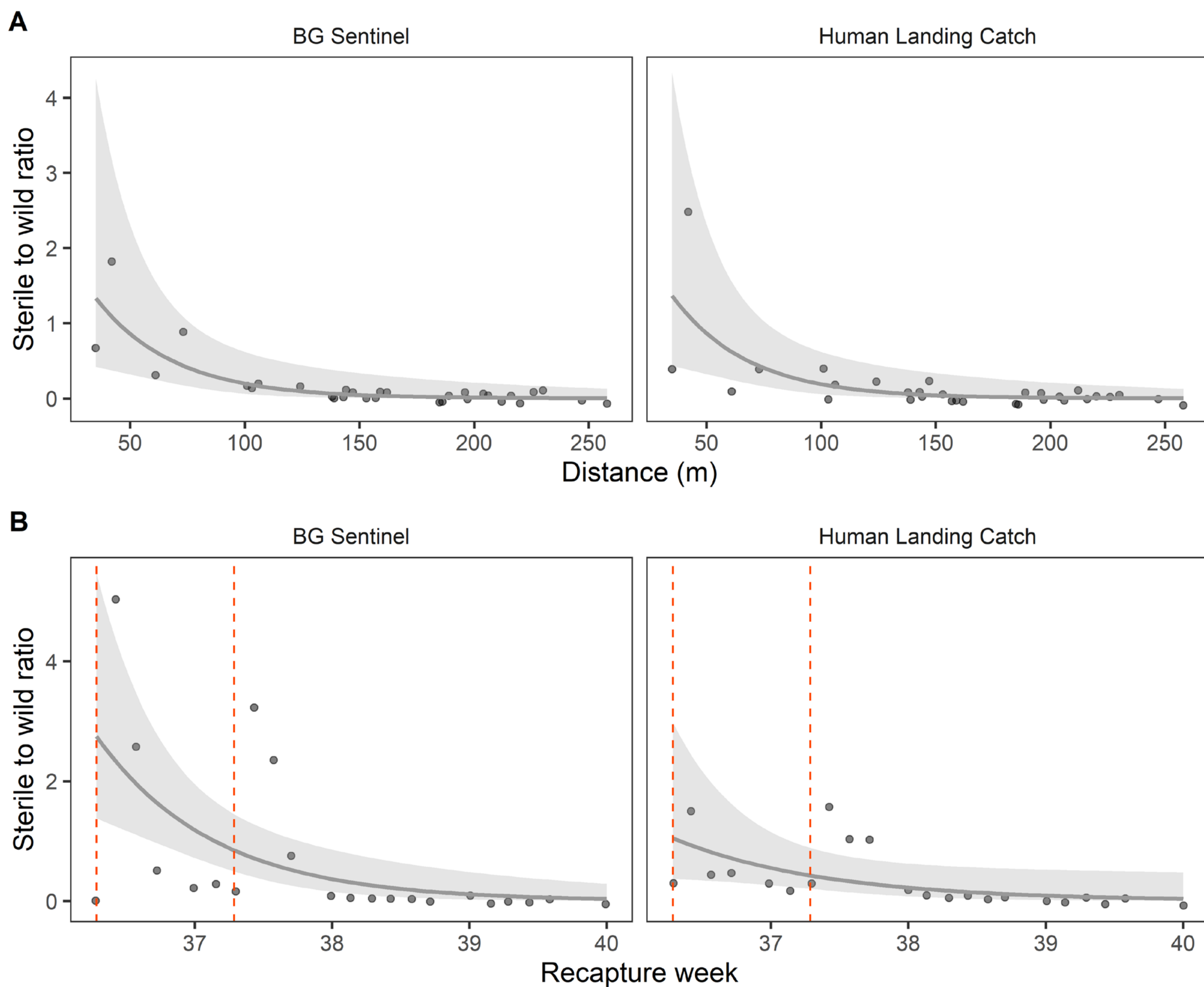

Figure 8. Dynamics of the ratio of sterile to wild males as a function of the distance to the release point (A) and time (B). Source: Velo et al. 2022.

4. Mark-Release-Recapture protocol for aerial release using drones

4.1 Mosquito release

Aerial releases of mosquitoes are ideally carried out with unmanned aerial vehicles (UAV) or drones. Stationary release points are used to estimate dispersal whereas line releases (Figure 9 and Figure 10) are used to estimate competitiveness. Mortality rates can be estimated in both scenarios. Point releases should be carried out at different altitudes (25-100m) to determine the optimal altitude regarding mortality and dispersal homogeneity considering the environmental characteristics and local regulations. The swath widths between release lines must be parametrised as the median dispersal. Line releases can also be carried out from the ground when no drone is available using this monitoring setting and, in this case, the distance between release points along the lines should be equal to the distance between lines. We recommend using batches of at least 10,000 sterile males for point releases by drone and 50,000 for line releases in a 20ha area.

The releases should be done early in the morning, especially in tropical climates, to avoid mortality and clumping of the mosquitoes.

Figure 9. Release area of 20 ha in Carnaìba do Sertão. The red point is the central release point, the red lines are the release lines with a swath of 80m and the yellow points are the monitoring stations (42 sites).

A. **B.**

Figure 10. (A) Aerial releases of sterile male Aedes aegypti *using a DJi Matrice 600 pro drone in Brazil (Photo WeRobotics) and (B) a custom-made drone in La Reunion island (Photo Pierre Marchal).*

4.2 Monitoring protocol

Prior to the day of the first release, battery operated BG-sentinel traps baited with CO_2 should be deployed in the study area, with a density of at least 2 traps/ha (as shown in Figure 9). In each of the trapping stations, one ovitrap is deployed in the vicinity of the BG trap at a distance of 10 - 20m.

In parallel, 5 ovitraps are deployed in the untreated control area (that should be located at least 2-5km from the release area) to assess the fertility of an untreated natural population during the release period.

Traps are checked every day starting the day following the release. Traps should be inspected every day during the same time frame, corresponding to 24h of operation. The collected samples are transferred to the laboratory. All mosquito catches are given an identification code corresponding to the relevant trapping station in order to calculate dispersal capacity. In the field, collected adults are immediately placed in an insulated storage container and placed in an ice box. They can be killed in a freezer overnight. Field collected samples are analysed, classified and data stored. Samples must be screened for colour under a UV-light stereomicroscope or using USB fluorescent microscope.

For a 20ha study area, traps should be checked by 4 teams simultaneously, each consisting of 2 staff and each team being responsible for monitoring 10 traps, depending on local conditions. Traps are monitored daily for a period of 2 weeks after the last release.

Eggs collected must be dried for 7 days and then hatched (ANNEX 1: PROTOCOL FOR EGG EMBRYONATION & HATCHING). Non-hatched eggs can be bleached or dissected to check for the presence of embryos. Release and recapture points must be geo-referenced using a Global Positioning System (GPS) device. All coordinates are entered into a Geographical Information System (GIS) to calculate the distances between release and recapture sites.

4.3 Lessons and recommendations

During a trial conducted in Carnaìba do Sertão, Brazil, it was shown that increasing release altitudes increased mortality of the released mosquitoes but also their dispersal. In France close to Montpellier (C. Jeannin, pers. Com) and in Vravrona, Greece (A. Michaelakis, pers. Com), good recapture rates (2-10%) were obtained with a release altitude of 25m. The release altitude might require adjustments based on local regulations for drone operations.

A density of 2 monitoring stations per hectare is enough to gather accurate data on the mortality, dispersal and competitiveness of sterile males. However, dispersal can be higher with drone release and the area where traps are set around the release point should be at least 20ha (or 250m of ray).

A MRR trial as the one described above will allow estimating the Fried index (Figure 6), but several recommendations can be made to improve it:

- The total duration of the releases should be at least 4 weeks;
- In the absence of a buffer area around the study area, with release of similar densities of sterile males than in the monitored area, the competitiveness is underestimated in this area due to the immigration of fertile wild females from the surrounding areas. Therefore, a buffer around the monitored section is necessary to prevent this bias. This buffer can be 250m to 1km depending on the environmental characteristics of the release area and on the dispersal capacity of wild females. Deploying ovitraps in this buffer will allow an assessment of the the emigration rate of sterile females outside the release area and conversely the immigration rate of wild females into the release area. Again, it is recommended to use a ratio of sterile males below 4 to avoid a strong underestimation of the competitiveness (Bouyer and Vreysen 2020).

Like for ground releases, it is recommended to deploy ovitraps in the control and release area at least three weeks before the first release to make sure that the fertility of the wild female population is similar in both areas.

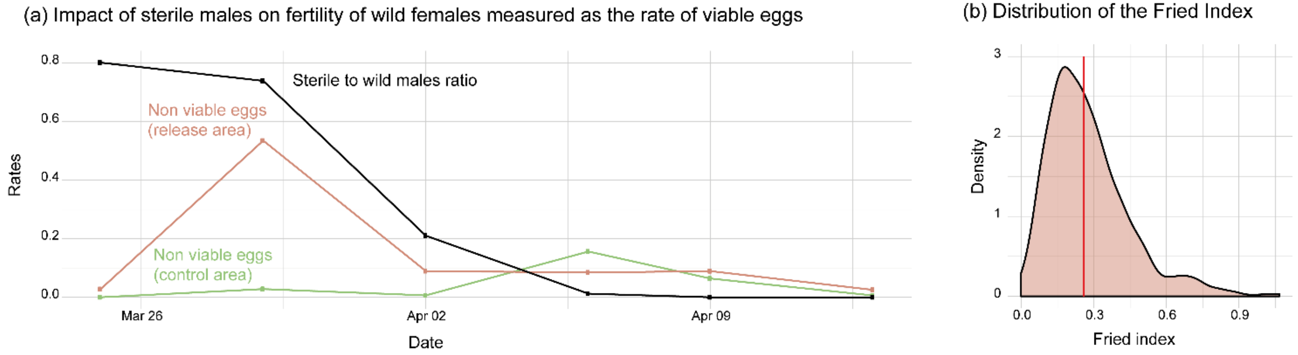

Figure 6. Induced sterility and sexual competitiveness of sterile males.
(a)Temporal dynamics of the sterile to wild ratio, and rates of viable eggs in the release and control areas. (b) Estimation of Fried index from 1,000 bootstraps in the distributions of sterile to wild males ratio in traps and viable eggs rates in ovitraps in the release and control areas (Density indicates the number of simulations with the given x value) Source: modified from (Bouyer et al. 2020c).

5. Statistical analysis

The preparation/organization of the data sets is of utmost importance for statistical analyses. Data sets must be accurate in order to allow precise and reliable statistical analysis. They can be analyzed using either an excel MS template spreadsheet (Carrieri 2020) or a customized R package (Muñoz 2021). The main parameters to be evaluated include:

i) **The Mean distance travelled (MDT)** of mosquito males, that can be estimated by drawing annuli 50m apart (depending to the dimension of the trial area) around the release site and applying a correction factor to accommodate for unequal catch densities in the calculation **CF.**
MDT = ∑(ER × d) /ERtot , where **ER** is the number of males recaptured in each annulus, **ERtot** is the total recapture and d the mean distance to the release point, **d = (Inner radius annulus + outer radius annulus)/2.** The estimated recaptures (**ER**) for each annulus is calculated as: **ER = (Fa/ nta)* CF,** where **Fa** is number of observed recaptures in the annulus and **nta** is number of traps in the annulus. **CF = (aa/at) * tt,** where **aa** is area of annulus; **at** is the total trapping area; **tt** is the total number of traps.

ii) **The mean dispersal distance (MDD)** is a simple estimation method calculated as: **Σ ni di / Σ ni,** where **ni** is the number of insects in trap **i** and **di** is the distance between trap **i** and the point of release.

iii) **The Flight Range (FR)** can be estimated through the linear regression of the cumulative estimated recaptures performed within each annulus (x-axis) on the **$\log_{10}$ (annulus median distance + 1)**. The FR50 and FR90 indicate the distance that comprehends the maximum flight distance reached by 50% and 90% of the individuals. These parameters are calculated from the equation of regression as the values 50% and 90% of the largest value of x, respectively.

iv) The survival rate of sterile males can be estimated by the linear corrected method (Harrington et al. 2001, Buonaccorsi et al. 2003) as follows: $\boldsymbol{\theta} = e^{a} / (N + e^{a})$, $\boldsymbol{S} = e^{b} / (1-\theta)^{1/}$d, where a and b are the regression coefficients of the linear regression of the log-

transformed captures as a function of time, N is the number of individuals released, θ is the recapture rate, d is the number of days after release, and ***S*** the survival rate. **The probability of daily survival (PDS)** is estimated by regressing $\log_{10}(x+1)$ of the number of recaptures against the day of recapture where the antilog_{10} of the slope of the regression line is the PDS (Muir and Kay 1998). Average life expectancy (ALE) is calculated from the PDS as $1/-\log_e$ PDS.

v) **The wild male population size** can be estimated using the standard Lincoln index $P = a(n+1)/(r+1)$, where a is the number of originally marked males, n is the total number of recaptures and r is the number of recaptured marked males.

vi) **The field competitiveness** can be assessed through the calculation of the Fried competitiveness Index: **C = ((Ha - Ee)/ Ee) / R**, where Ha = natural fertility in the control site (determined during the MRR feasibility study period), Ee = observed fertility rate, R = ratio of sterile over wild males. Also, a nonparametric bootstrap approach (Efron 1979) can be applied to obtain a confidence interval for the estimate of the Fried index as described in (Bouyer et al. 2020c). In brief, the data on fertility and ratio of sterile male over wild one are resampled without replacement, and for each set of resampled data, the Fried index is computed (1000 simulations). Assuming a symmetric distribution, the basic percentile method to get a 95% confidence interval is used. This method however, assumes that R is homogeneous over the release area which is possible only in the case of line releases. If R is heterogeneous, pulling the trap data will lead to the underestimation of C.

Moreover, the data obtained as presented above, can be used to estimate other parameters such as the diffusion coefficient, the diffusion time, the direction of dispersion, and the capacity to induce sterility (CIS) index. In addition, the data can be used to analyse:

- correlations between mosquito samples collected with BG traps and HLC;
- correlations between ovitrap data and females collected by BG traps and HLC;
- correlations between ovitrap data and males collected by BG traps and HLC;
- spatial heterogeneity of C;
- effect of time of the day on samples with HLC;
- estimates of trap efficiency per hectare per day for each trapping system.

Finally, it must be noted that the analyses presented here are based on the following assumptions (that are rarely fulfilled):

- marked and unmarked insects have equal probability of being trapped;
- marked insects intermingle with the rest of the population e.g. they are not territorial, and they are not altered by the marking;
- the marking does not adversely affect survival;
- the population is isolated (no immigration or emigration within the period of study) and stable (no births or deaths within the period of study);
- the marking is not lost during the experiment;
- sufficient time is required between release and recapture to enable random dispersal for all marked individuals through the wild population;
- recapture rates are high enough to make an accurate assessment.

To obtain better estimations of the parameters, the information generated in such MRR trials may be used to feed simulation models that can be much more flexible and may include an explicit representation of space, that can be modelled using remote sensing data (Haramboure et al. 2020, Cecilia et al. 2021, Douchet et al. 2021). This is the only method that will allow an optimized estimation of the various parameters altogether, since estimating mortality and dispersal independently for example is quite problematic and relies on hypotheses that are not necessarily fulfilled (like the isolation of the population under study). It is therefore advised to involve biostatisticians and/or modellers from the beginning of the study design to ensure a proper analysis afterwards.

ANNEX 1: PROTOCOL FOR EGG EMBRYOGENESIS & HATCHING

Eggs collected in ovitraps (on germination paper) are initially placed in plastic containers on sheets of absorbent paper to remove excess water.

Eggs are then counted directly on the substrate (germination paper) under a stereomicroscope 20-30X.

Stripes of oviposition paper containing eggs are cut. The stripes are placed inside 50 mL Falcon tubes with the lid unscrewed to allow air to enter, and humidity to escape, for one day. When all water has evaporated in the tube, but the paper remains humid, the tube is sealed. Plastic tubes containing the eggs are maintained at room temperature (23 °C) for seven days before starting the hatching procedure as follows:

- Field collected eggs are left to embryonate on their substrate for one week.
- In the case of *Aedes albopictus*, a given volume of hatching solution is prepared in the afternoon (18:00) according to the number of tubes. It consists of a solution of dechlorinated water with 0.36g/L of Nutrient Broth (CM0001 Oxoid Ltd., Hampshire, England) and 0.07g/L of Brewer's yeast (YBD-1KG, Sigma-Aldrich, St. Louis, MO).
- In the case of *Aedes aegypti,* the hatching solution consists of dechlorinated (or dechlorinated boiled-cooled) water with 2mL of 4% larval FAO/IAEA diet.
- The Falcon tubes containing the egg stripes are filled with the solution, tightly sealed and maintained overnight in laboratory condition or climatic room (ca. 28 °C).
- The following day the tubes can be opened, and eggs will be discriminated and assigned to three possible categories: 1-intact eggs (normally shaped); 2-hatched eggs; 3-collapsed eggs.
- The intact eggs need to be double checked by dissecting them with a mounted needle under a stereomicroscope to ascertain the presence of an embryo. Embryonated or hatched eggs are considered fertile whereas empty or collapsed eggs as sterile.

ANNEX 2: PROTOCOL FOR FIELD TRAPPING

Before starting:

1. Check whether all necessary equipment is prepared (cf. checklist in ANNEX 3: EQUIPMENT LIST)
2. Prepare labels for trap and HLC catches
3. Prepare labelled germination paper for ovitrap replacement

On field station:

1. BG trap are deployed in a shaded area protected from rain where possible, in the vegetation or nearby houses for *Aedes albopictus*, and can be deployed indoor for *Aedes aegypti* :

    a. Mount and deploy the trap
    b. For *Aedes albopictus*, place 1 kg of dry ice in a closed isotherm box (but with 2 small holes, diameter 5-6mm) and place the box inside the BG trap. A CO_2 dispenser based on compressed gas can also be used.
    c. Place the battery inside the BG trap and connect or plug in to a 220V source
    d. Check whether the fan works well
    e. Close the trap
    f. Place a label (piece of paper, 2×4cm) with station ID and start date
    g. Add the nets

    To collect the samples:

    h. Take off the net containing mosquitoes and close it, remaining at the entrance/sucking zone of the trap (without disconnecting the trap)
    i. Place the net in the dry ice box, avoiding direct contact with dry ice with a paper or plastic sheet
    j. Restart from bullet c.

2. Deploy an ovitrap

    a. Place an ovitrap in a shaded area at low elevation from the soil, 10-20m away from a BG trap
    b. Write station ID and start date on the seed germination paper sheet/paddle
    c. Place the germination paper in the ovitrap, sticking on its internal side
    d. Fill the ovitrap with water up to 2/3 level

    To collect:

    e. Collect the germination paper/paddle and place it in a plastic bag
    f. Filter the water with a coffee filter of a 100µm sieve
    g. Add end date and trap code on the plastic bag

h. Restart from bullet b
i. Check the presence of ants and take necessary action to minimise egg damages.

3. Perform HLC
    a. Place a black bag (ideally a 100L black plastic bag containing an empty cardboard box)
    b. Add station ID and start date on aspirator tube (catch chamber)
    c. Connect hand mechanical aspirator to its battery
    d. Catch all male mosquitoes flying around the bag and people during 15 minutes (do NOT shake bushes/vegetation in the surrounding)
    e. Perform this at proximity (in between) BG trap and ovitrap
    f. Place the catch chamber in dry ice box (to kill the mosquitoes and avoid longer flying in the tube)

Back at office/laboratory:

1. Store the samples adequately until counting and identification.
2. Clean up and store the field equipment safely
3. Recharge the batteries
4. Count and identify samples
5. Report data on forms and then in database.

ANNEX 3: EQUIPMENT AND MATERIALS LIST

- BG traps (x)
- Batteries for BG traps (2x)
- BG battery charger (check presence of adequate number of sockets or presence of multiple plug sockets
- 100mL urine cups (sugar feeding)
- Coffee filters (sugar feeding)
- Emergence bowls
- Larval diet
- Hand held sieves
- Counters
- Balance
- Irradiation canisters
- Irradiator
- Fluorescent dust (yellow, pink, green, orange, white, blue – DayGlo)
- USB Fluorescent microscope
- Tape (labelling) + Sharpies + forceps
- Petri dishes and forceps (QC)
- Ziplock bags
- Insulated containers (specimen collection)
- Dry ice (BG traps and for collecting samples) in the case of *Aedes albopictus*
- BG lure
- Hand held fluorescent light
- Stereomicroscopes
- Mouth aspirators
- Weather vane and anemometer (weather conditions)
- Killing boxes plus ethyl acetate (if authorized and no cooling system available to keep the collected specimen)
- Ovitraps and paper substrate
- Coffee filters of 100µm sieves
- Container where to maturate the eggs (see protocol hatch maturation)

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