Alma Mater Studiorum - Università di Bologna

DOTTORATO DI RICERCA IN
SALUTE, SICUREZZA E SISTEMI DEL VERDE

Ciclo 35

Settore Concorsuale: 07/D1 - PATOLOGIA VEGETALE E ENTOMOLOGIA

Settore Scientifico Disciplinare: AGR/11 - ENTOMOLOGIA GENERALE E APPLICATA

DEVELOPMENT OF SEXING SYSTEMS FUNCTIONAL TO MASS PRODUCTION OF AEDES ALBOPICTUS STERILE MALES

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Esame finale anno 2023

Abstract

Aedes albopictus is a vector able to transmit several arboviruses. Due to its high impact on human health, it is important to develop an efficient control strategy for this pest. Nowadays, control based on chemical insecticides is limited by the number of available active principles and the occurrence of resistance. A valuable alternative to the conventional control strategies is the sterile insect technique (SIT) which relies on releasing sterile males of the target insect. Mating between wild females and sterile males results in no viable offspring. A crucial aspect of SIT is the production of a large number of sterile males with a low presence of females that can bite and transmit viruses.

The present thesis aimed to find, implement and study the most reliable mechanical sex sorter and protocol to implement male productivity and reduce female contamination. In addition, I evaluated different variables and sorting protocols to enable female recovery for breeding purposes. Furthermore, I studied the creation of a hyper-protandric strain potentially able to produce only males. I also assessed the integration of artificial intelligence with an optical unit to identify sexes at the adult stage. All these applications helped to realise a mass production model in Italy with a potential weekly production of 1 million males.

Moreover, I studied and applied for aerial sterile male release in an urban environment. This technology could allow the release of males in a wide area, overcoming environmental and urban obstacles. However, the development and application of drone technologies in a metropolitan area close to airports, such as the Bertalia Area in Bologna, must fit specific requirements.

Lastly, I carried out a Short Term Scientific Mission inside the AIM-COST Action framework at Réunion Island, France, Indian Ocean, where I studied the Boosted SIT application. Coating sterile males with Pyriproxyfen may help spread the insecticide into the larval breeding sites.

Contents

1. Introduction

Aedes albopictus (Skuse), commonly known as the Asian tiger mosquito, is a mosquito species native to Southeast Asia that has spread to several continents mainly due to the international used tire trade (Battaglia et al., 2016; Benedict et al., 2007; Reiter and Sprenger, 1987).

The distribution of this species is influenced by environmental and demographic factors such as temperature, humidity, rainfall and urbanisation (Brown et al., 2014; Lounibos, 2002). Global predictory distribution maps calculated on such variables can accurately predict mosquitoes occurrence (Bhatt et al., 2013; Gething et al., 2011; Hay et al., 2006; Hijmans et al., 2005; Pigott et al., 2014a, 2014b).

This species has established in several countries (Kraemer et al., 2015) thanks to its high adaptive potential and the ability of its diapausing eggs to overcome winter seasons (Romi, 2001). Furthermore, its distributions rapidly expand thanks to climatic change (Kraemer et al., 2015; Liu et al., 2019) (Figure 1).

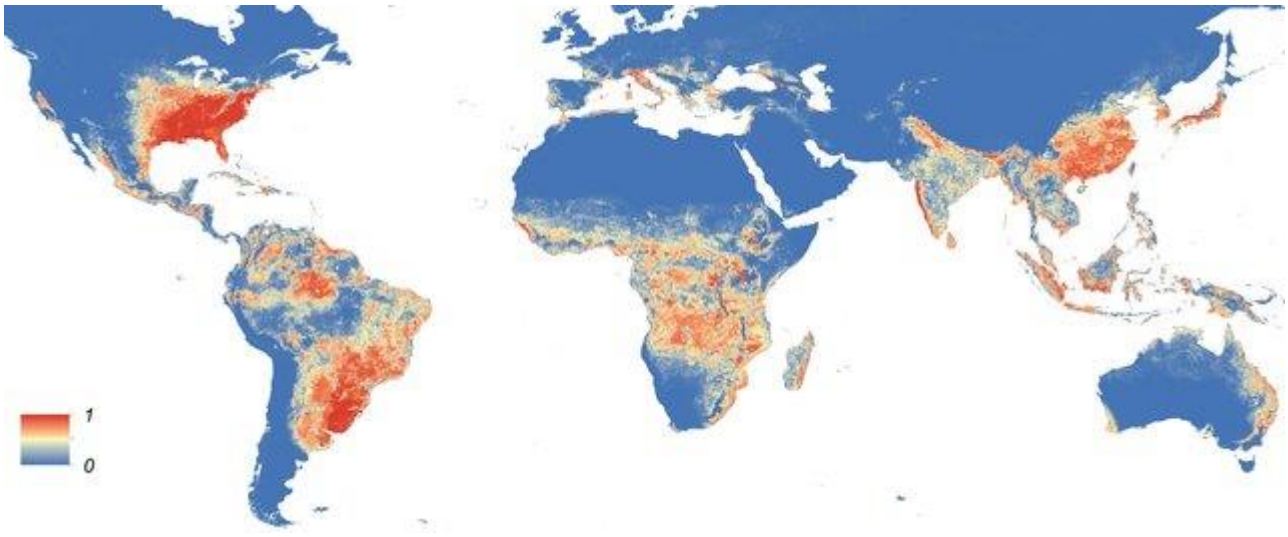

Figure 1. Global map of the predicted distribution of *Ae. albopictus*. The map depicts the probability of occurrence (from 0 blue to 1 red) at a spatial resolution of 5 km × 5 km. DOI: 10.7554/eLife.08347.009

This mosquito being anthropophilic and feeding during daylight hours can strongly influence the use of green spaces, especially within urban contexts which have the ideal conditions for its proliferation. Furthermore *Ae. albopictus* is a vector able to transmit viruses, such as Dengue, Chikungunya and Zika, to vertebrates, including human beings. For these reasons, this insect represents an element of considerable importance that strongly influences human health (Bhatt et al., 2013; Gardner et al., 2012; Staples et al., 2009; Weaver and Lecuit, 2015).

The primary control strategy of *Ae. albopictus* aims to reduce its field population density through insecticide application (Larramendy and Soloneski, 2012) using chemicals, microbial larvicides, and growth regulators (Achee et al., 2019; Bellini et al., 2009; Poopathi and Abidha, 2010).

Mosquito control using insecticides and potential larval breeding site removal and information campaigns gave only partially satisfactory results (Bonizzoni et al., 2013) (Figure 2).

Figure 2. Larval removal, adult, and larval treatments (CAA).

Furthermore, the increasing insecticide resistance strongly affects insecticide effectiveness (Moyes et al., 2017; Poopathi and Abidha, 2010). For these reasons, other control techniques are needed. The integrated approach of conventional vector control and biocontrol could offer a valuable strategy to maintain mosquito population density under control. Traditional biocontrol approaches such as larvivores, fish and copepods, bacterial and fungal pathogens, and endosymbionts could be used (Huang et al., 2017; McGraw and O'Neill, 2013). Another interesting biocontrol approach could be the mosquito genetic control strategies (GCSs) (Achee et al., 2019) (Figure 3). These technologies have become an important research area because of their species-specificity, track record in targeting agricultural insect pests, and environmentally non-polluting nature (Papathanos et al., 2018).

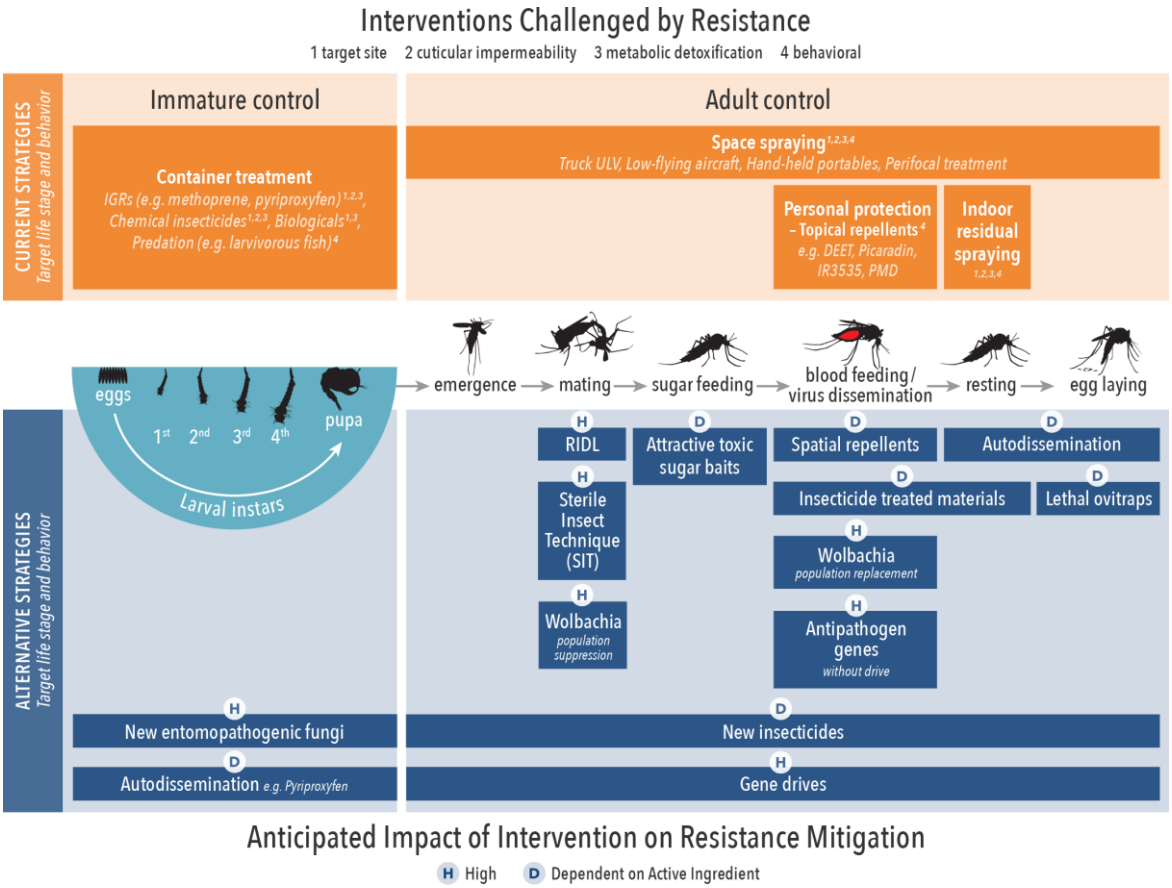

Figure 3. Genetic control strategies (GCS). Numbers indicate the kind of resistance that could occur for each specific treatment. Letters indicate the resistance mitigation of each specific GCS (Achee et al., 2019).

Since 2000, research projects on the application and development of the sterile insect technique (SIT) on *Ae. albopictus* have been ongoing in Italy (Bellini et al., 2007).

SIT involves the mass rearing, sterilisation and systematic area-wide release of sterile males of a target pest, in this case, *Ae. albopictus*. Mating with wild-type females by sterile males results in no offspring, leading to population decline (Dyck et al., 2021) (Figure 4).

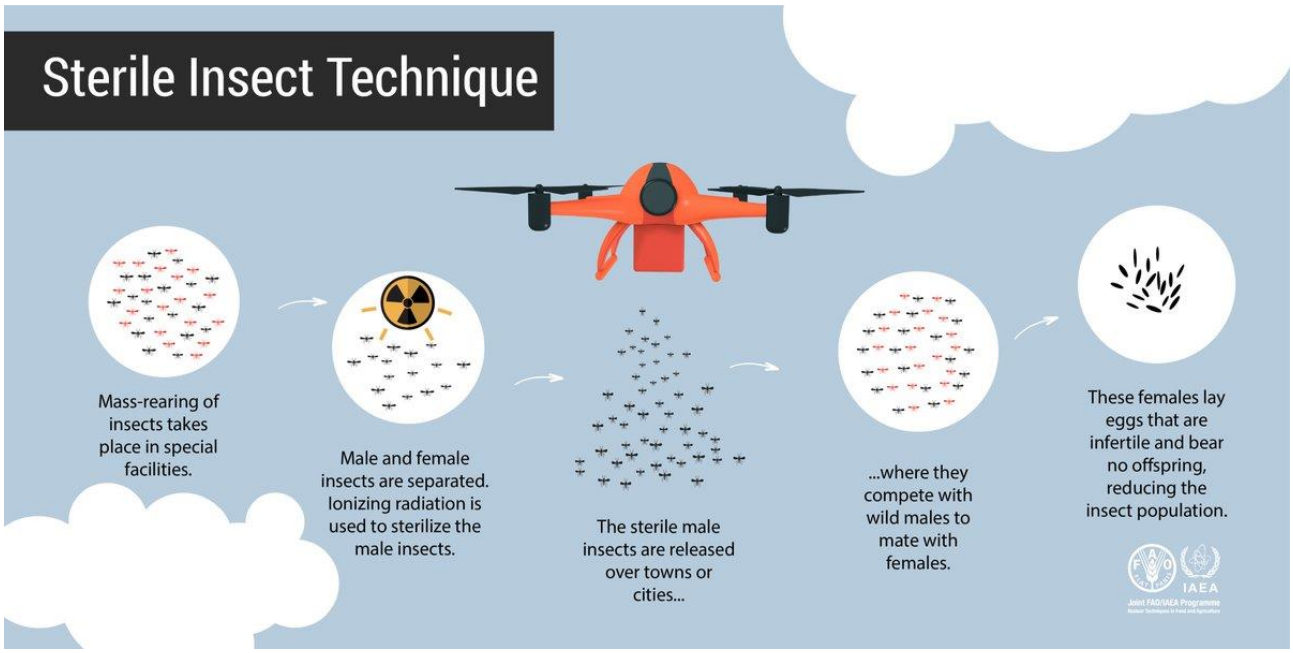

Figure 4. Sterile insect technique scheme by the International Atomic Energy Agency (IAEA).

Sterile male mass production encompasses several steps to properly rear the insect, including sex separation, which is crucial. The presence of females in the SIT release campaigns must be avoided or strongly reduced. Indeed, even if wholly sterilised, the females maintain their feeding activity and vectorial capacity (Aldridge et al., 2020; Cunningham et al., 2020).

Sex separation of *Ae. albopictus* can be effectively achieved by exploiting different biological traits at either the pupal or adult stage, such as protandry (Bellini et al., 2018) and dimorphism (Fay and Morlan, 1959; Focks, 1980; Sharma et al., 1972) or by

investigating classical genetic or transgenic methods (Gilles et al., 2014; Papathanos et al., 2018; Sakai and Baker, 1974).

Classical genetic or transgenic methods are based on the establishment of genetic sexing strains (GSS), which are laboratory strains of target insects whose genetics have been manipulated to allow efficient sex separation (Lutrat et al., 2019; Papathanos et al., 2018). They can be created using classical mutagenesis to select a marker such as a cuticle colour (McInnis et al., 2004), chemical or temperature sensitivity (Ndo et al., 2018; Yamada et al., 2012) to induce males production exclusively or by causing an androgenisation process (Aryan et al., 2020; Lutrat et al., 2022). GSSs have been generated in dozens of insect species, including mosquitoes, such as *Anopheles albimanus* (Wiedemann) (Kaiser et al., 1978), *An. arabiensis* (Patton) (Yamada et al., 2015), *Ae. albopictus* (Lutrat et al., 2022) and *Ae. aegypti* (Linnaeus) (Aryan et al., 2020) using insecticide resistance translocations that make males insensitive to the treatment (Lutrat et al., 2019; Papathanos et al., 2018) or transgenic expression of NIX locus (Aryan et al., 2019; Hall et al., 2015). However, environmental problems related to insecticides, inefficient selection, low production and the need for a mother colony have so far prevented their development (Yamada et al., 2015).

The difficulty in generating mutants using classical mutagenesis, in terms of time and laboratory activities, is shifting the focus to recombinant technologies. These systems are only used on a small scale, and questions about their scalability, stability, accuracy, productivity and operating costs remain unanswered (Lutrat et al., 2019; Panjwani and Wilson, 2016; Papathanos et al., 2018).

Even if the methods described allow effective sexing theoretically, changes and improvements must be made from the perspective of mass production.

These methods must comply with European regulations, which limit genetic manipulation, and must allow maintenance of the breeding line required for continuous production (Panjwani and Wilson, 2016).

Nowadays, the most employed sexing methods are based on mechanical sexing tools. These tools exploit the protandry, according to which males develop earlier compared to females, and rely on sex size dimorphism present at the pupal stage, whereby females are more prominent than males (Bellini et al., 2007; Gilles et al., 2014; Gouagna et al., 2020; Hamady et al., 2013; Iyaloo et al., 2020; Kavran et al., 2022; Mikery-Pacheco et al., 2015). The developmental rate of males, females and larvae is not synchronous, and it is time-related, determining the simultaneous presence of pupae of both sexes and larvae.

Mechanical sexing, and larval removal, can be performed using sieves, which are characterised by specific mesh sizes (Bellini et al., 2007; Sharma et al., 1972). An alternative is the Fay-Morlan separator (Fay and Morlan, 1959), with its adaptation (Focks, 1980) and mechanisation (Baton et al., 2020; Lees et al., 2021).

These tools help to collect male pupae with the lowest number of females. The fixed mesh size of the sieve, the thickness adjustment of the Fay-Morlan device, the operating procedures, and the sample variability are delicate aspects that can affect their efficiency.

We investigated and compared the efficiency of sieve and Fay-Morlan separators, testing their efficiency with different variables, such as adopted strains with varying numbers of generation and larval diets.

Country-based colonies were initiated from field-collected eggs to prevent concerns about the possible introduction of exotic genetic backgrounds (Benedict et al., 2009). Strains were mass-reared to sustain male mass production for each SIT program.

Each colony had different breeding generations and was reared on different larval diets during the period considered. In particular, the IAEA-BY diet (Balestrino et al., 2014), a standard diet used for rearing, was compared with two other diets, based primarily on bovine or swine liver powder, due to poor availability and high costs of IAEA-BY diet ingredients.

As already observed by Puggioli and colleagues (Puggioli et al., 2013), diet may influence male productivity and sex ratio. Possible interaction or effect by colonised strains has not been studied yet.

Strains from distinct countries and of different breeding generations could show variable productivity or residual female presence levels. In addition, a strain fed on different diets could modify its development, influencing the sex sorting operation(Puggioli et al., 2013; Sasmita et al., 2019).

Sexing tools used to separate immature stages with different degrees of development can recover only part of all the developed males since pupation dynamics are affected by rearing conditions(Medici et al., 2011).

During SIT programs, the maintenance of the breeding colonies could be based on different rearing routines and time schedules (Parker, 2005; Vreysen, Hendrichs and Enkerlin, 2007). At the Centro Agricoltura Ambiente (CAA) laboratory, the individuals employed to sustain the breeding lines came from the discarded material of the sex sorting procedure to better fit space and labour costs without maintaining a mother colony.

The extensive rearing and selection process could lead to inbreeding and laboratory adaptations affecting the quality of the reared strains (Drouin et al., 2022; Gargan et al., 1983; Ross et al., 2019). The strains, over generations, could vary their productivity affecting both male and female productions.

Other aspects such as genetic background, synchronous growth, temperature, humidity, larval density and diets (Couret et al., 2014; Kavran et al., 2022; Malfacini et al., 2022; Mamai et al., 2020; Sasmita et al., 2019) may determine a variation in the development parameters compromising the quantity and quality of sorted males.

Finding optimal larval density in rearing should be possible to increase male productivity yield (Couret et al., 2014; Lyimo et al., 1992; Mamai et al., 2018; Puggioli et al., 2017). Increased male productivity in a standard rearing protocol may increase

male production and decrease egg consumption. Then, I analysed two different larval densities, one lower than the other, hypothesizing that a reduced number of larvae may allow better growth and increase productivity.

The overall feasibility of SIT is not only connected to male production and female contamination. Indeed, the self-sustainment of mass rearing should be guaranteed. Mosquito egg production should be guaranteed to sustain the larval colonies. The balance between eggs and sterile male production must be appropriately managed if a mother colony is not available or egg production is not optimised (Vreysen et al., 2007).

Mother colony management requires dedicated operators, space, facilities and a separate line of larvae production. In addition, adults reared in a mother colony must produce eggs for mother colony sustainment and male mass production. Indeed, adopting a mother colony can reduce the adverse effects of colonization. Still, its scale-up is not easy and requires a significant investment in money, facilities and technicians (Hendrichs and Robinson, 2021).

For these reasons, mass rearing usually produces males for field release and adults, both males and females, to sustain the breeding lines; this, during scale-up processes, allows cost reduction.

During sorting operations, female presence affects the residual female presence and can also limit cage production. Indeed, females placed into cages make eggs necessary to restart the larval cycle.

Usually, the mechanical sorting method is carried out 24 hours from the onset of pupation. This time can provide enough males for the release but not enough females for breeding purposes. This lack forces the larvae's reintroduction into rearing and increases labour, water, diet, and time waste. In addition, further sexing must be carried out after 24 hours to recollect females.

In this case, males' and females' productions should find a balance directly at the first sorting. Sex sorting must be delayed increasing the female amount. This fact leads many pupae to emerge. Adult emergence can consequently affect productivity but also female workplace contamination.

Since the improvement of egg production has not been achieved, which would lead to a reduced number of females required, other solutions are needed. It could be possible that sufficient females were produced by adopting a delayed sorting time of 48 hours from the onset of pupation. In this case, male productivity and female contamination would be affected.

The work here presented investigated sorting at 24 and 48 hours from the onset of pupation. Understanding if male productivity and residual female presence, in combination with female production for breeding purposes, could be effective for SIT application and sustainment.

Finding solutions that lead to a cost reduction, may result in an improvement in mass production quantity and quality. Due to the technological and technical advancements obtained during the thesis period, I improved male production and guaranteed good levels of productivity and female contamination.

Other applications, such as exploiting the protandry, ensuring a level of female contamination suitable for field release, or exploiting the high dimorphism present in adult antennas, may be valuable tools to achieve sex separation.

The use of natural protandry in *Ae. albopictus*, as the only method of sexing, does not allow to achieve an acceptable sex-ratio. However, targeted cross-breeding makes it possible to obtain a hyper-protandry strain in which premature males' development over females is higher, ensuring a level of female contamination suitable for field release. Using this strain would be possible to separate sexes based only on pupation timing avoiding any dimensional sorting system (Bellini *et al.*, 2018). Therefore, we started a new strain and continued the cross-breeding selection until sixteen generations to observe if the selection process could be effective and replicated.

Recognizing sex in many mosquito species during embryonic and early larval stages is highly demanding. On the other hand, pupal stages present morphological differences in the last abdominal segments between males and females detectable by microscope (Herbert H. Moorefield, 1951). However, this method requires a lot of time and experience and can increase mortality due to operator manipulation.

The adult stage has different characteristics that allow sexes identification: antennae, palps and the last abdominal segments shape. Again, sorting made by skilled technicians would be prohibitive in terms of mass breeding.

An automatic system able to sort sexes based on morphological differences at the adult stage would permit us to overcome issues connected to larval rearing, such as synchronous growth and pupation timing (Tuda and Luna-Maldonado, 2020). The realisation of an automatic machine able to sort males and females through an optical system could permit the revisitation of a mass production facility. The device must respect and process the adult stage, so chilling conditions should be ensured to keep the adults immobilised (Gómez et al., 2022; Sasmita et al., 2022; Zhang et al., 2020). As a recognising system, the crucial aspect of the machine, we focused on integrating artificial intelligence with an optical unit.

If this machine would be able to discriminate the sex correctly in the adult stage, this device would be potentially able to recover all the males from the rearing.

Since males' mass production has grown thanks to all the efforts reported (Figure 5), the manual release of many males into the field is reaching its limits due to the high labour cost, space storage required and dispersion inefficiency.

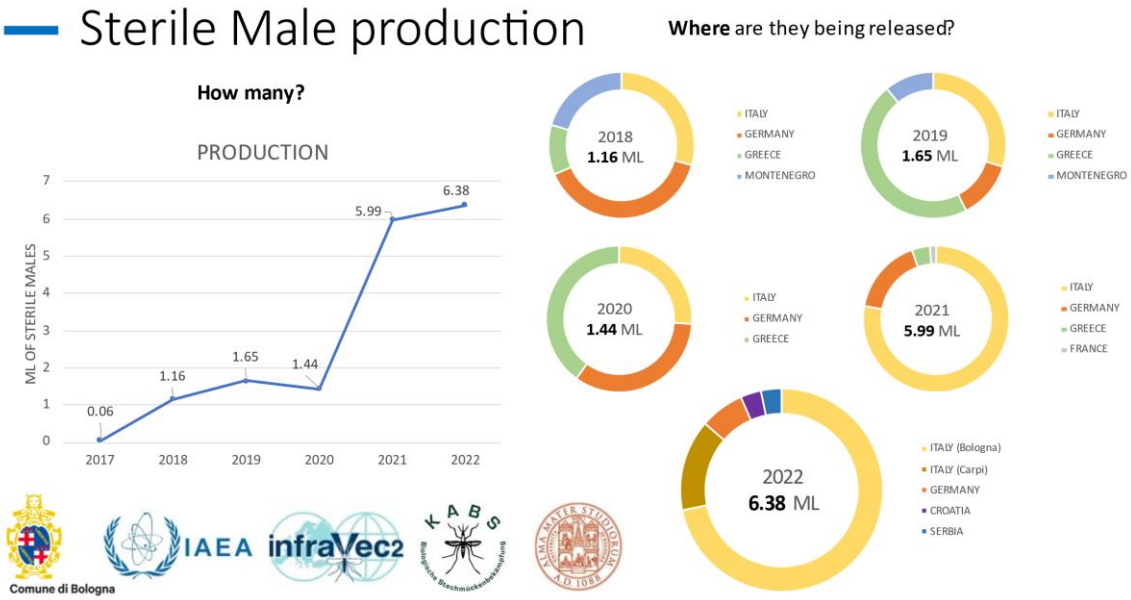

Figure 5. Sterile Male production over the years by Centro Agricoltura Ambiente. Different logos represents financer and supporters (CAA).

Currently, sterile males are released by releasing tubes. These devices, immersed in water, hold the pupae after irradiation. Adults can directly emerge inside the tube without manipulation. Once all adults have emerged, tubes can be removed from the water and transported to the release points, where they are opened. Field release when tubes reached a high number was carried by car (Figure 6).

New releasing alternatives must be adopted to reduce cost, time, space and labour.

Some field trials have been conducted with drones (Bouyer et al., 2020; Marina et al., 2022). In this case, an uncrewed aerial vehicle can be equipped with a release system. The drone can be a viable alternative to cover homogenously a wide area. In addition, drones can overcome environmental and urban obstacles. However, to permit sterile male loading, the males must be chilled under refrigerated conditions (Sasmita et al., 2022; Zhang et al., 2020).

Figure 6. Adult mosquito releasing tube system and car release (CAA).

However, drone use in urban areas, where mosquito control is carried out, has strong limitations to drone flight. Even if release efficiency is good, still a strong effort must be carried out to make it well accessible and functional for practical use.

Indeed, different authorisations, operational risk evaluation and drone conformity must be assessed. In addition, drone pilots must have specific licenses to carry out the flight.

The drone's male release is a complex activity that has to be appropriately intercalated in a well-defined urban context. The realisation of specific drones with specific features that allow the overflight of inhabited areas could enormously expand the potential of this technique.

We tested a releasing prototype developed by the International Atomic Energy Agency (IAEA) in laboratory and field conditions. The experiments that we carried out highlighted the potential of drone release and opened the doors to the first aerial release of sterile males in urban areas in Italy (Figure 7).

Figure 7. Aerial field release of *Ae. albopictus* sterile males in Bologna City (Orsi, S., 2022)

During my PhD, I won a grant to carry out a Short-Term Scientific Mission (STSM) inside the AIM-COST framework action (*Aedes* invasive mosquito - European Cooperation in Science and Technology) at Réunion Island, France. The STSM entitled 'Improvement of technologies serving the Sterile Insect Technique against *Aedes albopictus*' aimed to improve my knowledge and the collaboration between Italy, France, and the International Atomic Energy Agency. La Réunion Island is an overseas department of France located in the Indian Ocean.

The host institution was represented by CIRAD, at the Pole De Protection Des Plantes, in Saint-Denis and Saint Pierre. CIRAD is a French research institution focused on Environmental and Agricultural research, where I joined the REVOLINC team, a workgroup focused on applying the Boosted SIT against *Aedes* species.

The Boosted SIT project aimed to strengthen the SIT technique by coating released sterile adult males with pyriproxyfen (PP). PP-coated sterile males mating with wild females or even only touching them can contaminate the females and their larval habitats, improving SIT to control the target population.

Since a full larval treatment of the breeding sites is hard to achieve, especially in private yards, Boosted SIT may help in an urban environment, to vehiculate PP into larval breeding sites not easily accessible by pest control companies. The integration of Boosted SIT, also with biological insecticide, may offer an effective tool for integrating the control of *Ae. albopictus*.

Covering sterile males with insecticide, even if active only in the larval stage, may affect the quality and survival of the adults. The work presented was so mainly focused on estimating and comparing the effects of Boosted SIT and standard SIT in field conditions.

The presented thesis aimed to find, implement and study the most reliable mechanical sex sorter and protocol to implement male productivity and reduce female contamination. I evaluated different variables and sorting protocols to enable also female recovery for breeding purposes.

The studies conducted during my PhD program not only aimed to find, test and improve alternative methods of sexing. As can be seen in the results and discussion sections, different parameters can affect sex separation. Mechanical sexing tools relying only upon size dimorphism do not consider other parameters that may affect the separation. Other aspects such as genetic background, synchronous growth, temperature, humidity, larval density and diets (Couret et al., 2014; Kavran et al., 2022; Mamai et al., 2020; Sasmita et al., 2019) may determine a variation in the development parameters compromising the quantity and quality of sorted males.

We also studied a new method of sterile male distribution with the drone that could ensure a more effective releasing system, especially when productivity will increase

and handling a large number of males will be highly required. I also had the opportunity to investigate the integration of insecticide with SIT technology.

All the studies presented here aim to offer new solutions to simplify the implementation of SIT on a large scale. These project technologies are advanced and widely used in different industrial sectors. Fusing these with a genetic control program could significantly reduce *Ae. albopictus* presence.

2. Materials and Methods

2.1. Evaluated parameters and statistical analysis

The effectiveness of different mechanical sex separation methods, according to the reared strains and used diets, was evaluated by the male productivity yield and the residual female presence together with the sorted males.

The productivity yield was calculated as the percentage of male pupae collected on the original number of first instar larvae. The productive yield, been related to the total number of larvae, was multiplied by two, assuming an equal proportion of males and females, to obtain the male productivity yield on the total number of reared males (Crawford et al., 2020).

$$\text{Male productivity yield } \% = \left[\frac{\text{No. males}}{\text{No. larvae}} * 100\right] * 2$$

'No. males' is the number of male pupae after sex sorting, and 'No.larvae' is the number of larvae present in the trays at the beginning of rearing. The ratio of 'No.males' to 'No.larvae' represents the productivity of male pupae over the reared larvae.

Residual female presence was estimated by evaluating the sex ratio in a randomly collected sample of about 300 pupae in each batch of male pupae collected after each mechanical sorting session.

$$\text{Residual female presence } \% = \left[\frac{\text{No. females}}{\text{No. pupae}} * 100\right]$$

Where 'No.females' is the number of females present in the sample and 'No. pupae' is the total number of pupae checked. The sex ratio observations were made by using a binocular stereomicroscope to analyse the tenth abdominal segment of each pupa (Herbert H. Moorefield, 1951). The numbers of total pupae and females were counted separately. The number of females found was divided by the total number of pupae

checked, and the result was multiplied by 100 to give the percentage of residual females.

In this study, the male productivity has been corrected based on the residual female presence to offer more truthful data on productivity, as reported below.

$$\text{Corrected Productivity \%} = \left[\left(1 - \frac{\text{R. f. presence}}{100}\right) * \text{M. p. yield}\right]$$

Where 'R.f.presence' is the residual female presence and 'M. p. yield' is the male productivity yield.

2.2. Mechanical sex sorting tools and procedures

After 24 or 48 hours from the onset of pupation, according to tested condition, larvae and pupae were sexed using calibrated metal sieves, Fay-Morlan glass plate separator (Guangzhou Wolbaki Biotech Co. Ltd) or an automatic version of Fay-Morlan separator (Centro Agricoltura Ambiente Srl).

When sieve sorting was employed, the material collected from each tray was transferred into buckets filled with tap water at 34 °C. The set temperature encouraged pupal emersion, while larvae tended to accumulate in the basal part of the bucket. A sieve with a 1,400 µm mesh size was placed inside the bucket over the water surface and left for 3 minutes. Male pupae pass through the sieve net while larvae and female pupae remain under the sieve. A large number of small larvae were able to pass the sieve together with the male pupae. The males passed through the sieve were then collected (Bellini et al., 2007, 2013b; Medici et al., 2011), while larvae present between males were manually removed (Figure 8).

Figure 8. Metal sieve and sieving procedure (CAA).

The Fay-Morlan separator (Guangzhou Wolbaki Biotech Co. Ltd) comprises two adjustable paired sheets of glass. Modifying the distance between the two sheets allows the separation of male and female larvae pupae by size. Samples were introduced in the upper part of the instrument, and through water washing, it was possible to spread the biological material through the glass plate. Following the set distance, it was possible to differentiate and separately collect larvae, male pupae and female pupae (Fay and Morlan, 1959; Focks, 1980) (Figure 9).

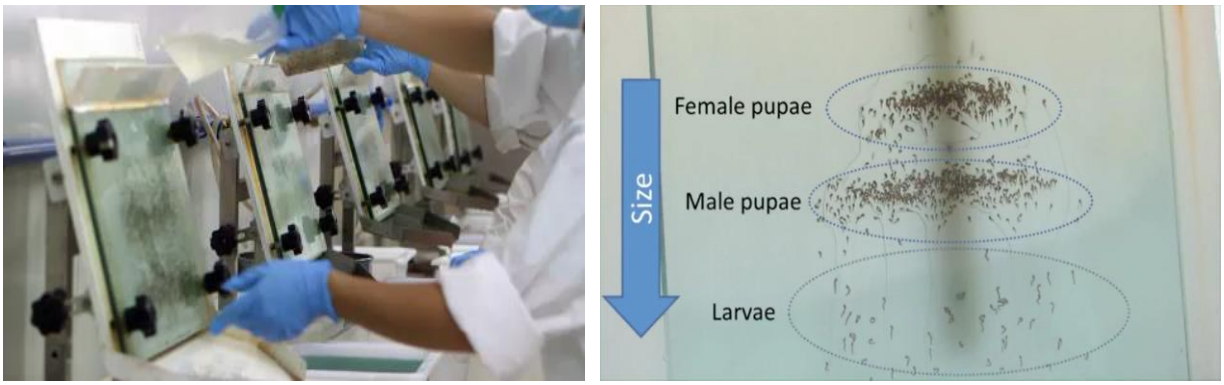

Figure 9. Fay-Morlan separator and material distribution (BRASIL).

The automatic glass plate separator is composed of the Fay-Morlan device of two glass sheets moved by a motor that repetitively alternates the distances through two supports in the bottom part of the glass. The two upper screws could be regulated only manually.

The machine is electrically supplied, and a graphic interface is present to set up the sorting parameters as product distribution, washing cycles, glass distances and speed (Figure 10).

Figure 10. Automatic Fay-Morlan separator front and back (CAA).

The device hosts a larvae and pupae blender, where the material is expelled directly in the opening present above the glass's sheets through a supply valve. After the first step of material distribution, two water jets clean, allow sample distribution and remove

larvae which come out of the glass and are kept by a collection tank and eventually conveyed into a dedicated tray.

The distances between paired glasses are then automatically adjusted following the set distance parameters to allow the male pupae collection. The collection tank changes position to gather the pupae into a different dedicate tray. Another washing cycle is then performed, and males are conveyed into the proper tray.

Distances between the glasses increase, and all the present material flows out of the separation table, falling into the collection tank, which conveys the female into their tray. The sorting cycle can then start again from the beginning.

All the material sorted as larvae, male and female pupae, are conveyed in trays in a collection tank at the rear of the machine. The trays present a fine net which allows water to pass, retaining the biological material. The filtered water is collected by the water tank and recycled by a water pump to wash the material between the paired glasses.

2.3. Sexing tool comparison with different strains

Ae. albopictus strains were established by collecting eggs from the field in Italy, Germany, Montenegro, and Greece. Samples of a few thousand eggs were provided by the German Mosquito Control Association, the University of Montenegro, and the Benaki Phytopathological Institute.

Each strain was reared in 40×40×40 cm Plexiglas cages under standard conditions at 28 ± 1°C, 85% RH, and a photoperiod of 14:10 (L:D) at the Centro Agricoltura Ambiente “G. Nicoli” Srl laboratory (Figure 11).

Figure 11. Larval rearing adopted.

Adults were constantly supplied with 10% sucrose solution, with the females offered swine blood daily through a thermostat-controlled device. Eggs laid on wet wrinkled paper were removed from the adult cages and placed, when dry, in sealed plastic boxes to maintain relative humidity close to 100% (Bellini et al., 2013b).

Eggs were counted using ImageJ software in order to precisely hatch the corresponding amount of L1 larvae for each rearing tray, considering a hatching rate of 85%. The desired number of eggs was then put overnight in sealed containers with nutrient broth solution and brewer yeast to induce hatching, as reported by Balestrino *et al.*, 2010 (Balestrino et al., 2010). First instar larvae were transferred to 35×25×10 cm plastic trays at a density of 2 larvae/ml of deionised water (Puggioli et al., 2017) and fed with the IAEA-BY 5% wt:vol diet (developed at the FAO/IAEA Insect Pest Control Laboratory)—36% bovine liver powder (MP Biomedicals, Santa Ana, CA, USA), 50% tuna meal (T. C. Union Agrotech, Thailand), 14% brewer yeast (SigmaAldrich, St. Louis, MO, USA), and, as an additive, 0.2% w/v of Vitamin Mix (Vanderzant Vitamin

Mix, Bio-Serv, Frenchtown, NJ) (Balestrino et al., 2014; Damiens et al., 2012; Puggioli et al., 2013).

A slurry of larval diet was prepared by manually mixing the solid components, consisting of small particles, in tap water. Using a volumetric cylinder diet was introduced in each rearing tray.

Since hatching, an increasing amount of larval diet was provided daily for each rearing tray (Bellini et al., 2013b; Puggioli et al., 2017).

Four different strains, ME F2-8, GR F1-8, DE F9-18 and IT F68-74, were reared on IAEA-BY 5% diet and sexed 24 h from the onset of pupation using a sieve or a Fay-Morlan separator.

Pupae so collected were volumetrically counted using a graduated cylinder with a net underneath to allow water drainage. The graduated cylinders were previously calibrated by counting and measuring male samples from each reared strain in order to avoid any possible influence due to dimensional differences in each strain. In addition, the sex ratio was checked. Data so obtained were used to calculate the male productivity yield and the residual female presence.

Two-way ANOVA analysis was carried out, with the male productivity yield and the residual female presence adopted separately as dependent variables while reared strain and sexing tool were adopted as the fixed factors. The interaction effect between the fixed factors was also analysed.

The analysis investigated differences in male productive yield and residual female presence using a sieve and a Fay-Morlan separator. Hypothesising that different strains with different breeding generations could influence productivity and residual female presence, I also studied the strain variable and its interaction with the sexing tool used.

2.4. Larval diet and strain influence with the Fay-Morlan separator

Ae. albopictus strains were established by collecting eggs from the field in Italy, Germany, and Greece. Samples of a few thousand eggs were provided by the German Mosquito Control Association, the University of Montenegro, and the Benaki Phytopathological Institute.

Each strain was reared in 40×40×40 cm Plexiglas cages under standard conditions at 28 ± 1°C, 85% RH, and a photoperiod of 14:10 (L:D) at the Centro Agricoltura Ambiente "G. Nicoli" Srl laboratory. Adults were constantly supplied with 10% sucrose solution, with the females offered swine blood daily through a thermostat-controlled device. Eggs laid on wet wrinkled paper were removed from the adult cages and placed, when dry, in sealed plastic boxes to maintain relative humidity close to 100% (Bellini et al., 2013b).

Eggs were counted using ImageJ software in order to precisely hatch the corresponding amount of L1 larvae for each rearing tray, considering a hatching rate of 85%. The desired number of eggs was then put overnight in sealed containers with nutrient broth solution and brewer yeast to induce hatching, as reported by Balestrino *et al.*, 2010 (Balestrino et al., 2010). First instar larvae were transferred to 35×25×10 cm plastic trays at a density of 2 larvae/ml of deionised water (Puggioli et al., 2017) and fed with one of the IAEA- BY 5%, BLP-BY 5% or SLP-BY 5% larval diets so composed:

1) IAEA-BY 5% wt:vol diet (developed at the FAO/IAEA Insect Pest Control Laboratory)—36% bovine liver powder (MP Biomedicals, Santa Ana, CA, USA), 50% tuna meal (T. C. Union Agrotech, Thailand), 14% brewer yeast (SigmaAldrich, St. Louis, MO, USA), and, as an additive, 0.2% w/v of Vitamin Mix (Vanderzant Vitamin Mix, Bio-Serv, Frenchtown, NJ) (Balestrino et al., 2014; Damiens et al., 2012; Puggioli et al., 2013).

2) BLP-BY 5% wt:vol diet—86% bovine liver powder (MP Biomedicals, Santa Ana, CA, USA), 14% brewer yeast (SigmaAldrich, St. Louis, MO, USA), and, as an

additive, 0.2% w/v of Vitamin Mix (Vanderzant Vitamin Mix, Bio-Serv, Frenchtown, NJ).

3) SLP-BY 5% wt:vol diet—86% swine liver powder (Mucedola Srl, Settimo Milanese, Milano, IT), 14% brewer yeast (Mucedola Srl, Settimo Milanese, Milano, IT), and, as an additive, 0.2% w/v of Vitamin Mix (Mucedola Srl, Settimo Milanese, Milano, IT).

A slurry of larval diet was prepared by manually mixing the solid components, consisting of small particles, in deionised water. Using a volumetric cylinder diet was introduced in each rearing tray.

Since hatching, an increasing dose of larval diet was provided daily for each rearing tray (Bellini et al., 2013b; Puggioli et al., 2017).

Each GR F1-8, DE F9-18 and IT F68-74 strain was reared on IAEA-BY 5%, BLP-BY 5% and SLP-BY 5% larval diet and sexed 24 h from the onset of pupation using a sieve or a Fay-Morlan separator.

Pupae so collected were volumetrically counted using a graduated cylinder with a net underneath to allow water drainage. The graduated cylinders were previously calibrated by counting and measuring male samples from each reared strain in order to avoid any possible influence due to dimensional differences in each strain. In addition, the sex ratio was checked. Data so obtained were used to calculate the male productivity yield and the residual female presence.

This analysis focused on evaluating the effects on male productivity yield and residual female presence determined using different strains and larval diets. Male productive yields were calculated for each strain reared with a specific diet, and the obtained data were then analysed. Residual female presence values for each replication of the different strains reared with different diets were calculated and used for the analysis.

Two-way ANOVA analysis was carried out, with the male productivity yield and the residual female presence adopted separately as dependent variables, while reared strain

and larval diets were adopted as the fixed factors. The interaction effect between the fixed factors was also analysed.

2.5. Sorting time influence using Automatic Fay-Morlan separator

Ae. albopictus strain colonies were established by collecting eggs from the field in Emilia-Romagna Region in 2020. The so-called 'RER' strain was reared in 80×80×20 cm mass production cages (Figure 13) (Balestrino, 2018, IT Patent No. 102018000002696; produced by A Zeta Model Sas), under standard conditions at 28 ± 1°C, 85% RH, and a photoperiod of 14:10 (L:D) at the "Experimental Module – Biological Control of the Tiger Mosquito" of the Centro Agricoltura Ambiente "G. Nicoli" Srl (Figure 12).

Figure 12. "Experimental Module – Biological Control of the Tiger Mosquito" of the Centro Agricoltura Ambiente "G. Nicoli" Srl (CAA).

Adults were constantly supplied with a 10% sucrose solution, with the females offered defibrinate swine blood daily through collagen sausage casings. Eggs laid on wet wrinkled paper were removed from the adult cages. When dry, eggs were gently

brushed and sieved in order to remove unwanted residues. Eggs were stocked into stainless steel cups in sealed plastic boxes to maintain relative humidity close to 100% (Bellini et al., 2013b).

The larval rearing was conducted in mass-rearing rack units holding fifty FAO/IAEA mosquito mass-rearing trays (Figure 13).

Figure 13. Adult and larval rearing (CAA).

To obtain a larval density of around 2.2 larvae/ml, the corresponding amounts of eggs placed in each rearing tray were directly encapsulated with larval diet (Zheng et al., 2015). Capsules were directly inserted into the rearing trays for the hatching, and data time was collected. Since hatching, an increasing dose of larval diet was provided until pupation. The sex sorting procedure was carried out 24 or 48 hours from the onset of pupation, using an automatised Fay-Morlan separator (Centro Agricoltura Ambiente "G. Nicoli", Bologna, Italy).

Pupae so collected were volumetrically counted using a graduated cylinder with a net underneath to allow water drainage. The graduated cylinders were previously

calibrated by counting and measuring male samples from each reared strain in order to avoid any possible influence due to dimensional differences in each strain. In addition, the sex ratio was checked. Data so obtained were used to calculate the male productivity yield and the residual female presence.

Sorting males 24 hours from the onset of pupation is impossible to achieve a good number of females to sustain the breeding lines. This sorting time necessitates re-inserting the residual larvae into the rearing and performing another sieving 24 hours later to collect more females for the adult rearing.

The analysis aims to asses if male productivity and residual female are affected by the two different sorting times, at 24 and 48 hours from the onset of pupation. Relying on a single separation at 48 hours would be possible to increase the number of females collected directly. If the sex ratio and productivity were acceptable, this would reduce labour costs.

One-way ANOVA analysis was carried out, with the male productivity yield and the residual female presence adopted separately as dependent variables, while sorting time was adopted as grouping variables.

2.6. Larval density influence using automatic Fay-Morlan separator

We used the same strain and laboratory conditions as the previous test in this trial. We evaluated the effect of two different larval densities on the rearing trays. In particular, we tested 1.8 larvae/ml and 2.2 larvae/ml. The corresponding amount of eggs was directly encapsulated with the proper amount of larval diet and directly inserted into the rearing trays. An increasing dose of larval diet was provided until pupation for both densities.

The sex sorting procedure was carried out 48 hours from the onset of pupation using an automated Fay-Morlan separator (Centro Agricoltura Ambiente “G. Nicoli”, Bologna, Italy).

Pupae so collected were volumetrically counted using a graduated cylinder with a net underneath to allow water drainage. The graduated cylinders were previously calibrated by counting and measuring male samples from each reared strain in order to avoid any possible influence due to dimensional differences in each strain. In addition, the sex ratio was checked. Data so obtained were used to calculate the male productivity yield and the residual female presence.

Increased male productivity, determined by using a lower larval density in the rearing tray, may lead to saving eggs, thus reducing the need for eggs could lead to a labour cost reduction.

The analysis aims to assess if male productivity and residual female are affected by the two different larval densities. Relying only on a single separation at 48 h would be possible to increase the number of females collected directly. If the sex ratio and productivity were acceptable, this would reduce labour costs.

One-way ANOVA analysis was carried out, with the male productivity yield and the residual female presence adopted separately as dependent variables, while larval density was adopted as grouping variable.

2.7. Implementation of a hyper-protandry strain through classical breeding

An *Ae. albopictus* strain was established from eggs collected in the Emilia-Romagna region, Italy. The colony was reared in 40×40×40 cm cages at 28 ± 1°C, 85% RH and 14:10 (L:D) photoperiod. Adults were constantly provided with a 10% sucrose solution. Blood was offered to females three times a week through a thermostat-controlled device. Eggs laid on wet wrinkled paper were removed from the adult cages and placed, when dry, in sealed plastic boxes to maintain relative humidity close to 100% (Bellini et al., 2013b).

The eggs were maintained for hatching for four hours in jars with water containing the hatching solution, constituted by nutrient broth and brewer's yeast. Jars were left closed overnight before the introduction of the eggs.

The larvae were introduced and reared in 35×25×10 cm plastic trays at a density of 1 larva/ml and were fed for four days from L1. An increasing diet amount was daily provided for each rearing tray (Bellini et al., 2013b; Puggioli et al., 2017).

At each generation, 200 first pupating male pupae and 200 last pupating female pupae were collected, sexed under the stereomicroscope and placed into 40×40×40 cm cages for mating and egg production (Figure 14).

Figure 14. Pupal sex identification (CAA).

The intermediate pupating pupae were discarded. At generations F1, F6, F11 and F16, we checked the number of males and females present at 24, 48 and 72 h from the onset of pupation. This way, we calculated the male and female productivity yields.

The use of natural protandry in *Ae. albopictus*, as the only method of sex sorting, does not allow achieving an acceptable sex separation in SIT application. However, through targeted crossbreeding, it is possible to obtain a hyper-protandry strain in which

premature males' development over females is higher, ensuring a level of female contamination suitable for field release at 24 h (Bellini et al., 2018). Using this strain would be possible to separate sexes based only on pupation timing avoiding any dimensional sorting system. This trial wanted to investigate further and assess if the selection process could be repeated with a different strain. Percentage data before the statical analysis have been transformed through arcsine transformation. Male and female productivity yields between other numbers of breeding generations have been compared, through one-way ANOVA, at 24, 48 and 72 h from the onset of pupation. Also, linear regressions have been performed to assess the presence of tendencies through the generations.

2.8. Feasibility study for the recognition of male/female adult mosquitoes with an optical system

This study wanted to analyse the effectiveness of developing an automated sexing system to discriminate sexes based on morphological differences at the adult stage.

The exploitation of the high dimorphism present in adult antennas may be used. Indeed, males have a strong plumage that could offer an effective trait for the optical system. We have then identified and calibrated an optical system associated with artificial intelligence (AI) (Figure 15).

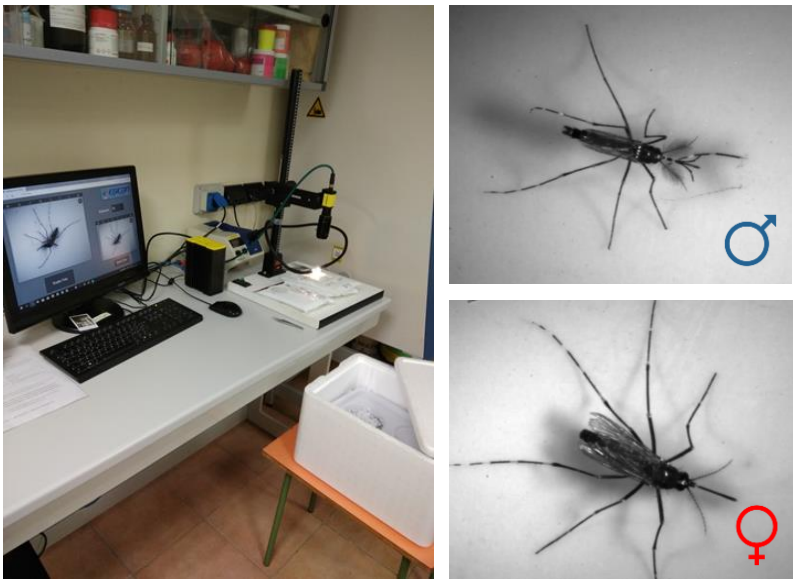

Figure 15. Recognition system and adults' sex classification.

For image acquisition, a two-megapixel vision system was set up and used with the same condition of light. The image collection was carried out for 500 adult male mosquitos and 500 adult female mosquitoes in different positions; live samples were immobilised using low temperature (8°C) in the cooling cell. A ceramic plate, conditioned at eight degrees to immobilise adults, was placed under the camera as an image collection field.

The collected images were used to train artificial intelligence in collaboration with EGICON Srl and SPECIAL VIDEO Srl.

This study aimed to assess if the integration of artificial intelligence with an optical system could offer a valuable alternative or improvement as a sex separator.

2.9. Prototype drone releasing tests for aerial distribution of sterile males

2.9.1. Laboratory test

Improving the release of sterile males using a remotely piloted aircraft system (RPAS) could offer an efficient tool to overcome problems related to urbanisation, ensuring a more efficient distribution of sterile males. However, loading sterile males through a dedicated RPAS system must fit specific technical requirements to be suitable for drone releases. High numbers of sterile males must be piled under refrigerated conditions to limit the number of flights required to cover the treated area, reducing labour costs.

We tested a releasing prototype developed by the International Atomic Energy Agency (IAEA) under laboratory conditions (Figure 16).

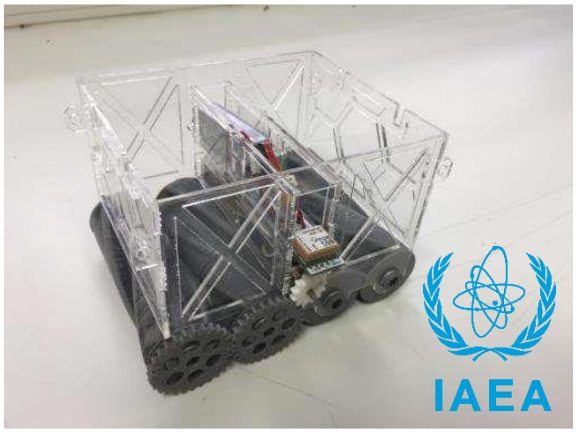

Figure 16. Releasing prototype mechanism developed by IAEA.

The release machine was composed of four different parts:

- Insect holding chamber: where chilled insects are loaded and kept until released.
- Release mechanism: two parallel 3D-printed cylinders at the bottom of the mosquito chamber turn in opposite directions by means of a stepper motor and a set of gears. The movement of the cylinders smoothly sucks the insects to the tiny groove in between them.

- Electronic board: the customised electronic board includes, among others, a microprocessor, a GPS antenna, a micro-SD slot, a micro-USB port, a LiPo battery connection, one probe for temperature and relative humidity and two status LEDs.
- Insulation: The release machine is surrounded by a layer of 12 mm of EPS for thermal Insulation.

A first laboratory trial was set up. Seven tubes filled with 1250-1500 adult males were chilled in an 8°C cooling chamber for each replica, both for the tests and the controls.

The control tubes were removed from the cooling chamber and directly opened into four 30x30x30 cages, while chilled mosquitoes in tested tubes were previously loaded into the releasing device (Figure 17).

Figure 17. Cages and prototype releasing system adopted.

The device was turned on to simulate the release into the treated 30x30x30 cages, and a 10% sugar solution was provided. To estimate the mortality connected to the release, we directly dropped the chilled males into a plastic cup, dead males or adults still present after 24 hours into the cup were considered dead, and mortality was calculated.

This machine has been designed to release sterile insects from the air in predefined georeferenced positions using an unmanned aerial vehicle (drone). Because of the payload limitations that a light drone can carry, the design and components of the release machine have been optimised to minimise the weight.

The release mechanism has been designed to avoid mechanical damage to the insects during the dosage. The volume of the insect holding chamber can vary in the different models of the machine according to the number of sterile insects to be released in one flight, depending on the insect species and characteristics of the release area. Similar models of the release machine have been designed for mosquitoes, moths and tsetse flies.

Sterile insects must remain inactive during the release flight. The release machine can host three small sachets of phase change material to maintain low temperatures that will transfer the cold to the mosquito chamber while melting during the flight. The release machine is surrounded by a layer of 12 mm of EPS for thermal insulation.

2.9.2. Field test

After obtaining results in laboratory conditions, we assessed the efficiency of releasing the device in field conditions. The trial was carried out in collaboration with AERMATICA3D Srl in the private courtyard of CAA laboratories.

The drone operation was conducted in the ‘open category’ scenario following the Italian Civil Aviation Authority restriction as reported by D-Flight.

The IAEA prototype and the DJI Matrice 300 drone have been used for the trial. The drone weighed 6.3kg (batteries included), adding 150 g of the releasing system and 50 g of the Li-Po battery. The total weight was 6.5kg lower than the maximum take-off weight of the drone. Once loaded with chilled mosquitoes, the IAEA releasing system was mounted through four fixed points at the drone.

The releasing prototype was used in the ‘constant release rate’ mode, which consists of a fixed turning speed of the cylinders; thus, the insect release flow is kept. The latency period was fixed at 15 seconds after powering on the device to give enough time for the drone to reach the release point. The speed rotation was increased to 2.24 rpm to accelerate the expulsion speed of the adults in order to decrease the permanence period inside the releasing device.

Two different field conditions were tested. The first trial was carried out in a wide close room with a maximum height of 15 meters to limit environmental wind conditions and easily collect dead males on the ground. The second field release was carried outside, and a 30x30 meters net was placed on the ground to collect dead males where the drone had released the males.

Mortality, release height, temperature, and relative humidity data were collected.

After one hour from the release, dead or unable-to-fly mosquitoes were counted as dead mosquitoes and used to calculate the mortality (Figure 18).

Figure 18. Drone with the releasing device and its application in field condition (CAA).

2.10. Boosted SIT trial in Réunion Island

The REVOLINC project in la Réunion Island aimed to strengthen the SIT technique by releasing sterile adult males coated with pyriproxyfen (PP), a technique referred to as Boosted SIT. PP-coated sterile males mating with wild females or even only touching them can contaminate the females and, subsequently, their larval habitats, leading to the improvement of SIT to control the target population (Bouyer and Lefrançois, 2014; Douchet *et al.*, 2021).

A semi-field trial was carried out in Saint-Denis to estimate the effects of Boosted SIT as compared to Traditional SIT on *Ae. albopictus*. The experiment was set up by arranging eight cages, where different ratios of sterile males (SIT♂), sterile males coated with pyriproxyfen (BSIT♂), fertile males (Wild♂) and fertile females (Wild♀) were used (Figure 19).

Figure 19. Semi-field cages, n.4 ♂BSIT and n.4 ♂SIT cages were respectively separated (CIRAD).

Adults were chilled at 6 °C for 30 minutes. Adults were then placed into dedicated sealed plastic containers of 150 ml. The sterilised adults selected to represent BSIT♂ have also been gently rolled into the sealed plastic container where a proper amount of pyriproxyfen and fluorescent powder was weighed (Figure 20).

Figure 20. Pyriproxyfen and fluorescent powder treating procedures (CIRAD).

After the chilling, all the plastic containers were quickly opened into the cages following the reported schema:

1. ♂ BSIT Treated Ratio 1:1:1 with 200 BSIT ♂ : 200 Wild ♂ : 200 Wild ♀

2. ♂ BSIT Treated Ratio 5:1:1 with 1000 BSIT ♂ : 200 Wild ♂ : 200 Wild ♀

3. ♂ BSIT Control with 200 BSIT ♂: 200 Wild ♀

4. ♂ BSIT Control with 200 BSIT ♂

5. ♂ SIT Ratio 1:1:1 with 200 SIT ♂ : 200 Wild ♂ : 200 Wild ♀

6. ♂ SIT Ratio 5:1:1 with 1000 SIT ♂ : 200 Wild ♂ : 200 Wild ♀

7. ♂ SIT Control with 200 SIT ♂: 200 Wild ♀

8. ♂ SIT Control with 200 SIT ♂

At each replica, the cage position was changed, and different operators managed the ♂BSIT and ♂SITs to avoid any possible contamination.

In each cage, a 5% sugar solution was provided to the adults. Blood was offered to females four times a week through a thermostat-controlled device, and a wrinkled wet paper was placed into each cage to collect eggs. The collected eggs were left to dry for one week to allow eggs' maturation. After eggs hatching, the residual fertility was calculated. Hatching occurred in the same water where the wrinkled paper came from to preserve and evaluate any possible effect related to pyriproxyfen.

The mortality of hatched larvae was assessed daily until emergence. Flight ability and mating competitiveness were estimated (Bouyer and Vreysen, 2020; Culbert et al., 2020, 2018).

A Plexiglas chamber of 18x18x41cm constitutes the flight ability device with an inner cylindric structure where the upper part is filled with 40 flight tubes (25 cm high, inside diameter of 8 mm) (Culbert et al., 2018; Maïga et al., 2022). At the bottom of this structure, a holding chamber connected with the 40 tubes is present. The flight ability test was performed by aspirating 100 adult male mosquitoes, sterilised, PP-coated, and fertile. Adults were placed in the bottom of the cylindric structure through a 1 cm hole. Mosquitoes in a confined space can exit from the device through the 40 tubes above the holding chamber. Over the Plexiglas chamber, a 12V vent was switched on and directed towards the males. One small pellet of BG lure was also placed on the top to stimulate the flight.

The number of males that were able to exit from the cylinder was counted and divided by the total number of males placed in the holding chamber, thus generating the escape rate. Furthermore, male competitiveness was calculated. The competitiveness of a sterile male represents the odds of a wild female being mated with a sterile male compared with a wild male when exposed to both in equal numbers. A value of 1 indicates that sterile and wild males are equally competitive. A value of 0.5 indicates

that females are two times more likely to be mated with wild males (Bouyer and Vreysen, 2020).

To assess the effects of pyriproxyfen on larvae, we maintained and hatched them in the same water where the eggs were laid. We estimate the proportions of larvae developed into pupae, larvae developed into adults, and pupae developed into adults.

3. Results

3.1. Sexing tool comparison with different strains

The comparison of male productivity yields between strains and the sex sorting separator showed significant differences ($F_{3,53}=3.18$, $p<0.05$ *and* $F_{1,53}=5.75$, $p<0.05$), but their interaction effect was not significant ($F_{3,53}=1.88$, $p>0.05$).

When strains were compared pairwise, a significant increase of 8.6±3.19% was observed between ME and DE strains ($t_{53}=2.70$, $p<0.05$), but no significant differences were found between each other's strain pairwise ($p>0.05$) (Figure 21 and Table 1).

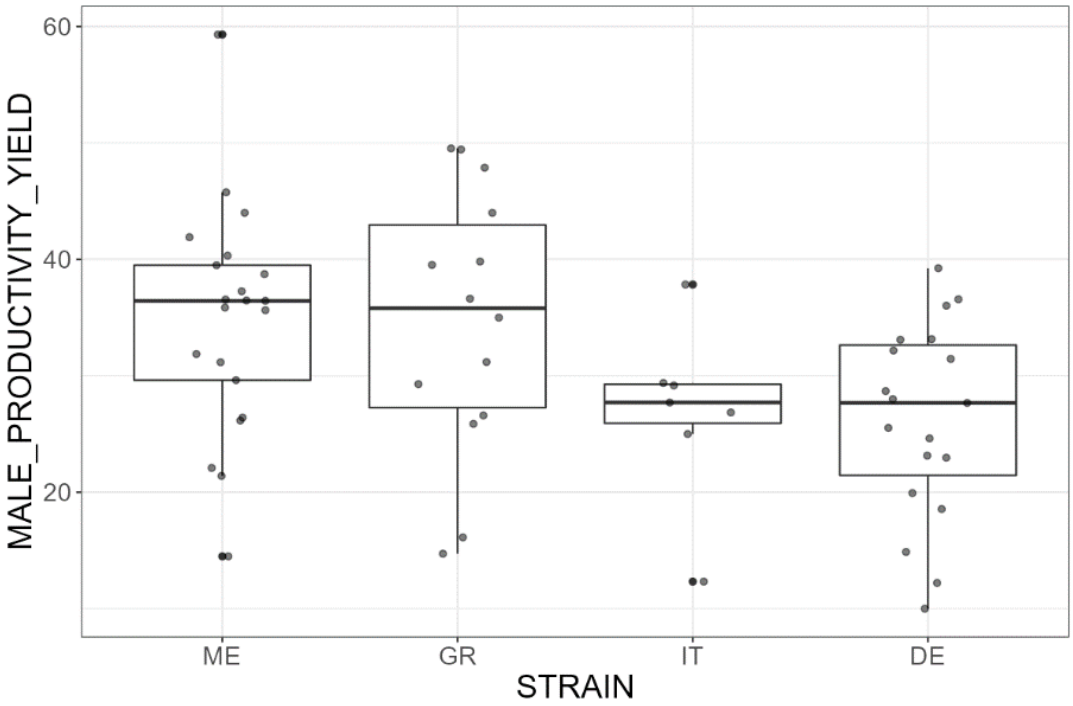

Figure 21. Male productivity yield of ME, GR, IT and DE strains. Boxplots present the median value and the quartiles, horizontal bars the 95% percentiles.

With the Fay-Morlan separator, the male productivity was 33.7±2.02%, compared to 27.4±1.68% for the sieve. Thus, the Fay-Morlan separator showed a mean significant improvement of 6.3±2.63% ($t_{53}=2.4$, $p<0.05$) (Figure 22 and Table 2).

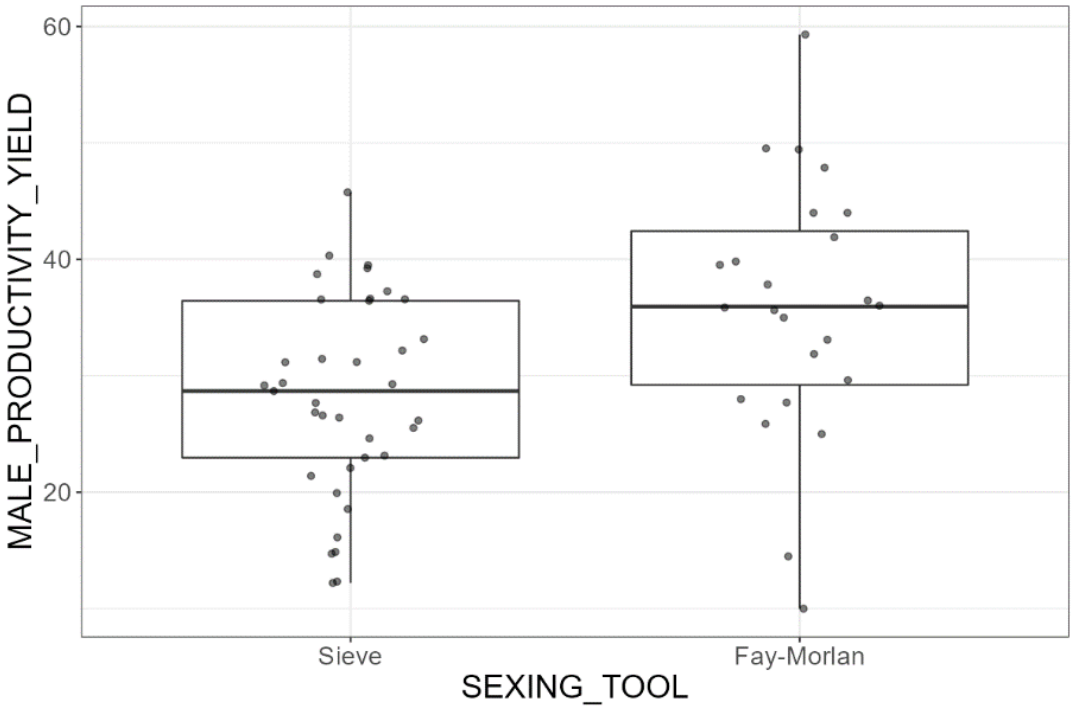

Figure 22. Male productivity yield of sieve and Fay-Morlan separator. Boxplots present the median value and the quartiles, horizontal bars the 95% percentiles.

Post hoc analysis between sexing tools for each reared strain showed a mean significant increase in male productivity of 15.6±4.82% with the Fay-Morlan separator, compared to the sieve, for only the GR strain ($t_{53}=3.24$, $p<0.05$). Other strains showed no significant differences between each other pairwise ($p>0.05$) (Figure 23 and Table 1).

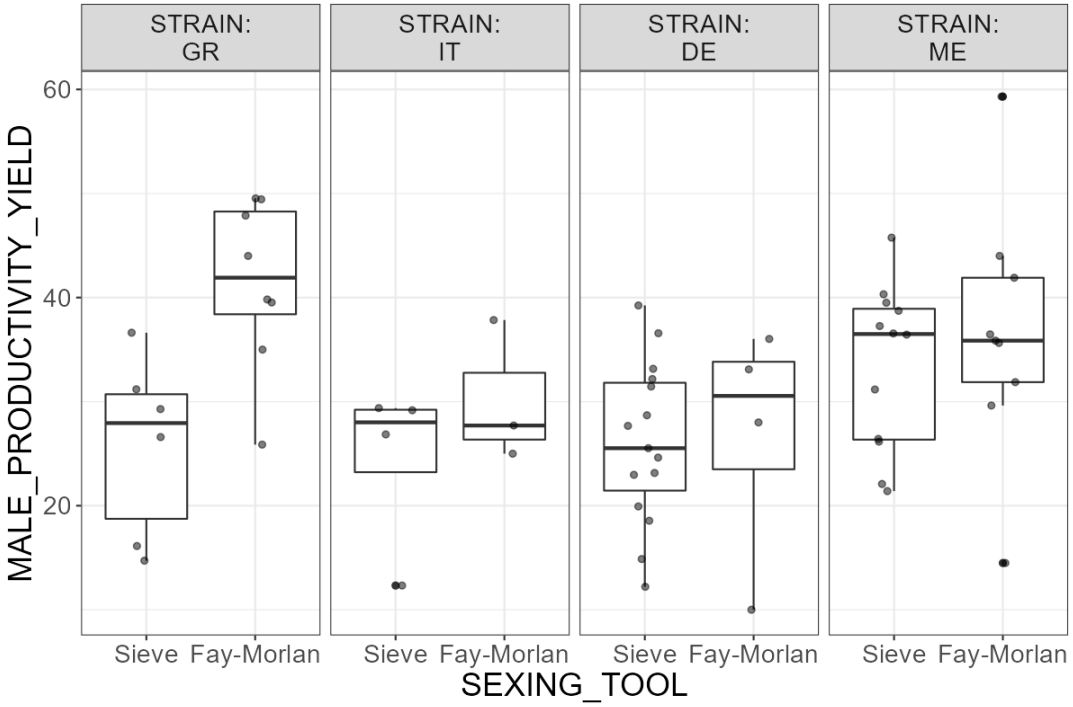

Figure 23. Male productivity yield considering each sorting device for every reared strain. Boxplots present the median value and the quartiles, horizontal bars the 95% percentiles.

The productivity of all the reared strains showed no significant difference between the two different sexing methods ($p>0.05$) (Figure 24 and Table2)

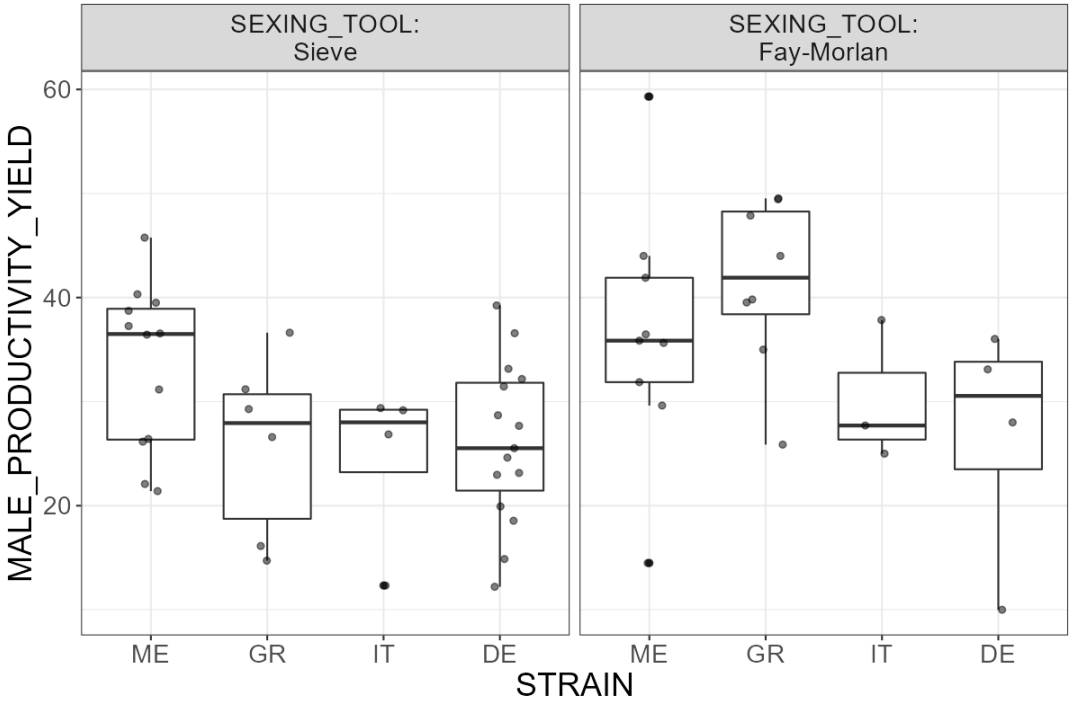

Figure 24. Male productivity yield considering each reared strain for the two sorting devices. Boxplots present the median value and the quartiles, horizontal bars the 95% percentiles.

Comparison of the residual female presence as a function of the strain and sexing tool showed a significant effect for the sexing tool variable ($F_{3,53}=70.79$, $p<0.001$). The sieve method gave the highest residual female presence of 4.52±0.29%, with a significant mean difference of 3.81±0.05% compared to the Fay-Morlan separator ($t_{53}=8.41$, $p<0.001$), which gave a residual female presence of 0.71±0.35% (Figure 25 and Table 2).

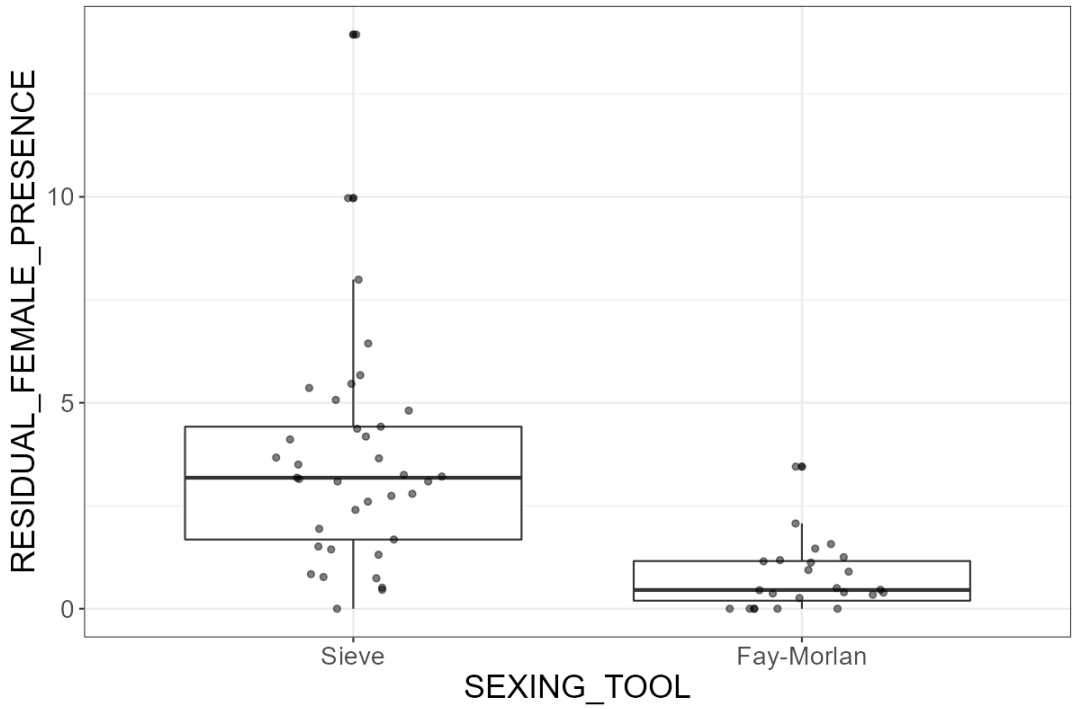

Figure 25. Residual female presence for the sieve and Fay-Morlan separator. Boxplots present the median value and the quartiles, horizontal bars the 95% percentiles.

The effect of the strains was also significant ($F_{3,53}=9.18$, $p<0.001$). In particular, the IT strain showed the highest mean level of residual female presence of 5.09±0.59%, with significant differences between this strain and each of the other reared strains; it showed significant mean differences of 3.46±0.72%, 3.25±0.73% and 3.20±0.68% compared to the GR, DE and ME strains ($ME_{t53}=4.72$, $MEp<0.001$; $DE_{t53}=4.45$, $DEp<0.001$ and $GR_{t53}=4.80$, $GRp<0.001$). No significant effect was found between strains pairwise ($p>0.05$) (Figure 26 and Table 1).

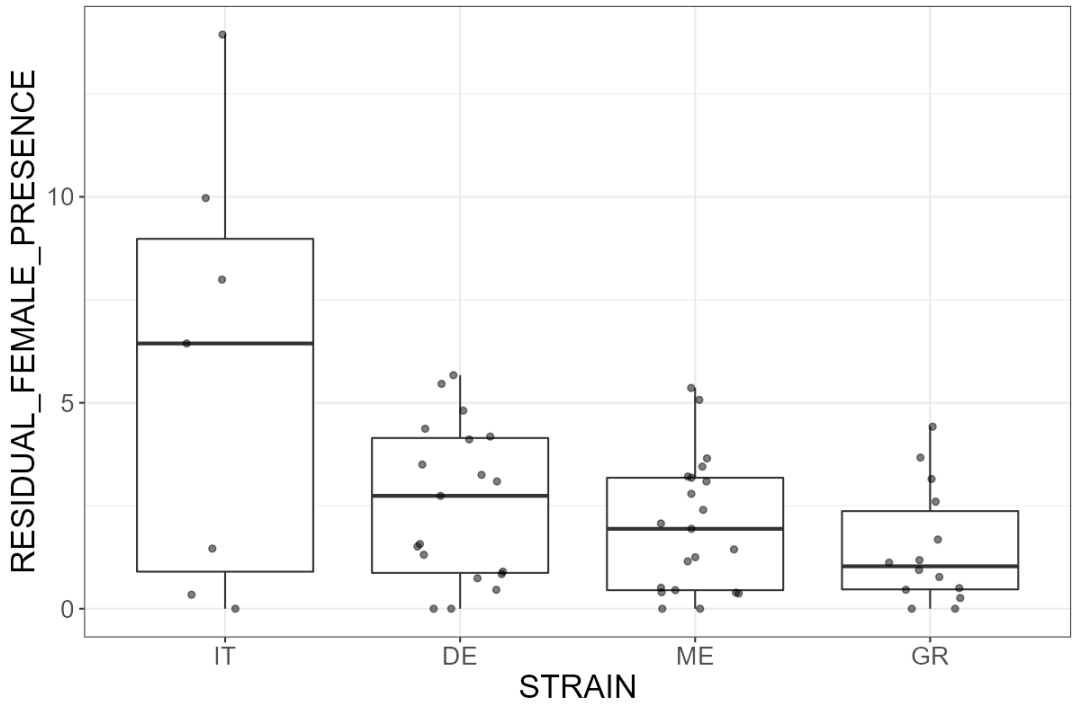

Figure 26. Residual female presence of each reared strain. Boxplots present the median value and the quartiles, horizontal bars the 95% percentiles.

The effect of the interaction between strains and sexing tools was also significant ($F_{3,53}=10.38, p<0.001$), suggesting that the other variable influenced a single variable's effect. Post hoc analysis of residual female presence between sexing tools for each reared strain showed significant differences only in the IT strain, with an increased mean difference of 8.99±1.18% ($t_{53}=7.64$, $p<0.001$). No significant differences between sex sorters were observed in the ME, GR, and DE strains ($t_{53}=2.45$, $p>0.05$, $t_{53}=2.59$, $p>0.05$ *and* $t_{53}=2.83$, $p>0.05$) (Figure 27 and Table 1).

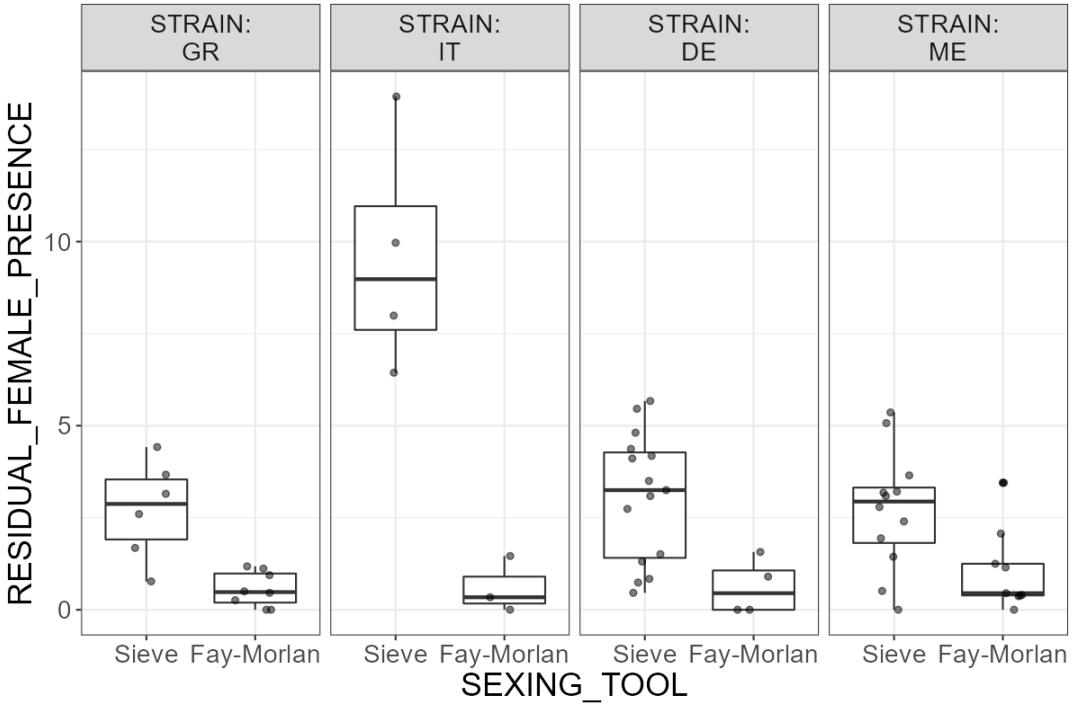

Figure 27. Residual female presence between sorting devices grouped by every single strain. Boxplots present the median value and the quartiles, horizontal bars the 95% percentiles.

Post hoc analysis of strains sexed with a sieve or a Fay-Morlan separator showed significant differences between the IT strain and other strains only when a sieve was used. The IT strain sex-sorted with a sieve showed a higher mean value of 9.59±0.77% of residual female presence, with significant mean differences of 6.52±0.87%, 6.87±0.89% and 6.87±0.99% compared with the DE, ME and GR strains. No differences were found between strains when sex sorting was done using the Fay-Morlan separator ($p>0.05$) (Figure 28 and Table 2).

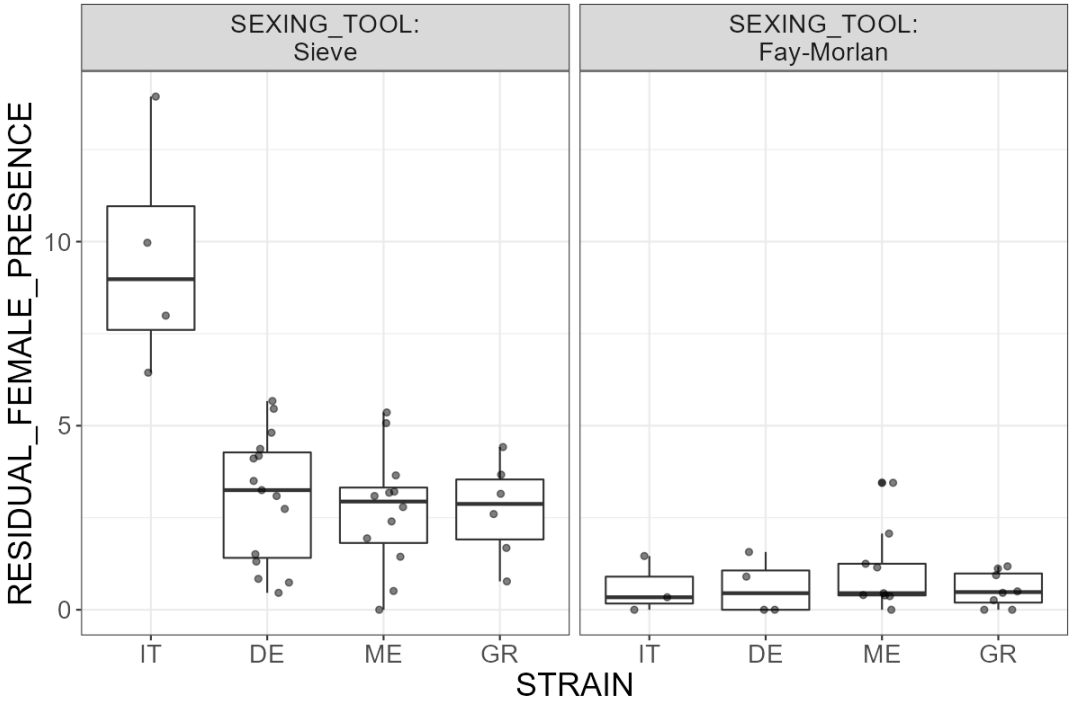

Figure 28. Residual female presence between strains grouped by sorting tools. Boxplots present the median value and the quartiles, horizontal bars the 95% percentiles.

Table 1. Male productivity yields and residual female percentages obtained with the two sexing tools for each strain.

			MPY		RFP	
STRAIN	**N**	**♀/♂ TOOL**	**Mean±SE (♀/♂ TOOL)**	**Mean±SE (STRAIN)**	**Mean±SE (♀/♂ TOOL)**	**Mean±SE (STRAIN)**
DE F9-18	4	Fay-Morlan	26.8±4.46^{a}	26.4±2.51^{b}	0.62±0.77^{a}	1.84±0.43^{a}
	15	Sieve	26.1±2.30^{a}		3.07±0.40^{a}	
GR F1-8	8	Fay-Morlan	41.4±3.16^{b}	33.6±2.41^{ab}	0.56±0.54^{a}	1.64±0.42^{a}
	6	Sieve	25.8±3.64^{a}		2.72±0.63^{a}	
IT F68-74	3	Fay-Morlan	30.2±5.15^{a}	27.3±3.41^{ab}	0.60±0.89^{b}	5.09±0.59^{b}
	4	Sieve	24.4±4.46^{a}		9.59±0.77^{a}	
ME F2-8	9	Fay-Morlan	36.6±2.98^{a}	35.0±1.97^{a}	1.06±0.51^{a}	1.89±0.34^{a}
	12	Sieve	33.5±2.58^{a}		2.72±0.45^{a}	

'STRAIN' indicates the origin of the strain, and 'F' is the range of the number of breeding generations. 'N' represents the replicates number. '♀/♂ TOOL' indicates the mechanical sexing tool adopted. 'MPY' is the male productivity yield percentage. 'RFP' is the residual female presence percentage. 'Mean±SE (♀/♂ TOOL)' is the marginal mean ± standard error of the values obtained with the two sexing tools for each strain. 'Mean±SE (STRAIN)' is the marginal mean ± standard error of the grouped values obtained with the two sexing tools for each strain. Tukey's mean separation test indicates different superscript letters within a column indicate statistical differences $p \leq 0.05$.

Table 2. Male productivity yields and residual female percentages for each reared strain for each sexing tool.

♀/♂ TOOL	N	STRAIN	MPY		RFP	
			Mean±SE (STRAIN)	Mean±SE (♀/♂ TOOL)	Mean±SE (STRAIN)	Mean±SE (♀/♂ TOOL)
Fay-Morlan	9	ME F2-8	36.6±2.98[a]	33.7±2.02[b]	1.06±0.51[a]	0.71±0.35[b]
	8	GR F1-8	41.4±3.16[a]		0.56±0.54[a]	
	3	IT F68-74	30.2±5.15[a]		0.60±0.89[a]	
	4	DE F9-18	26.8±4.46[a]		0.62±0.77[a]	
Sieve	12	ME F2-8	33.5±2.58[a]	27.4±1.68[a]	2.72±0.45[a]	4.52±0.29[a]
	6	GR F1-8	25.8±3.64[a]		2.72±0.63[a]	
	4	IT F68-74	24.4±4.46[a]		9.59±0.77[b]	
	15	DE F9-18	26.1±2.30[a]		3.07±0.40[a]	

'♀/♂ TOOL' indicates the mechanical sexing tool adopted. 'N' represents the replicates number. 'STRAIN' indicates the origin of the strain, and 'F' is the range of the number of breeding generations. 'MPY' is the male productivity yield percentage. 'RFP' is the residual female presence percentage. 'Mean±SE (STRAIN)' is the marginal mean ± standard error of the values obtained with the reared strain for each sexing tool. 'Mean±SE (♀/♂ TOOL)' is the marginal mean ± standard error of the grouped values obtained with all strains for each sexing tool. Tukey's mean separation test indicates different superscript letters within a column indicate statistical differences $p \leq 0.05$.

3.2. Larval diet and strain influence with the Fay-Morlan separator

The effects of strains and diets on male productivity yield were significant ($F_{2,45}=10.38$, $p<0.001$ *and* $F_{2,45}=8.85$, $p<0.001$), while the impact of their interaction was not significant ($F_{4,45}=0.43$, $p>0.05$).

The GR F5-F8 strain showed the highest productivity, with a mean male productive yield of 39.7±1.79%. The productivity of this strain was 11.9±2.96% higher in comparison with IT F73-F74 ($t_{45}=4.01$ $p<0.001$) and 10.0±2.82% higher than the DE F16-F18 strain ($t_{45}=3.54$, $p<0.01$). No significant difference was found between DE F16-F18 and IT F73-F74 ($t_{45}=0.58$, $p>0.05$) (Figure 29 and Table 3).

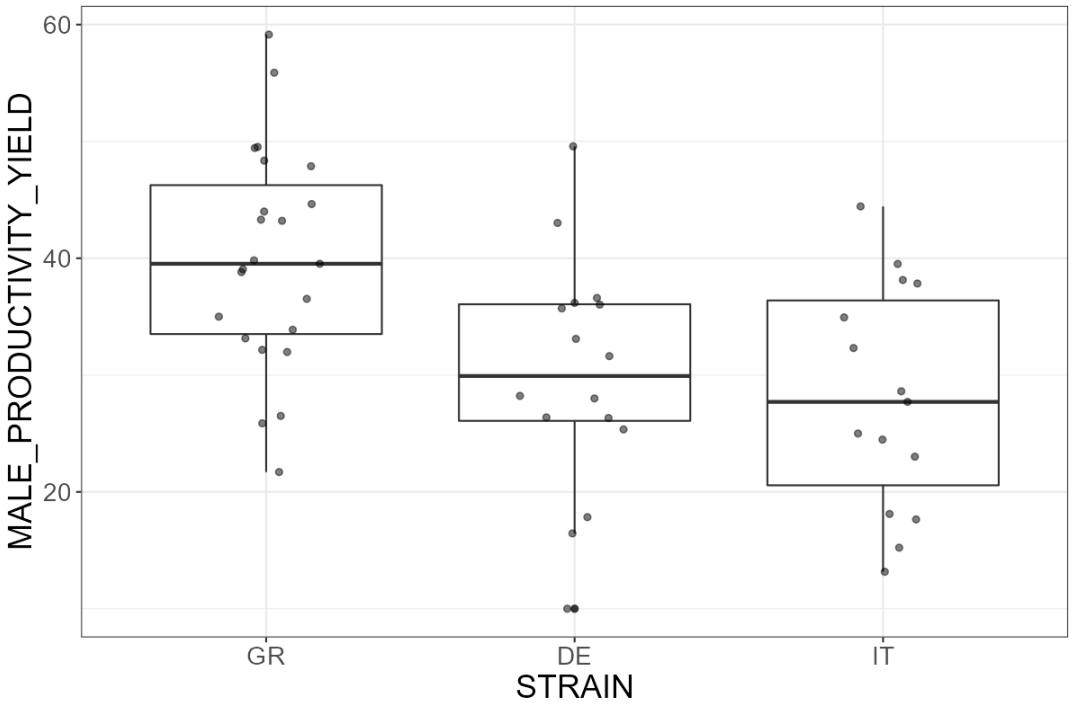

Figure 29. Male productivity yield between strains. Boxplots present the median value and the quartiles, horizontal bars the 95% percentiles.

The larval diet also influenced the male productivity yield, with a significant difference of 11.7±2.78% between SLP-BY and BLP-BY ($t_{45}=4.21$, $p<0.001$). The BLP-BY diet showed the highest male productivity yield of 38.0±1.88%. The differences between the IAEA-BY diet and either of the BLP-BY and SLP-BY diets were not significant ($t_{45}=1.71$, $p>0.05$ *and* $t_{45}=2.05$, $p>0.05$) (Figure 30 and Table 4).

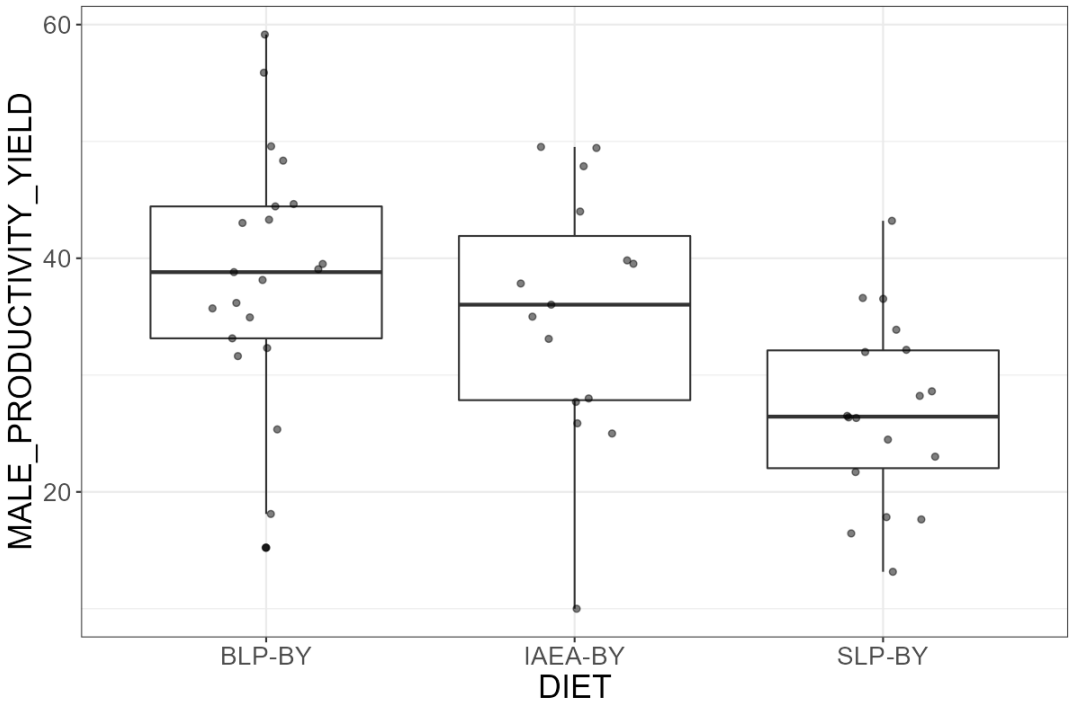

Figure 30. Male productivity yield between larval diets. Boxplots present the median value and the quartiles, horizontal bars the 95% percentiles.

Post hoc analysis of the three diets for each reared strain showed no significant difference ($p>0.05$). Interestingly, the mean values and data distribution of the male productive yield as a function of the diet followed similar trends in all reared strains (Figure 31 and Table 3).

A comparison of the productivity of each strain reared on the three diets showed no significant difference ($p>0.05$) (Figure 32 and Table 4).

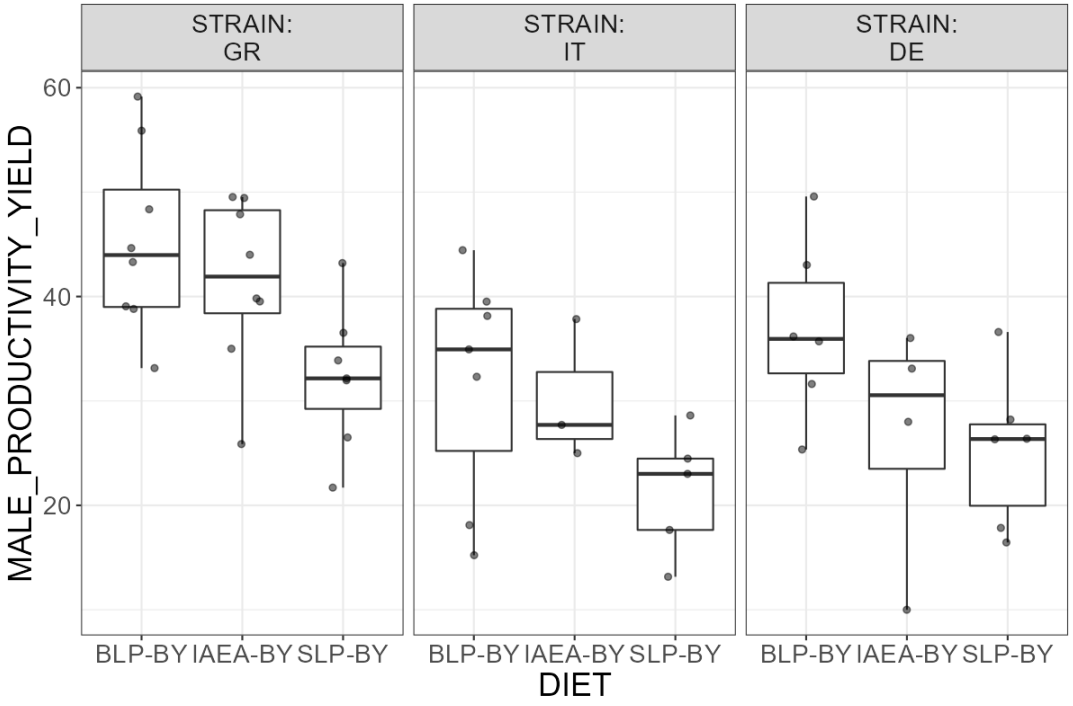

Figure 31. Male productivity yield between larval diets grouped by strain. Boxplots present the median value and the quartiles, horizontal bars the 95% percentiles.

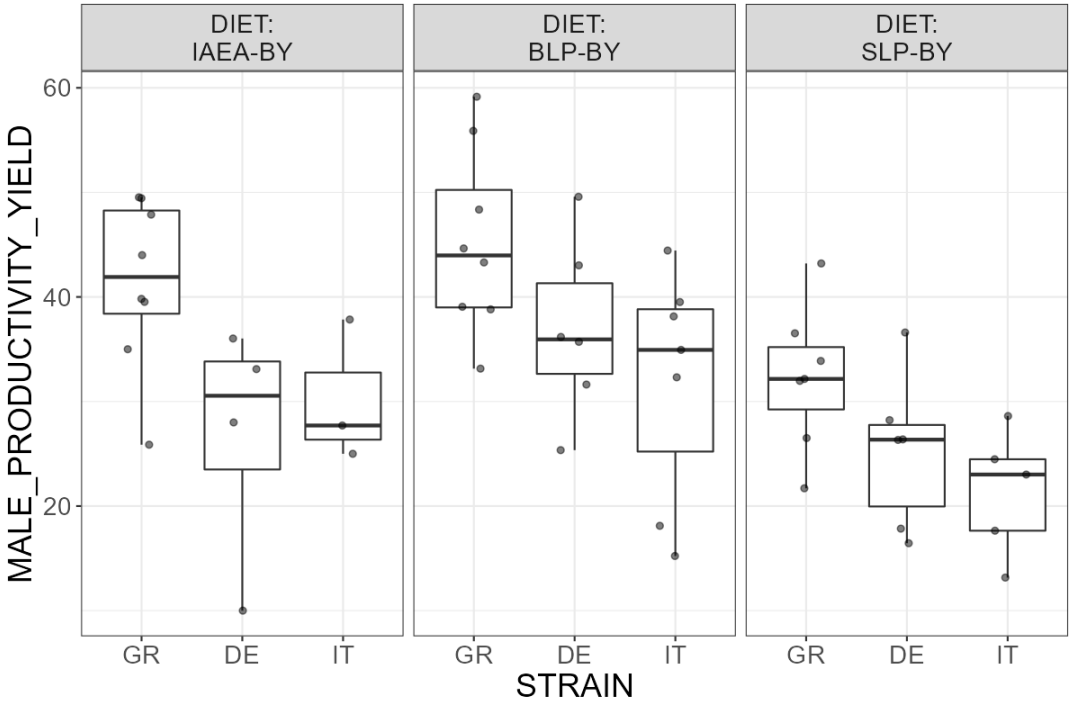

Figure 32. Male productivity yield between strains grouped by larval diets. Boxplots present the median value and the quartiles, horizontal bars the 95% percentiles.

Diets influenced the residual presence of females ($F_{2,45}=13.58$, $p<0.001$), but the strains showed no significant effect ($F_{2,45}=1.99$, $p>0.05$). An interaction effect between these two variables was not found ($F_{4,45}=0.73$, $p>0.05$).

The IAEA-BY diet gave the lowest value of the residual female presence of 0.59±0.34%. There were significant mean differences of 1.83±0.43% between IAEA-BY and BLP-BY and 1.3±0.45% between IAEA-BY and SLP-BY ($t_{45}=5.13$, $p<0.001$ *and* $t_{45}=3.80$, $p=0.001$). No difference was found between BLP-BY and SLP-BY ($t_{45}=1.33$, $p>0.05$) (Figure 33 and Table 4).

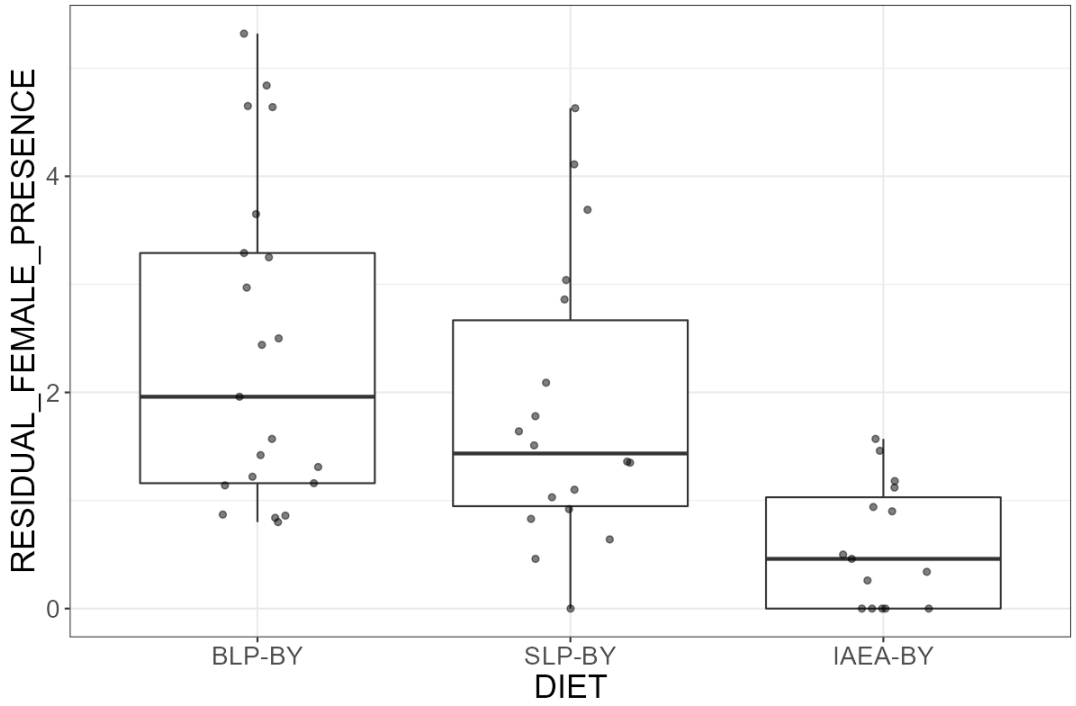

Figure 33. Residual female presence between larval diets. Boxplots present the median value and the quartiles, horizontal bars the 95% percentiles.

Post hoc analysis comparing residual female presence values obtained using the three diets for each reared strain showed no significant differences ($p>0.05$). As already observed for male productivity yield, the distribution of residual female presence showed the same trends for each strain according to the diet (Figure 34 and Table 3).

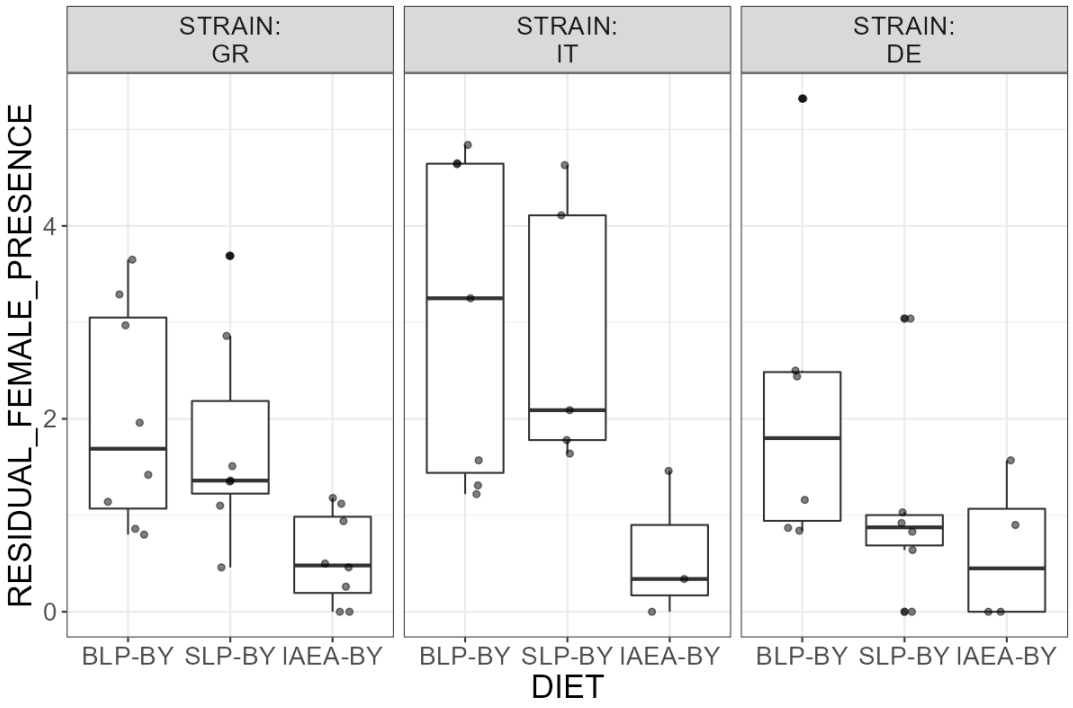

Figure 34. Residual female presence between larval diets grouped by reared strains. Boxplots present the median value and the quartiles, horizontal bars the 95% percentiles.

The residual female presence between diets according to the strain showed no significant differences (Figure 35 and Table 4).

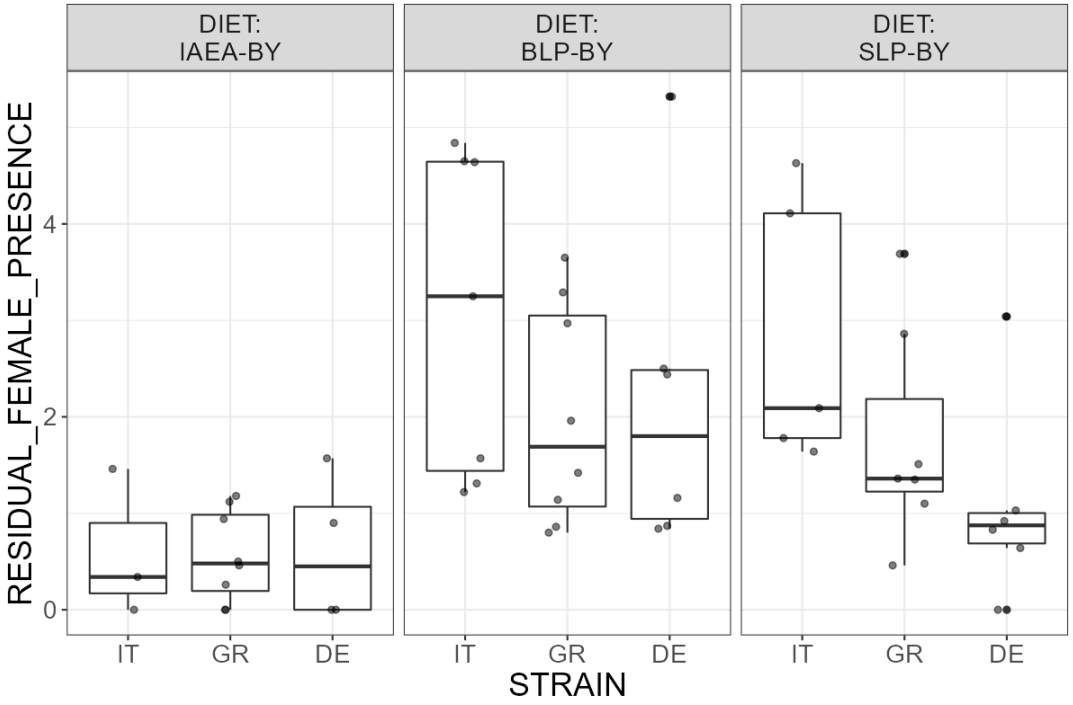

Figure 35. Residual female presence between reared strains grouped by larval diets. Boxplots present the median value and the quartiles, horizontal bars the 95% percentiles.

Table 3. Male productivity yields and residual female percentages obtained with the three larval diets for each strain.

STRAIN	N	DIET	MPY		RFP	
			Mean±SE (DIET)	Mean±SE (STRAIN)	Mean±SE (DIET)	Mean±SE (STRAIN)
DE F16-18	6	BLP-BY	36.9±3.47[a]	29.7±2.18[a]	2.19±0.49[a]	1.29±0.31[a]
	4	IAEA-BY	26.8±5.83[a]		0.62±0.61[a]	
	6	SLP-BY	25.3±3.01[a]		1.08±0.49[a]	
GR F5-8	8	BLP-BY	45.3±3.12[a]	39.7±1.79[b]	2.01±0.43[a]	1.44±0.25[a]
	8	IAEA-BY	41.4±2.89[a]		0.56±0.43[a]	
	7	SLP-BY	32.3±2.61[a]		1.76±0.46[a]	
IT F73-74	7	BLP-BY	31.8±4.17[a]		3.07±0.46[a]	
	3	IAEA-BY	30.2±3.91[a]	27.8±2.35[a]	0.60±0.70[a]	2.17±0.33[a]
	5	SLP-BY	21.4±2.70[a]		2.85±0.54[a]	

'STRAIN' indicates the origin of the strain and 'F' is the range of number of breeding generations. 'N' represents the replicates number. 'DIET' indicates the larval diet used. 'MPY' is the male productivity yield percentage. 'RFP' is the residual female presence percentage. 'Mean±SE (DIET)' is the marginal mean ± standard error of the values obtained with the three larval diet for each strain. 'Mean±SE (STRAIN)' is the marginal mean ± standard error of the grouped values obtained with the three larval diets for each strain. Different superscript letters within a column indicate statistical differences $p \leq 0.05$, Tukey's mean separation test.

Table 4. Male productivity yields and residual female percentages obtained with the three reared strains for each larval diet.

DIET	N	STRAIN	MPY		RFP	
			Mean±SE (STRAIN)	Mean±SE (DIET)	Mean±SE (STRAIN)	Mean±SE (DIET)
BLP-BY	8	GR F5-8	45.3±3.03[a]		2.01±0.43[a]	
	6	DE F16-18	36.9±3.50[a]	38.0±1.88[b]	2.19±0.49[a]	2.42±0.27[a]
	7	IT F73-74	31.8±3.24[a]		3.07±0.46[a]	
IAEA-BY	8	GR F5-8	41.4±3.03[a]		0.56±0.43[a]	
	4	DE F16-18	26.8±4.29[a]	32.8±2.41[ab]	0.62±0.61[a]	0.59±0.34[b]
	3	IT F73-74	30.2±4.95[a]		0.60±0.70[a]	
SLP-BY	7	GR F5-8	32.3±3.24[a]		1.76±0.46[a]	
	6	DE F16-18	25.3±3.50[a]	26.3±2.04[a]	1.08±0.49[a]	1.90±0.29[a]
	5	IT F73-74	21.4±3.84[a]		2.85±0.54[a]	

'STRAIN' indicates the origin of the strain and 'F' is the range of number of breeding generations. 'N' represents the replicates number. 'DIET' indicates the larval diet used. 'MPY' is the male productivity yield percentage. 'RFP' is the residual female presence percentage. 'Mean±SE (STRAIN)' is the marginal mean ± standard error of the values obtained with the three strains for each larval diet. 'Mean±SE (DIET)' is the marginal mean ± standard error of the grouped values obtained with the three strains for each larval diet. Different superscript letters within a column indicate statistical differences $p \leq 0.05$, Tukey's mean separation test.

3.3. Sorting time influence using Automatic Fay-Morlan separator

The effect of sorting time on male productivity and residual female presence were both significant ($F_{1,112}=5.90$, $p<0.05$ *and* $F_{1,112}=22.44$, $p<0.01$).

Table 5. Male productivity yields and residual female percentages obtained with the three larval diets for each strain.

SORTING TIME	N	MPY Mean±SE	RFP Mean±SE
24 h	61	27.8±0.88[a]	0.59±0.08[a]
48 h	53	31.1±1.06[b]	1.08±0.10[b]

'SORTING TIME' indicates the number of hours from the onset of pupation in which the sieving was carried out. 'N' represents the replicates number. 'MPY' is the male productivity yield percentage. 'RFP' is the residual female presence percentage. 'Mean±SE' is the marginal mean ± standard error of the values obtained with the two sorting times. Different superscript letters within a column indicate statistical differences $p \leq 0.05$, Tukey's mean separation test.

The sex sorting carried at 48 h from the onset of pupation showed a mean male productive yield of 31.1±1.1%, 3.32±1.37% ($t_{112}=2.43$, $p<0.017$), significantly higher with respect to the sex sorting carried at 24 h ($p<0.017$). The sex sorting carried out at 24 h showed a mean productive yield of 27.8±0.9% (Figure 36).

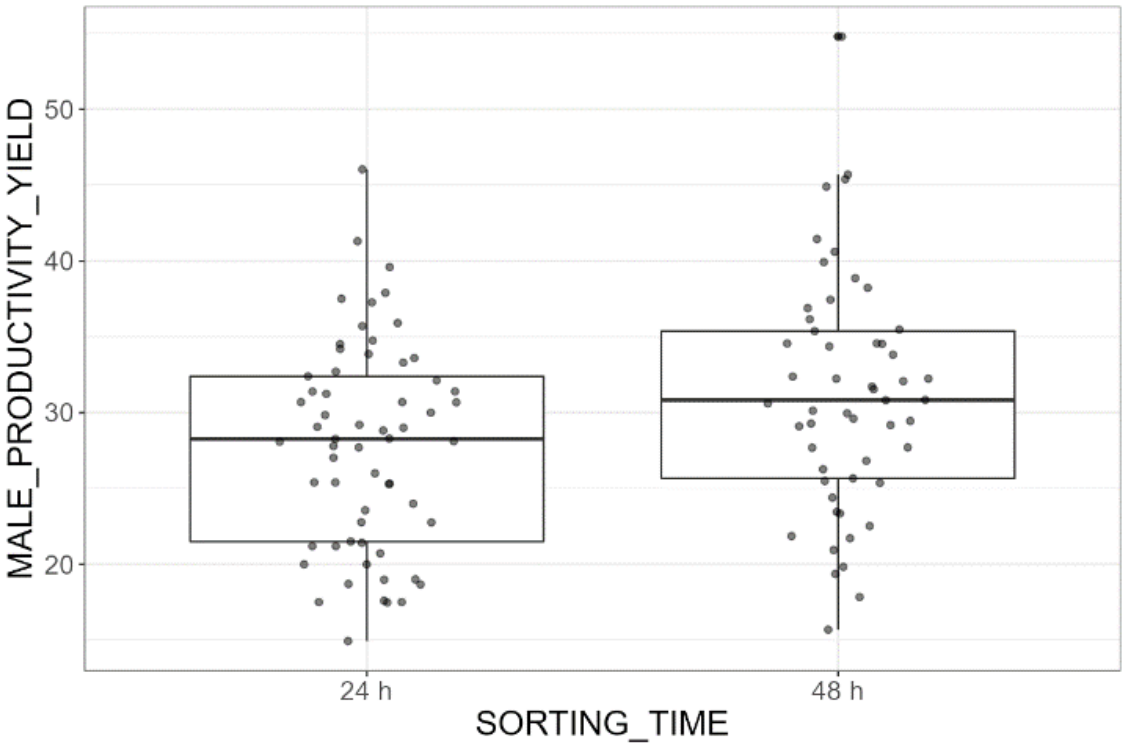

Figure 36. Effect of sorting time on the male productivity yield percentages. Boxplots present the median value and the quartiles, horizontal bars the 95% percentiles.

The sorting procedure at 48 h gave the highest contamination of females, with a mean residual presence of 1.08±0.09%. There was a significant difference of 0.50±0.13% ($t_{112}=4.74$, $p<0.001$) to the sorting carried at 24 h from the onset of pupation, which showed female contamination of 0.59±0.09% (Figure 37).

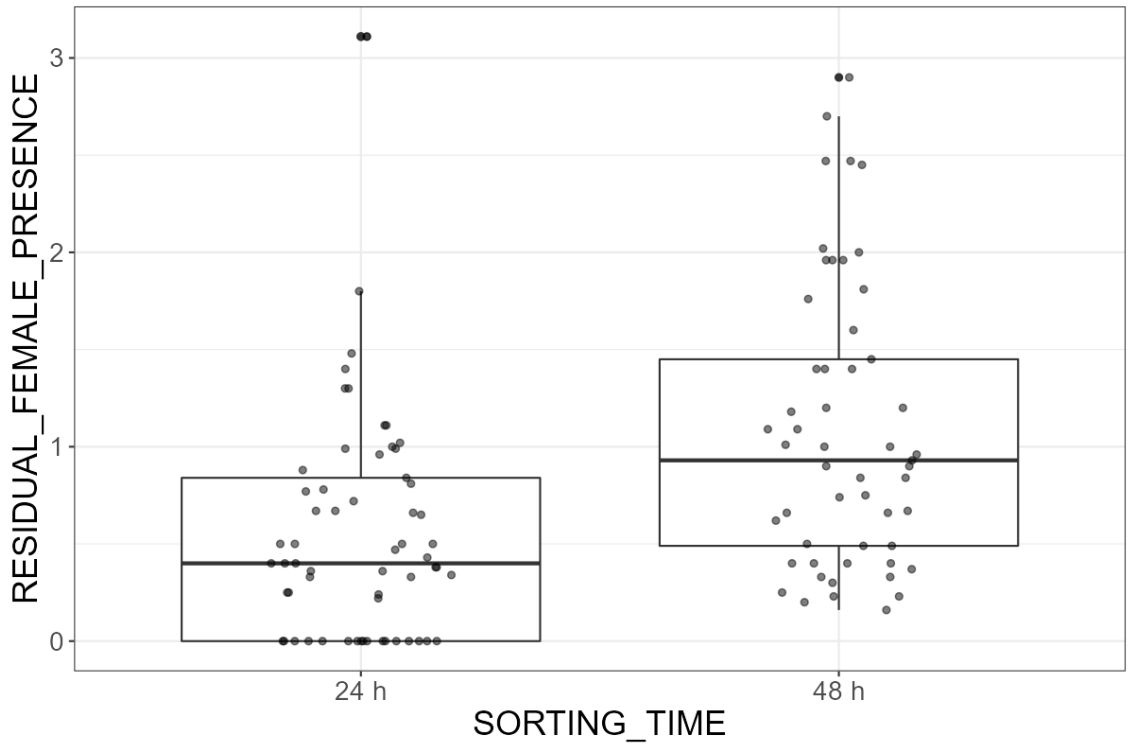

Figure 37. Effect of sorting time on the residual female presence percentages. Boxplots present the median value and the quartiles, horizontal bars the 95% percentiles.

3.4. Larval density influence using automatic Fay-Morlan separator

Mean male productivity of the 1.8 and 2.2 larvae/ml density were 34.02±3.45% and 29.74±1.01%, respectively. Instead, the mean residual female presence was 1.17±0.23% for the lower and 1.09±0.10% for the higher density.

The effect of the two tested densities on male productivity yield, and residual female presence was not significant ($F_{1,50}=2.212$, $p>0.05$; $F_{1,50}=0.211$, $p>0.05$) (Figure 38).

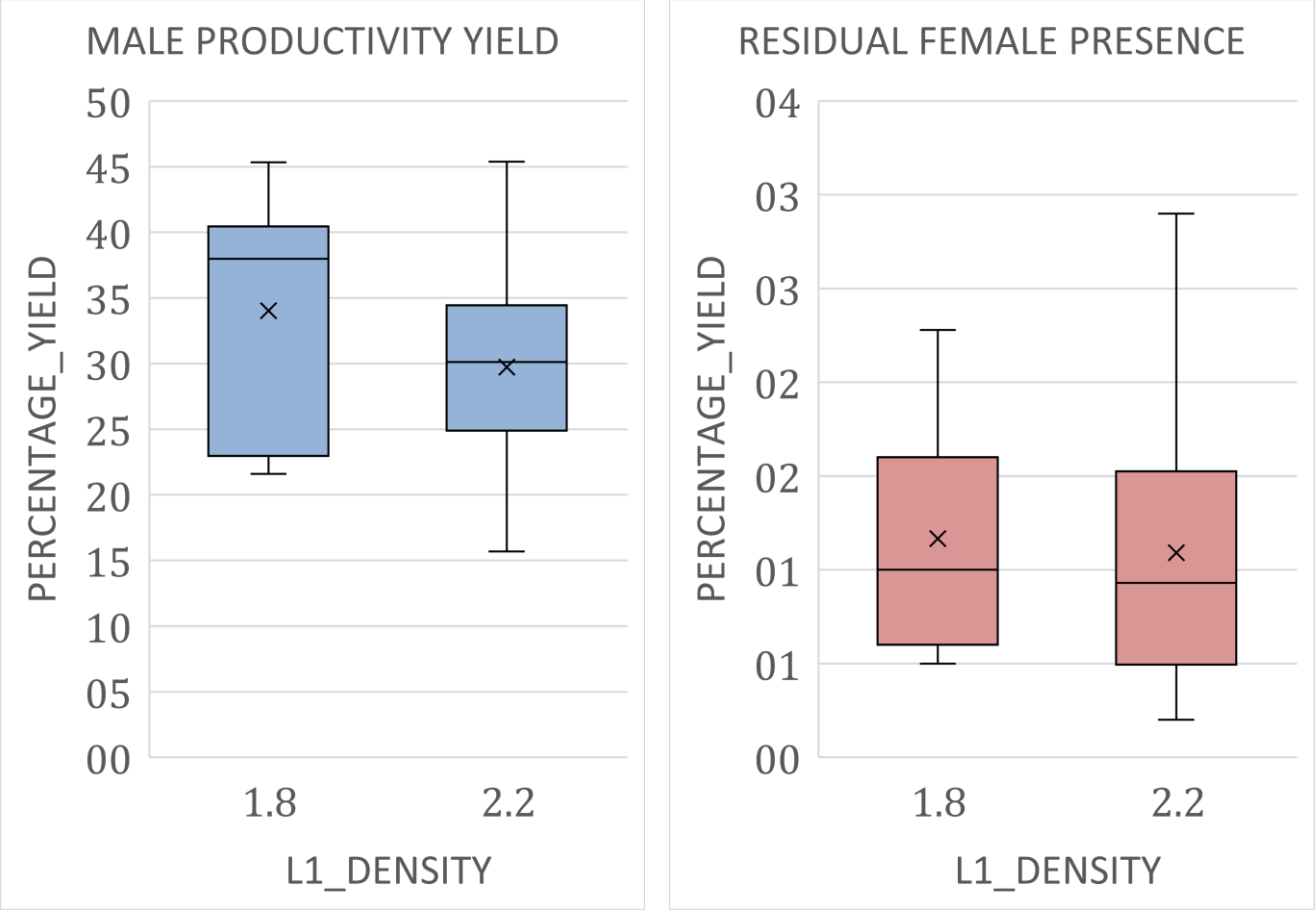

Figure 38. Effects on two adopted densities on male productivity yield and residual female presence. Boxplots present the median value and the quartiles, horizontal bars the 95% percentiles. X represent mean values.

3.5. Implementation of a hyper-protandry strain through classical breeding

The effect of the number of crossbreeding at 24 hours from the onset of pupation was not significant for the male productivity yield ($F_{3,4}=0.16$, $p>0.05$) and significant for the female presence ($F_{3,4}=16.67$, $p<0.05$).

Pairwise analysis showed significant differences in female presence between the F1 and all the other generations (*F6* $t_4=4.68$, $p<0.05$; *F11* $t_4=5.82$, $p<0.05$ *and F16* $t_4=6.33$, $p<0.05$), indeed F1 generation showed the highest female presence with mean female productivity of 7.62±0.74%. A significant linear regression was observed with the number of generations, showing a decreased female presence as the number of

breeding generations grows, reaching the lowest level of female presence of 0.10±0.04% with the F16 (*y=-4.7064x+16.364, R_2=0.72*) (Figure 39).

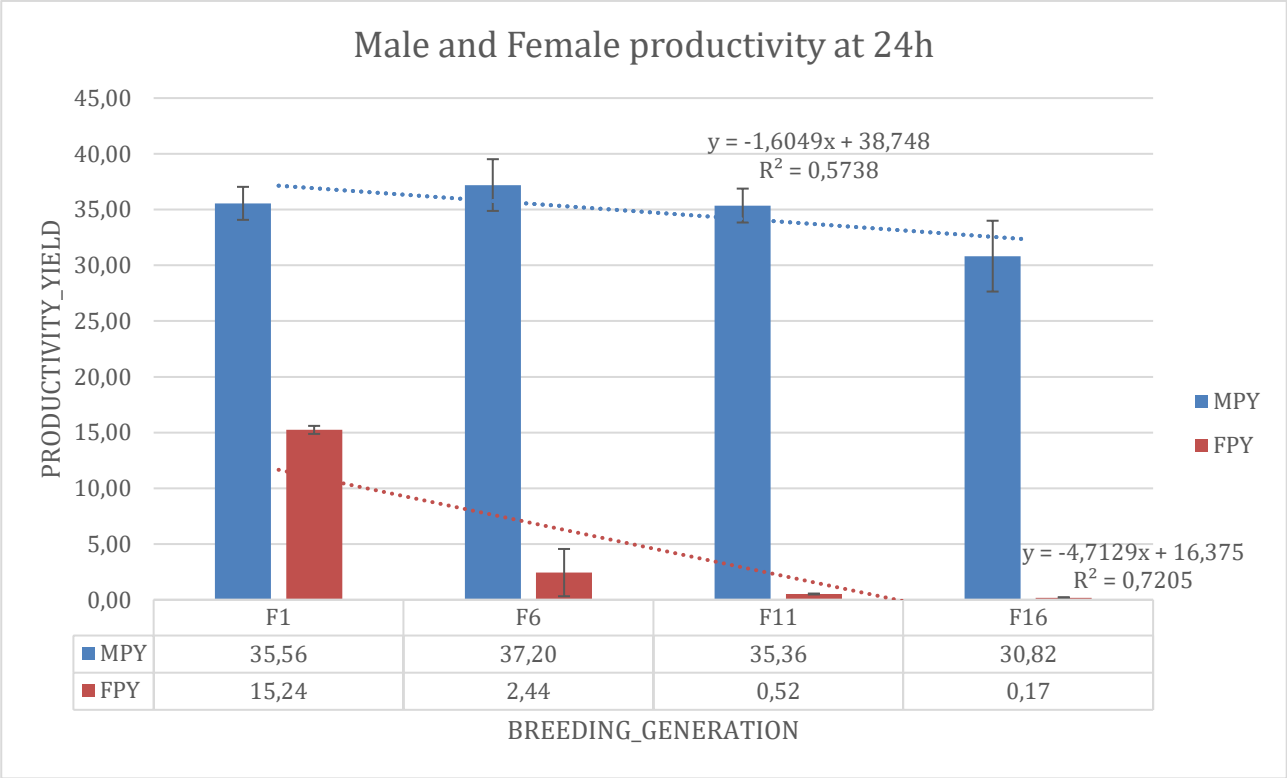

Figure 39. Effects of cross-breeding generations on male (MPY) and female productivity (FPY) at 24 hours from the onset of pupation.

At 48 hours from the onset of pupation, the number of breeding generations significantly affected the male productivity yield ($F_{3,4}$=74.58, p<0.01), while the residual female presence was not affected ($F_{3,4}$=0.21, p>0.05). The male productivity of F1 and F6 were 4.44±1.00% and 8.24±0.96%, and no significant difference was found between them (t_4=0.91, p>0.05). F1 and F6 were both significantly lower with respect to F11 and F16 (F1-F11 t_4=10.7, p<0.01; F1-16 t_4=10.38, p<0.01; F6-F11 t_4=10.7, p<0.01; F6-16 t_4=9.73, p<0.01), F11 and F16 showed a mean male productive yield of 28.72±2.04% and 25.93±0.34% respectively, between them there were no significant differences (t_4=0.97, p>0.05). Significant linear regression in male presence

as it grows the number of breeding generations could be observed (y=8.7742x-5.8037, R2=0.80) (Figure 40).

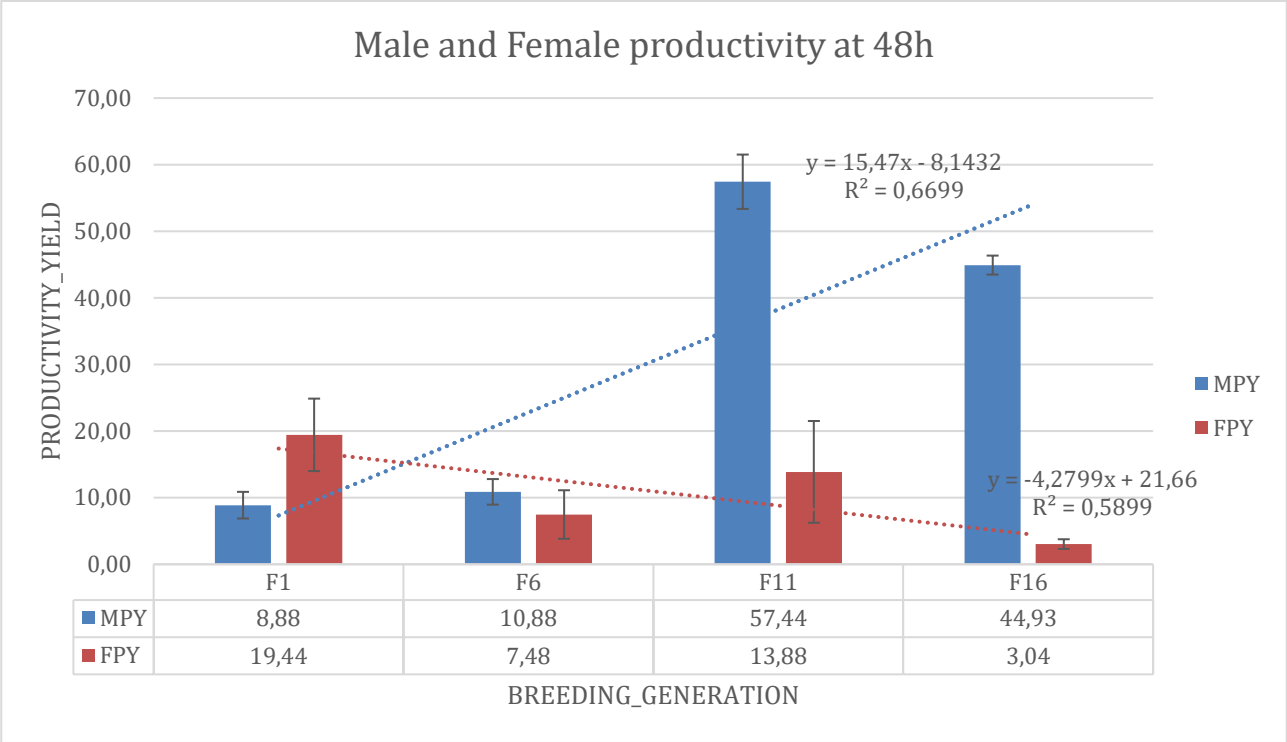

Figure 40. Effects of cross-breeding generations on male (MPY) and female productivity (FPY) at 48 hours from the onset of pupation.

After 72 hours from the onset of pupation, the number of breeding generation significantly affect male productivity and residual female presence ($F_{3,4}=13.3$, $p<0.015$; $F_{3,4}=48.8$, $p<0.01$).

Male productivity at the F16 generation was 16.38±1.55% and significantly higher than the F1 and F6 generations (*F1-F16* $t_4=5.96$, $p<0.05$ *and F6-16* $t_4=4.63$, $p<0.05$), which showed mean male productivity of 0.20±0.08% and 2.24±2.08% respectively. Between each other pairwise, there were no significant differences ($p>0.05$). Significant linear regression within generations was obtained for male productivity yield ($y=5.1915x-6.8692$, $R^2=0.8661$, $t=3.77$, $p<0.05$) (Figure 41).

The F1 and F6 generations' female productivity was 3.36±0.16% and 6.68±1.16%. Their yields were significantly lower than the F11 and F16 generations (*F1-11* t_4*=8.00, p<0.01 and F1-16* t_4*=11.36, p<0.01*), which have female productivity yields of 11.26±0.82% and 15.54±1.06% respectively. The further pairwise comparison did not show any other significative differences (*p>0.05*). A significant linear regression was found for female productivity yield between generations (*y=4.1106x-1.0676,* R^2*=0.9959*) (Figure 41).

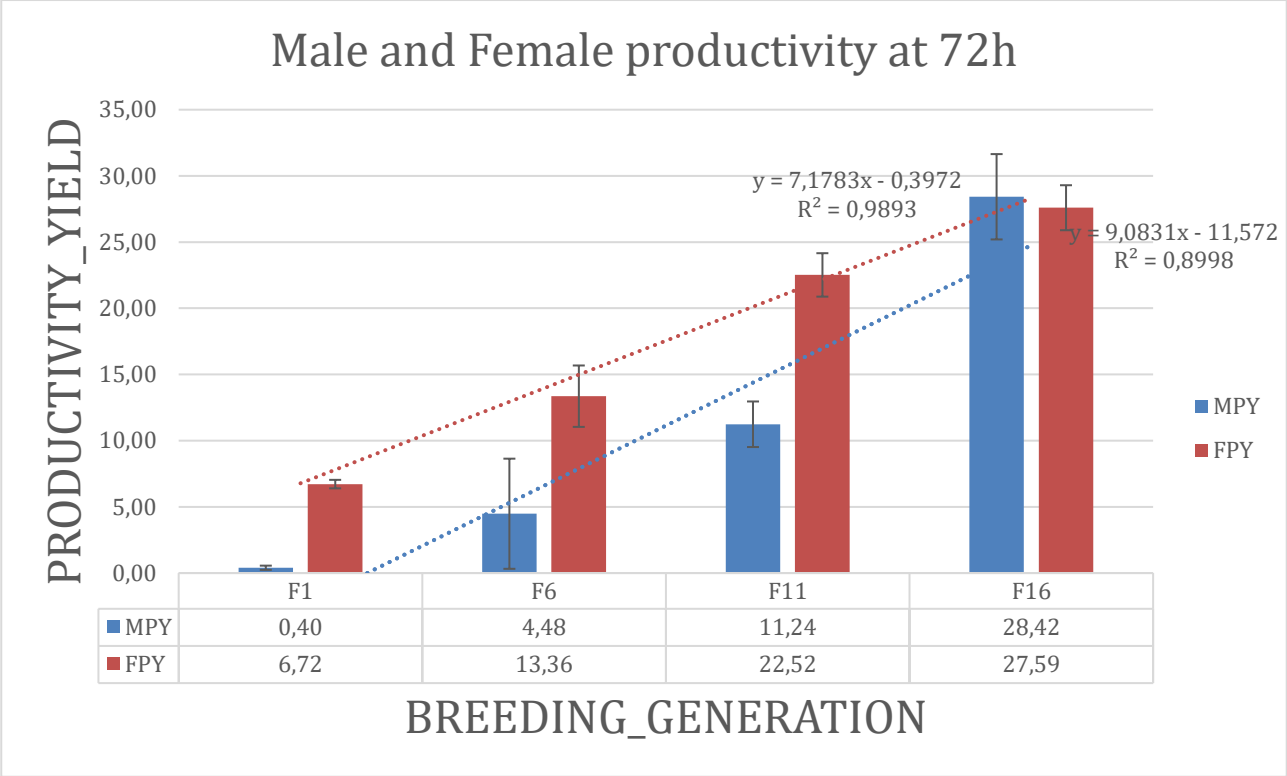

Figure 41. Effects of cross-breeding generations on male (MPY) and female productivity (FPY) at 72 hours from the onset of pupation.

3.6. Feasibility study for the recognition of male/female adult mosquitoes with an optical system

Of the total 1027 adult images collected, 616, divided into 303 females and 313 males, were used to train the artificial intelligence.

Once trained, the 616 images were mixed with 411 new adult images and submitted to the AI to assess if it could recognise the two sexes. Of the total, 1027 images recognised 1019 individuals correctly, 99.22% of the total. The images used to train the AI were also used to test it. In this case, of the 616 images, 614 were correctly identified (99.68%).

New 411 images were submitted to the AI for analysis, and 405 adults were correctly recognised (98.54%).

Of the total, seven males were unproperly identified as females, while only one female was incorrectly identified as male (Table 6).

Table 6. Sample image distribution and results of the AI process.

Total images used

	Total Images	**Training Images**	**Test Images**
M - Males	522	313	209
F - Females	505	303	202
TOTAL	**1027**	**616**	**411**

Result TEST OK

	Total Images OK	**Training Images OK**	**Test Images OK**
M - Males	515	311	204
F - Females	504	303	201
TOTAL	**1019**	**614**	**405**

Result TEST KO

	Total Images KO	**Training Images KO**	**Test Images KO**
M - Males	7	2	5
F - Females	1	0	1
TOTAL	**8**	**2**	**6**

'Total Images' represents the total number of images divided by sex. 'Training Images' represents the number of images used to instruct the AI. 'Test Images' indicates the number of images used to test the discrimination capacity of the AI. 'M – Males' represent the number of male images. 'F- Females' represent the number of female images.

3.7. Prototype drone releasing tests for aerial distribution of sterile males

2.9.1. Laboratory test

Once expelled into the cages, the adult chilled male mosquitoes began to recover in both control and test after a few minutes and started to fly. After half an hour, most adult males were flying or placed over the surfaces. At 24 hours, the dead males were counted, and mortality was calculated for each cage (Table 7).

Table 7. Number of adult dead males and mortality.

SORTING TIME	N	N adults	N dead	Mortality %
Control Tubes	7	8750-10500	544	6.2-5.2
Control Tubes	7	8750-10500	653	7.4-6.2
Drone Tubes	7	8750-10500	488	5.6-4.6
Drone Tubes	7	8750-10500	596	6.8-5.7

No substantial differences between the control and treated replicates were found, indicating a lack of significant effect due to the release mechanism. Adults showed the same mortality rate over time.

2.9.1. Field test

The release prototype mounted on the DJI Matrice 300 drone was tested in two different field conditions in the nearby pilot facility of the CAA.

The first release was carried out into a building at 5-7 meters. The machine expelled males in visible blocks due to the impulse movements of the rotatory cylinders. As the mosquito blocks fell, they began opening, and some mosquitoes were able to recover and fly directly during the fall. Part of the males reached the ground and was pushed in different directions by the top-bottom thrust of the drone’s propeller. After a few

minutes, many mosquitoes were alive and leaning on the wall or ground surfaces. After one hour, the dead mosquitoes on the ground and inside the releasing machine were 590, and the mortality rate was 5.3% concerning the 11000 males released. The onboard probe reported a temperature of 31°C and a humidity of 36% RU.

The second release trial was conducted in open field conditions at 10-15 meters. The released mosquitoes, with the same blocks' distribution, followed the wind direction and part of them were directly able to fly away. In this case, having adopted a greater height, the drone's propeller did not significantly impact the biological material. Once they reached the ground, the released mosquitoes started almost instantly to recover and fly away. One hour after the release, 474 dead males were counted on the ground and inside the release machine, with a mortality of 4.3%. The onboard probe reported a temperature of 28.8°C and a humidity of 37% RU.

The vitality of males released in open field conditions was visually appreciable compared to males released into the building.

3.8. Boosted SIT trial at Réunion island

The escape rate obtained from the flight ability test showed no significant differences between control, boosted and sterile males (*p>0.05*) (Figure 43).

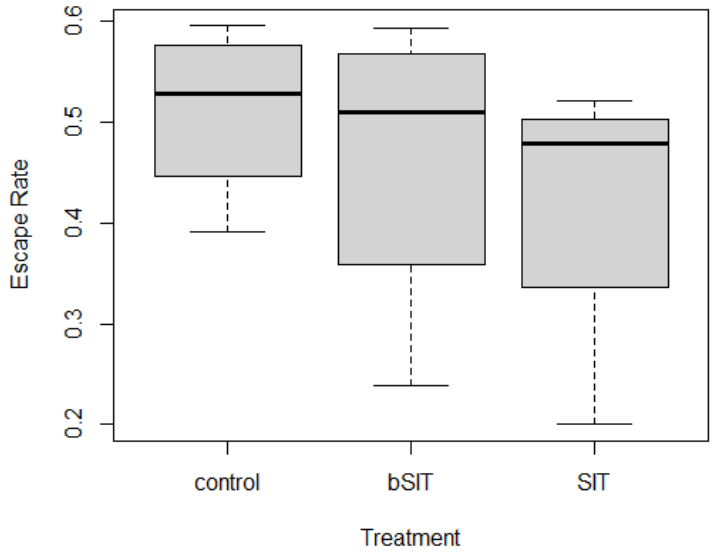

Figure 43. Escape rates among treatment conditions. Boxplots present the median value and the quartiles, horizontal bars the 95% percentiles.

Male competitiveness was not significative different from boosted and sterile mosquitoes (*p>0.05*). Despite this, we observed a tendency of decreased competitiveness for the ratio 5:1:1 as compared to classic SIT (Figure 44).

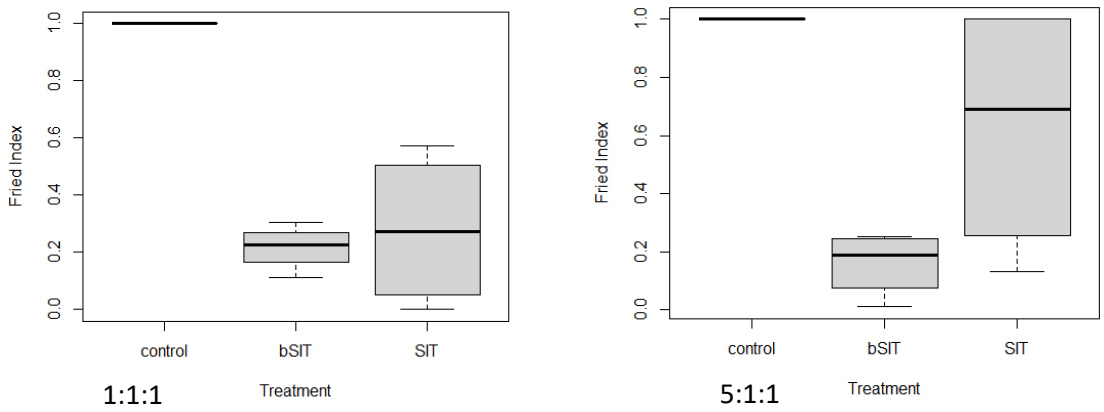

Figure 44. Competitiveness index of different treatments with different ratios: control, boosted and sterile. Boxplots present the median value and the quartiles, horizontal bars the 95% percentiles.

A significant reduction of the development rate of larvae into pupae for SIT and boosted SIT was observed as compared to control, with a significant effect of dose ($p<0.001$). No ratio effect was found ($p>0.05$) (Figure 45).

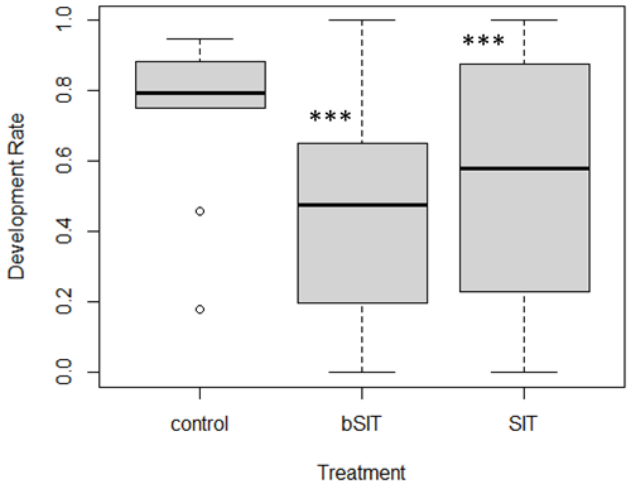

Figure 45. Development rate of larvae between treatments. Boxplots present the median value and the quartiles, horizontal bars the 95% percentiles and dots the minimal and maximal values. Significant differences between treatment and control groups are indicated (*** $p < 0.001$).

Results showed a significant reduction in the emergence of pupae into adults of boosted SIT compared to control and SIT ($p<0.001$). No effect of dose or ratio was observed ($p>0.05$) (Figure 46).

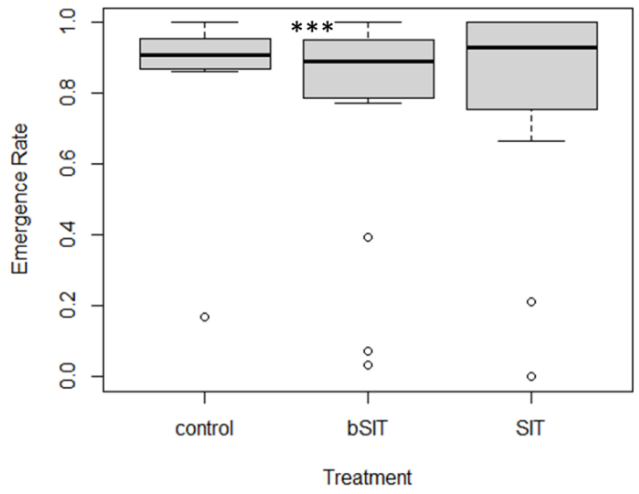

Figure 46. Emergence rate of adults between treatments. Boxplots present the median value and the quartiles, horizontal bars the 95% percentiles and dots the minimal and maximal values. Significant differences between treatment and control groups are indicated (*** $p < 0.001$).

Also, the development rate of larvae into adults was affected. We found a significant reduction of the development of larvae into adults of boosted SIT and SIT compared to control treatment with a significant effect of dose ($p<0.001$) but no impact of ratio ($p>0.05$) (Figure 47).

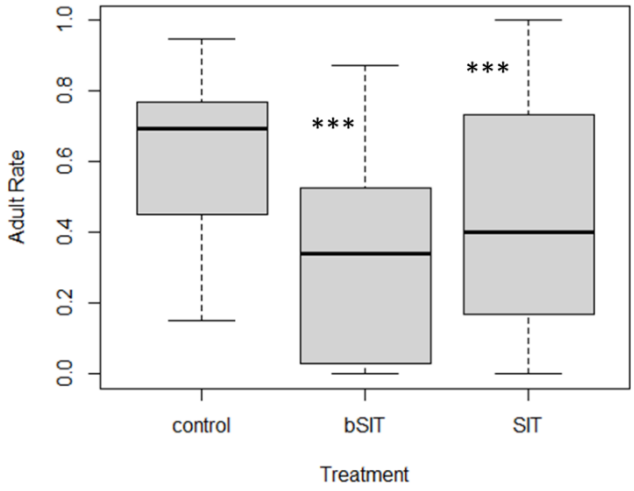

Figure 47. Development rate of larvae into adults. Boxplots present the median value and the quartiles, horizontal bars the 95% percentiles and dots the minimal and maximal values. Significant differences between treatment and control groups are indicated (*** $p < 0.001$).

4. Discussion

4.1. Sexing tool comparison with different strains

Mass production of sterile male mosquitoes is a complex activity strictly connected to larval rearing, which is affected by different variables, such as genetic background, synchronous growth, temperature, humidity, larval density and diets (Couret et al., 2014; Kavran et al., 2022; Mamai et al., 2020; Sasmita et al., 2019). The capacity to collect large quantities of males with the lowest residual number of females strongly affects the feasibility of SIT application (Bellini et al., 2013b; Madakacherry et al., 2014; Rull and Barreda-Landa, 2007; Zhang et al., 2016).

The Fay-Morlan separator represents a better alternative to the sieve as a mechanical sexing tool. The residual female presence obtained using the Fay-Morlan separator showed similar values to other SIT trials by Zheng *et al.*, 2019 in *Ae. albopictus* (Zheng et al., 2019) and by Carvalho *et al.*, 2014 in *Ae. aegypti* (Carvalho et al., 2014), using the same sorting method.

Besides the improved efficiency of the Fay-Morlan sexing separation for both productivity and female presence, there was reduced labour time and improved management, which led to a cost-benefit advantage for the whole production (Parker, 2005). The operating procedures connected to the use of sieves are highly time-consuming due to the management of the biological material (Lutrat et al., 2019; Papathanos et al., 2018). Indeed, the sieving method is quite laborious; the sample material from each rearing tray must be sieved into buckets filled with water at 34 ± 1°C (Bellini et al., 2007). The sieve must then be placed in the upper part of the bucket and left for 3 min to allow male emergence through the mesh (Sharma et al., 1972), and larvae still present between males have to be manually removed or treated with *Bacillus thuringiensis*. The treatment with *B. thuringiensis*, which leads to larval death without affecting pupae survival, may still determine the contamination of the larval rearing with consequent potential risks. Pupae passed through the mesh must be transferred to dedicated containers for final counting and sex ratio evaluation. Material

unable to pass through the sieve, comprised of larvae, female pupae, and some male pupae, must be collected and replaced with new material.

Furthermore, the water temperature must always be regulated during the whole sieving activity to encourage male emergence (Bellini et al., 2007). In addition, it is impossible to control the sieve mesh size to prevent female contaminations unless using a different fixed mesh size sieve. The Fay-Morlan separator has the distinct advantage of removing larvae from collected pupae.

The Fay-Morlan separator enabled the removal of larvae and male and female pupae consecutively in one step (Fay and Morlan, 1959; Focks, 1980). After introducing biological material, water is drained into the separator to allow sample dispersion over the glass plate. Through an initial regulation of the upper screws, it is possible to use only the bottom screws of the separator during the sorting operation, so adjusting the distances between the sheets of glass, pupae, and larvae can flow out easily through the separator step by step. To prevent female contamination, the operator needs to change the spaces between the sheets according to the pupal distribution and size dimorphism (Focks, 1980). Thus, in the present work, the material could be directly collected in dedicated trays for larvae, male and female pupae, directly placed underneath the Fay-Morlan separator.

Even if the Fay-Morlan separator is a better alternative to the sieve, the operating procedure connected with this tool is highly repetitive and demanding for the operators' well-being, especially for continuous and prolonged periods (Papathanos et al., 2018). Furthermore, the residual female presence highly depends on the operator's expertise and how the separation is carried out. The selection of smaller individuals reduces female contamination but simultaneously results in the loss of a more significant number of males (Zhang et al., 2015), affecting male productivity.

Automating sex sorting procedures and equipment can reduce labour costs and other issues, such as human error and microbial contamination, and boost the efficiency of space utilisation (Parker, 2005). The Wolbaki Company has already developed an

automatic sex sorter based on a robotised Fay-Morlan separator (Lees et al., 2021; Leppla, 2022), and some efforts on computer vision have also been conducted to separate males based on pupal (Zacarés et al., 2018) and adult dimorphism (Crawford et al., 2020).

Proper automation of the Fay-Morlan tool could offer an excellent advantage for extensive SIT programs. Still, the inherent limitations of exploiting sex size dimorphism may limit the achievement of optimal results since mass production is also affected by several other variables (Mamai et al., 2020; Medici et al., 2011; Puggioli et al., 2013; Sasmita et al., 2019).

My study demonstrated how different strains and mechanical sexing tools influenced the male productivity yield and residual female presence. The strains with different numbers of breeding generations (low colonisation versus high colonisation) may present other effects when sex-sorted with a specific sorting tool.

As reported by Hendrichs and Robinson, 2021, mass-rearing protocols for SIT are focused on producing males for field release. Using the material recollected after mass rearing permits the maintenance of the colony, but with this system, it is impossible to avoid highly selected genotype accumulation (Hendrichs and Robinson, 2021). In fact, under extensive breeding, modification of reproduction, development, and courtship behaviour has already been observed (Briceño and Eberhard, 2002; Rull and Barreda-Landa, 2007).

Strains with low generation numbers, i.e., the ME and GR strains, showed higher mean male productivity yields than the DE and IT strains with high generation numbers. An extensively reared strain, for instance, the IT strain, presented a significantly increased residual female presence compared to all the other strains.

When sex-sorted with the sieve, the increased female residual presence in the IT strain suggested that females may become smaller over generations, enabling them to pass through the fixed mesh size of 1400 µm of the sieve.

The extensive breeding procedure (high colonisation) might have influenced both the male productive yield and the residual female presence. It is plausible that the number of breeding generations of the reared colony is associated with the size variations, which affects the production yield and female presence once sorted. Size fluctuations have already been observed in adults in cages over generations (Hamady et al., 2013; Pudar et al., 2021) and in pupae when the strain was maintained or crossbred (Bellini et al., 2018; Schneider et al., 2011; Wijegunawardana et al., 2020).

During mass rearing, body size adaptation over generations could not exclude a loss of range dimensional dimorphism between males and females. Thus, a mechanical sexing system with a fixed mesh size, such as the sieve, could affect male productivity and residual female presence. Still, the Fay-Morlan device can be adjusted to fit the pupal dimensions better (Fay and Morlan, 1959; Focks, 1980), overcoming size-related problems due to the adaptation of the colony to cage breeding.

The correlation between pupal and adult body sizes in both sexes has already been shown (Steinwascher, 1982). In addition, size differences in adults, as already reported, influence productivity, mating competitiveness, survival, dispersal rate, fecundity and egg production (Blackmore and Lord, 2000; Hamady et al., 2013; Maciel-De-Freitas et al., 2007; Maïga et al., 2012; Ponlawat and Harrington, 2007; Pudar et al., 2021; Yuval et al., 1993). Furthermore, the effect of strain colonisation on vector competence has been observed (Drouin et al., 2022; Gargan et al., 1983). This suggests that not considering the possible variations in pupal size caused by different strains and extensive breeding processes could strongly affect the quantity and quality of male production (Benedict et al., 2009; Dyck et al., 2021; Vreysen et al., 2006).

The significant effects of the strains, also reared on different diets, on male productive yields confirmed our observation of the negative impact of continuous breeding in artificial conditions on sex separation outcomes. The GR strain, which had a low number of rearing generations, showed the highest male productive yield compared to the other strains. Differences in productivity between these strains could not be

attributed with certainty to the number of breeding generations. Still, the absence of any significant difference between the ME and GR strains reared on the IAEA-BY larval diet, which had similar ranges of breeding generations, suggests that a genetic feature specific to the ME strain might be missing, thus leading to increased productivity.

The absence of differences between strains, when considering the residual female presence, suggests that the Fay-Morlan separator was highly reliable in all strains. This absence also indicated that the decreased number of males obtained by continuous breeding could be attributed to a reduced pupation rate and size dimorphism loss. To achieve lower levels of female contamination, operators may have to increase selectivity using the Fay-Morlan separator, thus also reducing the male recovery rate and compensating for differences in residual female presence between strains (Zhang et al., 2015).

4.2. Larval diet and strain influence with the Fay-Morlan separator

Among larval diets, the BLP-BY diet gave a high production value, suggesting an increased capacity to promote pupation dynamics in the reared strain compared to the SLP-BY. The IAEA-BY diet showed an intermediate tendency to promote pupation dynamics than the other two diets, even if this was not supported by statistical analysis. Larvae fed with bovine liver powder, present in both BLP-BY and IAEA-BY diets, probably reached the critical weight needed for pupae formation earlier (Puggioli et al., 2013; Timmermann and Briegel, 1999).

Before the diet test, strains were all reared on the IAEA-BY; thus, some level of strain adaptation could not be excluded, even if the absence of interaction between strains with different generation numbers and larval diets suggests a lack of strain adaptation to diet.

The results given by the BLP-BY diet indicated that varying only a specific diet content and its composition could modify the effect of the diet on male productivity.

The larval diets also influenced the residual female presence, with the IAEA-BY diet giving the lowest contamination level, confirming that different diets affect the residual female levels differently (Balestrino et al., 2014; Mamai et al., 2020, 2019; Puggioli et al., 2013).

Different compositions of the diets may influence larval development, synchronous growth and size dimorphism. High values of residual female presence given by BLP-BY and SLP-BY diets did not necessarily amount to increased male productivity yields. This unaspected result suggests that high residual female presence does not always correspond to an increased male productivity yield. Other factors besides development speed probably influence female contamination and lack of carbohydrates affecting larval development (Briegel and Timmermann, 2001; Puggioli et al., 2013; Timmermann and Briegel, 1999). Without enough carbohydrates, the female larvae may develop into small-sized pupae, thus affecting the efficiency of sexing methods that cannot perfectly discriminate sexes based on size dimorphism.

In conclusion, the decreased productivity of highly colonised strains may be due to the adopted breeding scheme, which is determined by the need to collect pupating females one day from the onset of pupation (Briceño and Eberhard, 2002; Hendrichs and Robinson, 2021).

The Fay-Morlan separator allows the maintenance of similar levels of residual female presence for all reared strains and larval diet, resulting in reliable and properly managed sex sorting. The number of generations and laboratory involuntary crossbreeding selection must be controlled, particularly in places where SIT programs are to last for many years (Hendrichs and Robinson, 2021).

Collecting data on female productivity and its sex ratio would be necessary to better understand the male productivity of extensively colonised strains. After the sorting, understanding the overall number of males and females would better clarify male pupae

development and male recovery yield. The number of females at the sorting time may not be sufficient to sustain the breeding lines, forcing the re-entry of discarded larvae into the rearing, leading to an increased labour cost. An alternative may be the identification of a specific sorting time, higher with respect to the 24 hours from the beginning of pupation, that would provide a good production with low residual female presence, but at the same time enough females to sustain the adult rearing.

In addition, along with generations, male and female pupal size assessment may provide a quality measure to assess strain adaptation. Since adult body size affects adult quality parameters, the pupal body size measure could also provide an important tool to measure the quality of adult males.

Even if the IAEA-BY diet gave the best results in terms of male productivity and residual female presence, the adoption of a cheaper diet remains crucial. The Black soldier fly larval powder used by Mamai *et al.*, 2019 (Mamai et al., 2019) shows promising results and could ultimately reduce diet costs. Further studies on pupation dynamics, body size dimension and genetic background of both males and females in a mass-rearing context and in using other dietary components are ongoing.

4.3. Sorting time influence using Automatic Fay-Morlan separator

At 48 hours from the onset of pupation, the productivity was higher and female presence was enough to sustain the colony. Nevertheless, the sorting at 48 hours significantly determined an increase in residual female presence. The different growth curves of males and females probably begin to overlap, consequently increasing the number of females present at the sorting time (Carvalho et al., 2014; Mamai et al., 2020; Puggioli et al., 2017). The majority of the females present in the collected pupae were at a very early stage, which may indicate that the hardness of the cuticle may have affected the capacity of the sorting tool. Since females not ultimately formed were soft, they probably were not correctly retained by the two glass sheets. Along the separation table, males stratify downer respect than females (Fay and Morlan, 1959; Focks, 1980).

During the washing cycles, the water cleaning jets may have pushed softer females between males, affecting the female presence.

Good productivity, nonetheless, connected to an acceptable sex ratio of 1.08±0.09%, represents a valuable result demonstrating how sorting carried out at 48 hours reduces the labour cost. In fact, by adopting this time scheme, we could maintain male production for field application and female production for breeding purposes.

The sex sorting carried out 24 hours from the onset of pupation did not provide enough females to sustain the breeding lines. This situation forces the reintroduction of the larvae into the unit racks. Operators must fill the unit racks with water, count and aliquot the larvae, and add the larval diet. Furthermore, another sorting must be carried out after 24 hours to collect females to prepare the cages. It is clear that the 24 hour sorting procedure is highly demanding in terms of labour and costs.

After 48 hours from the beginning of pupation, the rearing of an entire larval unit rack enables the production of around thirty thousand females, the adequate number to start three rearing cages composed of ten thousand females and three thousand males (Mamai et al., 2020).

Three cages must be started after each sorting to sustain the cycle to maintain a constant weekly production of one mass-rearing unit rack. However, the adoption of this scheme presents obstacles.

Some adults have already reached the adult stage at 48 hours. This issue, in some conditions, may lead to a high adult presence in the facility, which could also be characterised by biting females. The significant presence of biting females is a nuisance affecting operators' well-being. The adoption of individual protection systems was necessary.

In this case, operational and technological improvement must be implemented to avoid adult emergence and protect the psychophysics operator’s health.

In addition, when the sorting was performed 24 hours from the onset of pupation, it was possible to leave the pupae for another 24 hours before the irradiation. This time allows pupal maturation to guarantee residual fertility lower than 1% (Balestrino et al., 2010). At 48 hours from the onset of pupation, some pupae have already reached the critical age to start the emergence phase. For this reason, delaying irradiation is impractical; waiting for another 24 hours, many adults would emerge, with a consequent loss of males, affecting SIT application. Furthermore, the early irradiation of some male pupae may also lead to increased residual fertility, which could affect the SIT field feasibility (Balestrino et al., 2022, 2010).

Adopting and developing new solutions to irradiate the material at the adult stage would allow overcoming this issue. Proper tools or dedicated containment cages would permit the adults' isolation from the pupae; in this case, it could be possible to irradiate the adults directly. Adult irradiation should be carried out under refrigerated conditions to prevent adults' movement and at a high adult density to limit the number of irradiations. Adult sterilisation does not affect induced sterility, longevity, and male mating competitiveness in *Ae. albopictus* in comparison to pupal sterilisation (Du et al., 2019). In addition, other studies on *An. arabiensis* have found that adult sterilisation induces a higher level of sterilisation with lower doses than pupal irradiation, suggesting that adult irradiation may maintain a better overall quality of the adults (Ndo et al., 2014).

4.4. Larval density influence using automatic Fay-Morlan separator

Larval density significantly affects male productivity (Puggioli et al., 2017). Since egg production is a crucial activity that may limit larval rearing, by finding a larval density with an increased male productivity yield we may produce the same number of male pupae using fewer eggs. The absence of difference in productivity terms between 1.8 and 2.2 Larvae/ml indicates that the higher larval density can provide a larger total

number of males from a single unit rack. It means that equivalent male productivity determines a higher net production for the higher density.

For a specific regime schedule, where a limited number of unit racks must be sieved each day, higher density offers an advantage in terms of male production, saving space and labour. The absence of differences in terms of residual female presence between the two densities also confirms the choice of 2.2 Larvae/ml as the better density.

However, even if not statically supported, the median higher tendency of lower density may indicate increased productivity that is not well highlighted. Further investigation with different larval densities may provide an improved setting which can increase productivity and help reduce egg consumption.

4.5. Implementation of a hyper-protandry strain through classical breeding

Since the actual mechanical sexing tool does not provide a complete effective sex separation and requires labour, new alternatives or integrations to automatic sexing tools must be developed. Creating a highly selected strain, where the low presence of females is related to high male productivity, may implement the SIT feasibility reducing cost and labour and reducing female contamination (Bellini et al., 2018).

Male productivity at 24 hours from the onset of pupation does not increase, while residual females significantly decrease among generations. Residual female presence at 24 hours follows a linear decrease, providing the lowest female development in the sixteenth generation. Already at the sixth generation, residual female presence significantly decremented; after eleven generations, it reached values of less than 1%.

Only male production was affected when comparing productivity between generations at 48 hours from the onset of pupation. After the eleventh generation, male productivity significantly increases, from 10.88% of the sixth generation to 57.44% of the eleventh generation. The sex sorting of this specific strain, at 48 hours from the onset of

pupation, may offer a tool to implement male productivity significantly. In addition, if we combine male and female productions at 24 and 48 hours for the sixteenth generation, we can observe a male output of 75.75% and a female production of 3.21% to the total number of larvae reared. Considering these productions for an entire unit rack with 663000 larvae, we may produce around 250000 males and 11000 females at 48 hours with a consequent unacceptable residual female presence of 4.4%. Carrying a single-sex sorting of this strain at the sixteenth generation, we may potentially exploit the high male presence. Indeed, the male separation will be unable to recover all the males present at the sorting time to avoid female contamination but can increment male productivity. In this case, sex separation efficiency should be studied to assess if this strain can properly contain female presence.

However, the number of females would be insufficient to guarantee cage production, forcing us to reintroduce the remaining larvae into the rearing unit to collect females at seventy-two hours from the onset of pupation; this procedure leads to a labour and cost increase (Vreysen et al., 2007). In addition, the characteristics of this strain may not be maintained during mass rearing if the selection is not perpetuated.

At seventy-two hours from the onset of pupation, an increasing trend for male and female productivity was found along crossbreeding generations. In the sixteenth generation, both male and female productivity increased. Considering the total male production for every 24 hours is possible to observe that almost every male has pupated.

Further studies must be conducted to evaluate if this strain's features are held with standard rearing protocol. Furthermore, the potential induction of diapausing eggs in this strain may permit storing many eggs produced during the winter, which could guarantee high production and labour reduction during the summer. Also, improvement concerning diapausing induction and hatching should be carried out (Huang et al., 2015; Pumpuni et al., 1992). Combining the productivity of this strain with an automatic sorting routine carried at 48 hours may increase male mass

production. Adopting a mother colony or different crossbreeding protocols, which allow male and female simultaneous production, may improve SIT application.

Currently, crossbreeding selection is ongoing, and application of this strain into the mass unit rack is already planned.

4.6. Feasibility study for the recognition of male/female adult mosquitoes with an optical system

The test of the optical unit integrated with the trained artificial intelligence showed high efficiency in sex identification (Crawford et al., 2020). It presented a total error rate of 0.88%. It must be mentioned that, in this case, the total error rate is comprehensive for both males and females. If we only look at female identification, the total error was 0.10%. The male discarding between females is accepted, and female presence should be maintained for releasing purposes. In this case, the presence of discarded males between females does not affect the process. Indeed, females must be placed into cages to allow egg production and a certain quantity of males is needed.

The adoption of this sexing method must guarantee specific environmental conditions. In our case, to maintain immobilised adults, the temperature must be 8°C (Gómez et al., 2022; Sasmita et al., 2022; Zhang et al., 2020). Adults could start to move or fly if the temperature was higher, affecting the system's efficiency. In addition, also low temperatures and related moisture could affect adult survival.

The development of this sorting system should be done under refrigerated conditions, which is a crucial design aspect. The machine should be provided with a collector tank where adults could be introduced. Through a vibrant convey belt, adults may be carried in the optical system, where they are selected and eventually removed if females. The females could be used to sustain the breeding lines. In the optic of adult chilling for shipment purposes, this technology should be integrated with an automatic supplier to

dispense the proper number of males into shipping containers. The packed adults may always be shipped or transported at the release points under refrigerated conditions.

An alternative to this method, where the fitness could be affected by the low temperature and manipulating procedure, is the creation of proper emerging containers where adults can emerge from the pupae. The natural research of light by the adults could be exploited, and the design of tunnels where the adults can walk inside may offer a valuable alternative to mechanical handling (Burkett and Butler, 2005). An optical unit with artificial intelligence can recognise the adults through the tube and eventually remove unwanted females. At the tubes' ends, adults may be spontaneously directly conveyed into proper adult cages where they could be handled after irradiation, shipment and release.

4.7. Prototype drone releasing tests for aerial distribution of sterile males

The results in laboratory conditions demonstrate how the releasing prototype does not increment adult mortality. After 24 hours from release, adults were still active and presented normal behaviour. The results encouraged the prototype test in operational field conditions. In indoor conditions, the released male from the drone showed mortality comparable to the first laboratory test in laboratory conditions. In this case, it was impossible to identify all the dead males due to the release; some of them may have been pushed away by the drone propellers. The proximity to the ground created turbulence that spread the males once they reached the bottom of the room. It may be possible that this phenomenon may damage the males.

The adults' presence over the surfaces should probably be connected to environmental conditions. Shady conditions and relative lower temperatures may have offered the optimal state for adult recovery and rest.

The release in full field conditions was carried out at a higher altitude, improving the male release performance. Males, in this case, were not spread by the downward airflow of the drone and gently followed wind direction. When they reached the ground, males presented more dynamic behaviour than in the previous test. In this case, most males left the ground surfaces very quickly. The field conditions, higher external temperature, and sun conditions have probably stimulated the adults' activity.

Males were packed into the releasing device under chilling conditions for both drone releases and maintained in the dedicated portable fridge at 8°C (Gómez et al., 2022; Sasmita et al., 2022; Zhang et al., 2020). The fridge allowed prototype transportation to the release points, where it was mounted on a drone as quickly as possible. In this case, when the releasing prototype, containing males, was removed from the fridge, no refrigerated gels were placed inside the releasing prototype to permit gradual temperature adaptations to the field conditions of the males. The onboard probe reported an internal temperature of 28-30°C and relative humidity of 36-37 RU%. These values, combined with the data obtained, suggest that even if the adult gradually started to regain activity during the release did not significantly affect the release's performance.

Further study must be carried out to evaluate the male quality, mating competitiveness, adult survival and sterility induction to assess the potential of this application (Bouyer et al., 2020; Marina et al., 2022).

Indeed, using drones to release sterile males could help field distribution. It would be possible through a drone release to overcome natural and artificial barriers such as rivers, streets, premises and hedges (Bouyer et al., 2020). This strategy would undoubtedly be an advantage, but inherent technical and aeronautical constraints must be respected and faced. In fact, different areas where drone operations can be carried out, respecting specific height and weight limits, are identified by each country's national aeronautical authorities. In Italy, the Italian Civil Aviation Authority (ENAC) provides indications, limitations and restrictions following guidelines expressed by the

International Civil Aviation Organization (ICAO) (*Normativa Droni*, 2021; *Rules or Guidance*, 2022).

In Emilia-Romagna, the Regional Public Health Service manage mosquito surveillance and control (Linee guida per gli operatori dell'Emilia-Romagna, 2022). At the same time, the municipalities carry out the assignment of tasks to different private companies or organisations, where specific tenders contain integrated pest management projects, which may include the SIT. This framework is where SIT is currently developed in Bologna Municipality, and only particular areas are identified as suitable for sterile male field release. Currently, field release of sterile males is carried out by car. A driver follows a specific path, and the technician releases males directly outside the car's windows, opening the tubes. This procedure strongly limits males' distribution and restricts the release to public areas.

Adopting and integrating an aerial release with standard releasing protocols would offer a tool to cover extensive areas in less time, reducing costs and permitting the release over private yards (Bouyer et al., 2020; Marina et al., 2022). The area where SIT is carried in Italy is currently located in the north part of Bologna municipality, a 100-hectare of surface in the proximity of the Bologna airport. Following ENAC instruction, the 100-hectare area is not prompt for the Open category, which allows flying up to 120 meters high with a maximum take-off mass of less than 2 kg (*Categoria aperta (Open Category)*, 2021).

Drones near Bologna airport need to be used in the specific category. The drone operator must ask for a specific authorisation based on operation risk assessment from the National Aviation Authority (*Categoria specifica (Specific category)*, 2022).

The risk assessment is a comprehensive analysis of all risks connected to the operations. Adopting proper emergence procedures, tools, compliance with a certain weight and inoffensive limits can lower the risk threshold until it is acceptable (Guglieri et al., 2014).

Usually, all the procedures connected to drones require to prohibit access to people not involved in drone operations. This fact can strongly limit the feasibility of using drones to carry out the sterile male adult release in an extended area. Exploiting characteristics that make the drone inoffensive, it could be possible to overflight uninvolved people. Developing a specific drone with an integrated releasing prototype, which maintains inoffensive features, could be possible to obtain a good tool suitable for field application.

4.8. Boosted SIT trial at Réunion Island

The sterile insect technique relies on releasing sterile males; these males must be in the proper quantity and quality to achieve the desired result. The efficiency of this technique is directly connected to the population density reached by *Ae. albopictus* during the summer season. Larval habitats, where *Ae. albopictus* can develop, are small, widely distributed, and often present in private yards. These larval habitats are not all accessible by pest control companies, so a 100% removal or treatment of larval breeding sites is challenging to achieve (Donati et al., 2020). Auto-dissemination characterises a possible approach to overcome this issue. Auto-dissemination relies on the coating of *Ae. albopictus* with pyriproxyfen (PP) (Seixas et al., 2019; Unlu et al., 2020). Pyriproxyfen is a juvenile hormone analogue (JHA) that prevents the metamorphosis of larvae into adults (Invest and Lucas, 2008). Through autodissemination stations (Devine et al., 2009), females involuntary cover their legs with pyriproxyfen. Females so contaminated can fly away and disseminate into the larval breeding sites during egg deposition. Pyriproxyfen may convey into the breeding sites and carry out its larvicidal action. The main problem of this strategy is that the amount of PP transported by females decreases with their density, making its efficiency strongly density dependent.

An integration of this approach with SIT, coating sterile males with pyriproxyfen, could enhance the impact of this strategy (Bouyer and Lefrançois, 2014). The meeting

of these males with wild females may determine female pyriproxyfen contamination. Females so sterilised not only lay unfertile eggs but can disseminate the larval regulator into the larval breeding sites where other fertile females have already laid fertile eggs.

Combining the two techniques may lead to an improvement in SIT application. However, the pyriproxyfen treatment as the sterilisation process may affect the quality of the released males.

Results showed that irradiation and pyriproxyfen covering did not affect the male flight capacity, suggesting that the overall quality of PP-coated males was not significantly affected compared to sterilised males and control.

Mating competitiveness is the capacity of a sterile male to compete with a wild male to mate with a female (Bellini et al., 2013a). The mating competitiveness of PP-coated males presented a similar mean value to only sterile males for the 1:1:1 test.

Surprisingly, in the 5:1:1 ratio, sterile males presented higher mating competitiveness compared to PP-coated males. Further studies must be carried out to confirm and interpret this result.

PP-coated sterile males presented lower variability than sterile males in mating competitiveness for both ratios. In addition, PP-coated males showed similar values between the two ratios.

Probably pyriproxyfen coating can limit movement or sexual attractiveness (Culbert et al., 2020). This result cannot be entirely connected to pyriproxyfen and may be related to the fluorescent powder, which becomes particularly visible at the increased ratio. In addition, we could not exclude that even the treating procedure may have affected the overall mating competitiveness. Indeed, PP-coated males are rolled into PP powder, at 5°C which is known to reduce male quality (Culbert et al., 2019).

The significant reduction of the developmental rate of larvae into pupae suggests that both sterile and PP-coated males affected larvae. The slight minor tendency of PP-coated males with respect to sterile males may indicate a possible effect led by

pyriproxyfen, even if not supported by statistical analysis. The absence of a ratio effect suggests that a significant accumulation of pyriproxyfen did not probably occur. The remaining effect might be related to the random mutations transmitted by sterile males.

A significant, even if marginal, reduction of pupae emergence was observed only in PP-coated males suggesting the possible role of pyriproxyfen contamination.

Also, the development rate of larvae into adults has been affected. Even in this case, a limited improved effect has been observed for PP-coated males with respect to control.

The absence of a ratio effect suggests missing a specific accumulation of pyriproxyfen in the breeding sites. However, minor improvements observed suggest a partial effect of pyriproxyfen. Testing different quantities of pyriproxyfen with respect to fluorescent powder or tools to cover the male body would increase the efficiency of boosted SIT.

Further study must be conducted to assess if PP may show adverse effects on other species. This preliminary study may offer the first step to assess the combination of the sterile insect technique with other biocidal compounds, such as *Bacillus thuringiensis* and crystallised Densoviruses (Bouyer, 2017; Perrin et al., 2020).

These technologies could improve standard SIT leading to an improved *Ae. albopictus* control.

5. Conclusions

Aedes albopictus is a vector able to transmit several arboviruses. Due to its high impact on human health, it is important to develop an efficient control strategy for this pest. Nowadays, control based on chemical insecticides is limited by the number of available active principles and the emergence of resistances. A valuable alternative to classic control strategies is the sterile insect technique (SIT) which relies on the release of the target insect sterile males. Mating between wild females and sterile males results in no viable offspring. A crucial aspect of SIT is the production of a large number of sterile males with a low presence of females that can bite and transmit viruses. Since productivity and female contamination during mass production are affected by different variables, we investigated mechanical sorting tools, strain, larval diets, sorting time and larval density in this thesis. It emerged that the use of the sieve could be limited by colony adaptation over breeding generations, while the Fay-Morlan separator, as well as its automatised version, could be a valuable tool to overcome this issue. Strains with different degrees of colonization and larval diets affect the productivity and the female presence; therefore, control of these variables could improve the feasibility and reduce the costs of SIT programs.

It emerged how to simplify the creation of mass production facilities, able to self-sustain the breeding colonies, the sieving at 48 hours allows not only good male production and female contamination but also allows the production of enough females to able to sustain the rearing. This approach avoids the larval reinserting after the sorting at 24 hours from the onset of pupation, when females were not enough for cage production, leading to a significant labour and cost reduction.

Currently, the larval rearing adopts a 2.2 larvae/ml density providing good net productivity. It could be possible that adopting different larval densities productivity may increase, potentially leading to the same net production but with fewer eggs.

The selection for an *Ae. albopictus* hyper-protandric can significantly improve sex separation in a mass-rearing facility. Through generation, female presence emerged

suitable for field release at 24 hours from the onset of pupation. However, proper larval removal still has to be performed. This method may support better productivity in mass-rearing facilities while allowing the release of males without any size selection.

The optical system integrated with artificial intelligence may provide a strong candidate for sex selection. This tool allowing adult recovery with low female contamination may be applied to the entire larval development. This tool not working only at a specific sorting time may lead to the process of all the material hatched, significantly increasing SIT feasibility.

Thanks to the results obtained, the increased male production has raised further problems. The manual handle of a high number of releasing tubes containing sterile males has moved the attention to finding a new releasing system able to spread the sterile males in a wide area to reduce costs. The work presented demonstrates how drone release is a valuable alternative that could be integrated into urban environments.

Furthermore, the Boosted SIT application in Italy may also strengthen the control of larval production in unreachable breeding sites.

All the efforts and results obtained with this thesis have allowed us to confirm and improve the applicability of this technique on a large scale. SIT programs will cover more and more cities with ever larger areas, and solutions here developed surely lay the foundation for further technological advancements.

Finally, the progress of the Sterile Insect Technique and its implementation in area wide control strategy would permit the control of *Ae. albopictus* under an acceptable sanitary and nuisance threshold. All this progress would safeguard people's health and improve the use of gardens and inhabited spaces strongly affected by the presence of this insect of medical interest.

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Acknowledgements

My deepest thanks go to Dr Romeo Bellini, Dr Arianna Puggioli, Dr Fabrizio Balestrino and to all the other colleagues of the Centro Agricoltura Ambiente, to Prof. Maria Luisa Dindo of the Entomology area of the Department of Agricultural Sciences of the University of Bologna and to Dr Jérémy Bouyer together with his research group, CIRAD Pôle de protection des plantes, Saint Pierre, La Reunion.