

GUIDELINES FOR MASS-REARING OF AEDES MOSQUITOES

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GUIDELINES FOR MASS-REARING OF AEDES MOSQUITOES

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FOREWORD

Sterile insects have been defined as beneficial organisms by the International Plant Protection Convention. The Sterile Insect technique (SIT) has been applied in more than 30 countries worldwide for pest suppression, eradication, containment and prevention. Current production of sterile fruit flies supporting pest control programmes is over 3 billion insects per week. Conversely to fruit flies and other plant pests, less than ten million sterile mosquitoes are currently produced per week in a small number of insectaries at the global scale with a low production capacity.

The number and production capacity of mass-rearing insectaries for mosquitoes is expected to increase in the coming years and this joint Food and Agriculture Organisation/International Atomic Energy Agency (FAO/IAEA) Insect Pest Control Laboratory (IPCL) guidelines for mass-rearing of *Aedes* mosquitoes has been developed to provide a description of procedures required for *Aedes aegypti* and *Aedes albopictus* mass-rearing. It is a summary of necessary steps of larval and adult mass-rearing as used at the FAO/IAEA. This is a temporary guide and will be continuously updated considering the improvements brought to the mass-rearing of mosquitoes.

If users need to consult more details of this or other processes do not hesitate in contacting the editors of this manual.

The Officers responsible for the publication were Hamidou Maiga, Wadaka Mamai, Hanano Yamada, Rafael Argilés Herrero and Jeremy Bouyer of the Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture.

Contents

1.	Intr	oduction6					
2. Larval stage							
	2.1-	Tray-rack set up6					
	2.2-	Rearing in large trays					
	2.3-	Feeding larvae					
	2.4-	Tray tilting					
3.	Pup	Pupal stage					
	3.1 Se	paration of male and female pupae12					
	3.2 Es	timating the number of pupae13					
4.	Adu	It stage14					
	4.1 M	ass-rearing cage description and setup14					
	4.2 Lo	ading of the mass-rearing cage17					
	4.3 Su	gar feeding solution17					
	4.4 Bl	ood feeding18					
5.	Egg	stage19					
	5.1 Eg	g collection-maturation-drying and storage19					
	5.2 Eg	g estimation20					
6.	Rel	evant and cited references					
7.	Арр	endix					

1. Introduction

The sterile insect technique (SIT) relies on producing sterile males of good quality in large enough numbers to achieve the target production level of males to be released. Therefore, the conditions and procedures under which mosquitoes are produced are of utmost importance for the overall success of the SIT activities. This document aims to provide a description of procedures required for *Aedes aegypti* and *Aedes albopictus* mass-rearing. It is a summary of necessary steps of larval and adult mass-rearing as used at the joint Food and Agriculture Organisation/International Atomic Energy Agency (FAO/IAEA) Insect Pest Control Laboratory (IPCL). This is a temporary guide and will be continuously updated considering the improvements brought to the mass-rearing of mosquitoes.

2. Larval stage

2.1- Tray-rack set up

Larvae are reared in a steel rack containing thermoformed ABS (acrylonitrile butadiene styrene) plastic trays (Glimberger Kunststoffe Ges.m.b.H., Austria) as described by Balestrino et al. (2012, 2014a). The trays are 100 \times 60 \times 3 cm in size and have a flat surface with two long ridges (each 32cm long, 2.5cm high) running along the major axis (Figure 1).



Figure 1. Thermoformed ABS (acrylonitrile butadiene styrene) plastic trays.

A fully loaded rack can hold 50 large plastic trays (Figure 2) and each tray must be checked carefully to have perfectly horizontal position before rearing. However, depending on the height of the rearing room, the rack can be designed to hold less than 50 trays. Each tray can be filled with 5L of water (water depth of 1.2 cm) and seeded with 18,000 first instar larvae (L1) corresponding to a larval density of 3.6 larvae/cm² (Zhang et al. 2017). Therefore, each rearing rack can hold 900,000 larvae at a time. Currently, the handling and maintenance of the larval rearing (e.g. adding food and water) are done manually, but a system to aliquot a set volume of diet into each tray using a dosing gun is under development. An automated prototype of the tray-rack is available allowing tilting the whole rack electrically (see **2.4 Tray tilting section**).



Figure 2. A full electrically automated rack holding 50 trays (a). The shelves that facilitate a sliding and tilting mechanism for the large plastic trays (b); the large plastic trays stacked in the steel rack complete with water, larvae and diet slurry (c).

2.2- Rearing in large trays

To prepare the large trays for rearing, each tray should be filled with 5L of water the day before adding the larvae to allow the water reaching ambient room temperature. Trays can also be filled with 5L of water the same day as larvae (water depth is between 1.2 and 1.5 cm). However, caution should be taken to ensure that water temperature is not lower than 27°C. Osmosis water should be used for mass-rearing larvae but tap water can be used exceptionally if its conductivity is below 50μ S/cm. *Aedes* larvae shall be maintained under controlled and stable conditions of temperature, humidity and photoperiod so that they develop in a predictable and reliable way every time eggs are hatched. A periodicity of lighting (light:dark cycle to mimic day:night) entrains larvae so that pupation and emergence is more synchronised and independent of season. *Aedes* larvae shall be reared in a climate-controlled room at $28\pm 1^{\circ}$ C, 70-80% RH with a 14h:10h light:dark photoperiod including 1 hour dawn and dusk.

Egg hatching

Although hatching *Ae. aegypti* and *Ae. albopictus* eggs is a simple task, it is influenced by factors including mosquito strain, egg age, dryness, storage conditions, temperature, humidity hatching media and oxygen levels (Hien 1975). Therefore, it is crucial to standardize the hatching protocol and eliminate factors contributing to data variability. After embryonation (see section 5.1), one-two week-old eggs are ready for hatching. However, a hatch rate greater than 80% can be obtained after 10 weeks of storage in plastic zip lock bags in *Ae. aegypti* and 11 weeks in *Ae. albopictus* (Zheng et al. 2015a). Loose and stored eggs can also be hatched with good results. Using oxygen-depleted water can help improving hatch synchronicity and can be obtained using a few different methods such as boiled cooled osmosis water only or with hatching solution (osmosis water with 0.25 g nutrient broth and 0.05 g brewer's yeast).

To be consistent with the protocol of Zheng et al. (2015b), we recommend, 2 week-old eggs that are brushed by using a medium-bristled brush (see Figure 22). From those eggs, 3 samples of 100-150 eggs are hatched overnight in the insectary conditions using a 50mL centrifuge tube (VWR, UK) filled with 40mL of either

boiled cooled osmosis water (*Ae. aegypti*) with 2mL of 4% larval FAO/IAEA diet or with hatching solution (*Ae. albopictus*). On the following day, the egg hatch rate of each sample is verified under a stereomicroscope. Once the hatching rate has been estimated, the required egg numbers are estimated using the equations described by Zheng et al. (2015b) (see section 5.2).

Egg batches for18,000 L1 are hatched separately. To do so, jam jars (IKEA of Sweden AB SE-343 81 Almhult, Germany) (Figure 3a) filled with 0.7L of boiled and subsequently cooled (deoxygenated) osmosis water stored at laboratory temperature are opened to add the eggs, before the jars are closed again to avoid re-oxygenation. A volume of 10 mL of 4% larval FAO/IAEA diet is added to jars to improve egg hatching and synchronization of larval development (*Ae. aegypti*). For *Ae. albopictus,* eggs are added to jam jars filled with 0.7L of hatching solution. All jars are kept overnight (for 20 hours) before their contents are sieved (Figure 3c) and transferred to the mass-rearing trays (Figure 3d) previously filled with 5L of osmosis water. If the jar content has to be transferred without sieving, make sure that the total volume of water in the tray is adjusted to 5L (0.7L of the jar content + 4.3 L of osmosis water).



Figure 3. Jar with air-tight lid filled with hatching solution (Nutrient broth and yeast) and brushed eggs (a) or with boiled cooled osmosis water (b) for hatching. Newly hatched first instar-larvae are sieved (c) before transfer to each rearing tray in the rack

Egg batches of 300,000 eggs/L can also be hatched together before dispensing L1 in the rearing trays according to the procedures described by Carvalho et al. (2014). To do so, stir hatched larvae in water using a magnetic stirrer and flea for sufficient time to take aliquots; take four aliquots of 1 mL each and count the number of live larvae present by eye; the use of a counter is recommended. The average number of larvae per mL is then used to calculate the volume of water to add on each tray to get the desired number of larvae per tray. This method is routinely used at the IAEA Collaborative Center, Moscamed, Brazil as another possibility of *Ae. aegypti* larval rearing in a mass-rearing facility.

The egg hatching procedures for *Ae. albopictus* as performed at the IAEA Collaborative Center, Centro Agricoltura Ambiente "G.Nicoli", Crevalcore, Italy require the eggs being left overnight in a glass jar with nutrient broth and yeast. However, the hatching duration can be reduced by leaving the nutrient broth solution overnight in sealed glass jars without the eggs, introducing the eggs in the next morning and resulting after 4 h with more synchronized larvae (Bellini et al. 2018).

2.3- Feeding larvae

Larvae are fed on an artificial liquid diet (4% w/v that was developed at the IPCL in Seibersdorf, Austria (Puggioli et al. 2013, FAO/IAEA, 2017). The diet is designed to adequately supply all necessary components for larval growth including fatty acids, proteins and vitamins. It consists of a combination of powdered tuna meal (50%), bovine liver powder (36%) and yeast (14%) (Table 1). However, as bovine liver powder is expensive and not widely available to all Member States, its potential replacement is under investigation at the IPCL.

As larval food availability and cost might be different from one country to the other, locally available and cheapest diets are used. However, it is recommended to ensure that its production is standardized with no contamination and fully assess the effect of each diet mixture on mosquito life history traits before using in mass –rearing. For example, Friskies dry adult cat food (Nestle S.A., Vevey, Switzerland) and rodent diet (PMI Nutrition International LCC, St. Louis, MO) are used in diet mixtures by some research and release programmes in Brazil (*Ae. aegypti*), Mexico (*Ae. aegypti*) (Bond et al. 2017) and Italy (*Ae. albopictus*) (Puggioli et al. 2013).

%	Component	Abbreviation	Potential supplier
50	Tuna Meal	ТМ	T.C. Union Agrotech (Thailand)
36	Bovine Liver Powder	BLP	MP Biomedicals (USA)
14	Brewer's Yeast	BY	MP Biomedicals (USA)

Table 1. Components of the IAEA reference diet

Ensure that the tuna meal is finely ground with a jar filled with balls (Figure 4a). The grinding jar is filled with larval diet mixture and inserted into the food grinder (Planetary Ball Mills, Model PM 100) (Figure b). On a daily basis, all ingredients are weighed with an electronic balance and diluted in deionised water to produce a 4% suspension (Figure 4c). Monthly use amount to be prepared can be estimated based on the daily use.



Figure 4. Grinding jars and balls for PM100 (a), larval food grinder (b), liquid diet (c).

The stock supply of the diet composition should be stored in an airtight container at 4°C to prevent loss in quality. For ease and speed of diet preparation, keep a store of aliquots of all the components in sealed containers in a fridge. The IAEA liquid diet, composed of 50% tuna meal, 36% bovine liver powder, 14% brewer's yeast (Damiens et al. 2012, Puggioli et al. 2013, FAO/IAEA, 2017) is provided daily to the larvae from the first day of rearing to achieve synchronised development. For example, when using IAEA large larval tray with 18 000 larvae, a daily volume of food provided per tray from first to ninth day are 50, 100, 200, 200, 150, 50, 50, 50, 50 mL corresponding respectively to 0.11, 0.22, 0.44, 0.44, 0.33 and 0.11 mg of ingredients per larva per day (Maiga et al. 2019). However, different feeding regimes can also be tested by laboratories to ensure good quality and maximum pupation rates on the first days. To prevent sedimentation of the liquid diet, shake vigorously the bottle containing the liquid diet before each aliquot, which should be distributed into both sides of the large tray. First pupation is usually recorded at day 6 from L1 introduction.

2.4- Tray tilting

The collection of larvae and pupae is done by tilting the trays in the rack on day 6 of development (after larval transfer). The pupae and the larvae stream down the vertical stainless-steel wall (Figure 5a) to the movable canal (Figure 5b) while tilting, are collected in the large baskets with sides made of 200 μ m mesh (Figure 5c). The larval water flows through the collection basket into a movable tank (Figure 5c).



Figure 5. Different parts of the rack tilting process comprising a vertical stainless-steel wall (a), a movable canal (b) and the collection basket on top of a movable tank (c).

Use the screw jack at the base of the rack to slowly create a slight and gradually increasing slope that will push the water slowly out of the trays (Figure 6). Care should be given not to tilt the trays too much at a time or too quickly to prevent water containing larvae and pupae splashing outside the collection basket.



Figure 6. The screw jack at the base of the rack (a); tray-rack in a tilted position (b); larval, pupae in the collection basket (c).

An automated electrical system (Figure 7) is available and can also be used for tilting. Up and down buttons allow a gentle tilting of the trays. Tilting speed can also be adjusted.



Figure 7. An automated electrical system used for tilting the rack.

As the rack at its full load holds around 250 L (for 50 trays) of water in total, the collection should be done slowly in batches of around 25 L per tilt. Pour the water from the collection basket (Figure 8a) into a 300 μ m sieve (Figure 8b) to concentrate the larvae and pupae and dispense them into small larval trays with 1-2L of osmosis water (Figure 8c). Repeat the tilting process (approximately 10 times) until all water from the trays is collected. Towards the end of tilting, many larvae and pupae remain at the edge of the rearing trays and need to be washed down with the collected larval water. All the remaining larvae and pupae should be collected and concentrated down to a few small rearing trays before the separation of larvae from male and female pupae.

The larval water collected in the movable tank is reused to rear the remaining larvae after pupae separation until the last sorting. However, performing more than one tilting/sorting event should be considered as a trade-off between pupae production, female pupae contamination and cost efficiency. In case of the fouling of the media, used larval water can be changed or partially replaced with osmosis water ($28\pm1^{\circ}C$) after the first sorting event.



Figure 8. A collection basket used to collect larvae and pupae (a); A 300 µm sieve on top of a water container (b) used to transfer larvae and pupae from the collection basket; Small rearing trays (c) to split larvae and pupae before the separation.

3. Pupal stage

3.1 Separation of male and female pupae

Because pupation is spread over time, separating pupae from the remaining larvae is needed. For rearing on a larger scale, it can be done using a mechanical device designed and manufactured for the separation of the developmental stages and sexes of mosquitoes (Focks, 1980).

The unit (Figure 9) consists of an approximately horizontal platen (1) supporting 2 glass panes that form between them an adjustable, downward-pointing, wedge-shaped space into which the contents of an aquatic insect culture are poured. The various forms can thus be separated (sexes, or developmental stages) based on size by regulating the thickness and angle of the wedge-shaped space by means of 4 control knobs (5). The lower opening is adjusted so that the larger organisms are retained in the tapering space between the panes of glass while the smaller forms (male pupae and larvae) drain through into a receiving container below. The operation is completed by opening the wedge and flushing the larger organisms (female pupae) into a second receiving container. The force with which the insects flow between the plates may be varied by adjusting the angle of the platen (3).

In addition to separating pupae by sex, the machine can be used to separate pupae from larvae. Repeat the pupal extraction on the following days until enough pupae have been produced to fill the required number of large cages.

To obtain higher male pupae rate, it is crucial to standardize the larval rearing conditions including homogeneous temperature in the climatic room (avoiding stratification) and larval diet dosages.



Figure 9. Detailed design of the sex sorter (left) (Focks, 1980) and larval and pupae mixture separated by size/sex (females, males and larvae) using a mechanical separator (right).

3.2 Estimating the number of pupae

The pupae production should be estimated before irradiation and release procedures. In the same way, before adding the pupae to a large cage, their count needs to be estimated so that the cage is filled with the correct density for optimal egg production. This is done volumetrically using a modified 10 or 50mL tube. First cut the tube horizontally. Then unscrew the cap and seal a fine mesh $(1 \times 1 \text{ mm})$ to that end. It should be small enough to hold the pupae but big enough to let the water flow through. To strengthen the mesh, cut a large hole in the cap and screw it back onto the tube (Figure 10a-b).

To estimate the number of pupae collected, let the modified tube stand upside down (on its cap) and add the pupae gently though the opening (Figure 10c). A small funnel can be used to pour pupae in the estimation tube. The water will flow through the mesh slowly, but the pupae will remain. For example, the level of 500 pupae is marked on the tube (Figure 10d) and thus allows daily estimation of the number of pupae. A measuring spoon with a mesh on the bottom, calibrated with 500 pupae or more can also be used to estimate the total amount of male and female pupae produced (Carvalho et al. 2014).

If higher numbers are to be estimated, caution should be taken to consider the estimation level that would not impact pupae quality (abnormal mortality due to high number of pupae per tube).



Figure 10. The net $(1 \times 1 \text{ mm})$ from outside (a) and inside (b) the tubes; Pouring pupae into tube using a pipette with a large opening (c); A modified tube calibrated for 500 male pupae (d).

4. Adult stage

Adults are maintained in a separate room under 26 ± 2 °C, 70 ± 10 RH%, 14:12h light:dark, including 1h dawn and 1h dusk.

4.1 Mass-rearing cage description and setup

Aedes mass-rearing cage (MRC) (Figure 11) of 100 litre-volume $(100 \times 100 \times 10 \text{ cm})$ for brood stock Ae. albopictus and Ae. aegypti colonies has been developed. The cage can be handled entirely from the outside, minimizing the risk of escape and facilitating the management of adult rearing colonies. Devices for the introduction of pupae (drainage system) (1), sugar feeding (2), blood feeding (3), egg collection and for the cleaning of the cage (4) are described.



Figure 11. Technical drawing with top, front, and side views (left) and picture of the Aedes mass-rearing cage (right). Devices for pupal introduction (inlet valve), and cleaning (outlet valve) (1), sugar feeder (2), blood feeders (3) and for egg collection and cleaning (4)

In addition to the general description made here, very detailed procedures for end users in given below:

<u>Hanging the cage up</u>

To minimize ant infestation, and to maximise use of insectary space, the adult rearing cages are designed to be suspended from the ceiling. The top side of the cage features two metal eyelets to which two carabineers can be attached (Figure 12). The carabineers can be hooked to two chains of appropriate length and position to suspend the cage (17kg) from the ceiling at a convenient height.



Figure 12. A carabineer attached to the top of the cage. The cage is suspended on a chain hanging from the ceiling.

<u>Drainage system</u>

Elbow plumbing fixtures are screwed to the stopcocks to serve as a movable spout, taking care to leave it loose enough to enable it to swivel either up or down (Figure 13a). It is suggested to ensure that the outlet stopcocks are in the "closed" position when not in use to avoid accidental spillage of water or pupae and/or floating eggs. Water is introduced at the cage bottom (Figure 13b) together with the pupae when the cage is set up. Drainage is performed by opening the bottom valve (Figure 13a) and flushing at the same time water from the inlet valve. Water can be poured inside the inlet valve with the use of a funnel (Figure 13c). The first drainage after pupal introduction should to be performed when all pupae have emerged (usually 48 hours after pupal introduction).

Water also needs to be replaced once or twice following egg papers collection to avoid fermentation of the dead adult which fell into the water. Fermentation can be also facilitated by sucrose solution drops from sugar device.



Figure 13. The drainage system. The stopcock with the elbow plumbing fixture down to drain water(a); View of the water outlet from inside the cage (b); The stopcock with the elbow plumbing fixture up to load the cage with pupae (c).

Internal sugar feeding device

This device is made of a stainless-steel cylindrical tube (6 cm in diameter) sealed on each end. On one side of the tube, at 5 cm from the end, an inlet pipe with a watertight stopper is connected for adding sugar solution from outside of the cage. This device is held in position along the length of the lower half of the cage by holders attached to the cage sides (Figure 14a). Three large cuttings are located along the tube on the same side as the inlet pipe and covered with 20-micrometer stainless steel mesh (Figure 14b). When filled with sugar solution (1.2L maximum capacity) (Figure 14c) and rotated by 180 degrees, the adults can feed through the mesh).



Figure 14. The internal sugar feeding device. The device spans the entire width of the mass-rearing cage (a). Sugar device mesh trough which mosquitoes can feed (b). The sugar solution is poured through the open elbow plumbing fixture into the tube (c).

<u>Net fitting</u>

The side panels of the cage must be made by cutting netting rectangles to size and lining them with Velcro[®] along all four edges. This is fitted by pressing the Velcro[®] against the corresponding Velcro[®] trimming around the metal frame of the cage (Figure 15a). The edges of the screen are sewn with strips of Velcro, which can be fixed on the cage frame on Velcro[®] strips previously glued (Henkel Central Eastern Europe GmbH, Austria). Care must be taken to ensure that Velcro[®] is neatly fitted (Figure 15b) on the cage frame and no gaps are present. Time should also be taken on a regular basis thereafter to inspect the netting for any damage. The adherence of the Velcro[®] to the metal frame should also be inspected and may be repaired with a contact adhesive.



Figure 15. The detachable netting system using Velcro® tape. A corner of an open cage aligned with Velcro® taping (a). The net is taped onto the cage by pressing it against the corresponding Velcro® trimming (b).

Blood feeding ports

The top of the cage has two round holes (3-5cm of diameter) to serve as blood feeding ports (Figure 16a). Two 17 cm long mesh socks are each hung vertically from these ports (Figure 16b) and allow the insertion of blood-filled sausage casings for blood feeding. The mesh socks can also be located either in the middle or at each end of the cage to increase the access to the blood meal.



Figure 16. The blood feeding ports (a). A 17 cm long mesh sock is hung vertically from one port for insertion of a sausage to provide mosquitoes with blood.

4.2 Loading of the mass-rearing cage

Through the inlet valve, it is possible to fill the bottom of the cage with water and to introduce the pupae when needed (Figure 17). The size of the initial batch of pupae to be loaded into the cage can be calculated as 20,000 (corresponding to 20 pupae /cm² according to the water surface available at the cage floor). This pupae batch size also corresponds to the maximum adult stocking density (Density Resting Surface) of 0.9 adult/cm² and taking into consideration the vertical resting surface available in the *Aedes* MRC (Balestrino et al. 2014b). The egg production of the cage can be assessed with 12, 000 and 4, 000 (ratio 3:1) female and male mosquitoes respectively corresponding to a Density Resting Surface of 0.8 adult/cm².

To load pupae in the cage, turn the spout into an upright position and open the stopcock (Figure 17), which will provide a small access to the inside of the cage. Place a large funnel into the opening and pour water containing pupae into it, allowing them to flow into the trough inside the cage. Add 2 litres of osmosis/distilled water to allow the pupae to spread evenly and prevent overcrowding through clustering.



Figure 17. Funnel used for loading massrearing cage with pupae

4.3 Sugar feeding solution

A 10% sugar solution is provided to the adults in the cage through the internal sugar feeding device. One litre of the 10% sugar solution is poured into the sugar feeding device through the open elbow plumbing fixture at the end wall (Figure 18) before loading the cage with mosquitoes.



Figure 18. The elbow plumbing fixture allows the sugar solution to be poured into the feeding device from outside.

4.4 Blood feeding

Aedes aegypti and Ae. albopictus mosquitoes are voracious feeders who readily feed from a variety of both mammalian and avian sources, with a strong preference for mammalian blood (Templis et al. 1970). Blood to be used in a mass-rearing facility must be collected from a slaughterhouse with controlled and certified veterinary system for animal diseases.

For the *Aedes* species mass-rearing cage described in these guidelines, blood sausages are used to feed females (Balestrino et al. 2014b). Two mesh socks are hung in the cage to hold collagen sausage casings (edicas 28NU, FIBRAN S.A., Girona, Spain) each filled with 150mL of defribrinated fresh swine blood (Figure 19a) heated in a water bath at 42°C for 10 min (Figure 19b-c) and the excess of water removed from the outside of the sausage using a paper towel (Figure 19d). The heated blood sausages can be inserted (Figure 19e) for blood feeding the caged females (Figure 19f). The immediate response of females to the blood source assures an effective engorgement rate after the first 20-30 min of exposure. During this time, the collagen sausage casing can maintain optimal temperature to trigger a blood feeding response before the same process is repeated to allow females to feed on warm blood for a total of 2 hours in each cage. The same feeding is repeated twice a week.

It is important to use a 25 cm-long sausage to ease recollection after feeding. Care should be taken for the quality of blood used to feed mosquitoes. Blood from animals submitted to some antibiotics or any other treatment should not be used as it might affect egg production and/or fertility.



Figure 19. Mass-rearing cage blood feeding process. Sausage casing filled with 150ml of blood (a); water bath set at 42°C (b); Heating blood sausages for 10 min (c); Rolling the blood sausage using a paper towel to remove excess of water (d); Insertion of blood sausage into hanging socks (e); females feeding on blood sausage through hanging socks (f).

5. Egg stage

5.1 Egg collection-maturation-drying and storage

The oviposition system created by the entire lower portion of the *Aedes* mass-rearing cage, promotes females to deposit their eggs in the areas covered by filter papers, confirming the ability of these species to lay eggs inside several water reservoirs using only the wet walls (Hien1975).

At the bottom of the cage, four 40cm-long and 3-mm-wide slots are created, two on each of the long sides (Figure 20a). The egg papers are inserted in the cage on the 3rd day following the first blood meal. Egg filter papers touch the bottom, with the lower part submerged in the water. Egg papers can be easily removed without any egg damage by removing the clip holding the metal plate that holds each egg filter paper against the cage side (Figure 20b-c). Egg filter papers can then be pulled out (Figure 20d-e) without adult escaping. Egg filter papers can be lined up in a tray using a paper towel (Figure 20e). When egg papers are collected from the cage, the remaining water is drained through stacked 300 and 50µm sieves (Retsch®, Haan, Germany) to collect floating eggs. The first egg papers are collected on the third day after females were offered to lay eggs. Further operations are performed following the schedule described in the appendix (see 7. Appendix).



Figure 20. Egg collection method in the mass-rearing cage. A 40 cm-long and 3mm-wide slot create created along the bottom part of the cage (a). A clip holding the metal plate that holds each egg filter paper against the cage side (Figure 20b). Egg filter papers pulled out (Figure 20c-d) and lined up onto a tray using a paper towel (Figure 20e).

Upon oviposition, the embryos of *Ae. aegypti and Ae. albopictus* are not yet fully developed and require an additional maturation period before hatching can occur (Hawley 1988). For this embryonation to take place, egg papers must be kept in a moist environment (80-100% RH) for a period of 36-72 hours at $25 \pm 1^{\circ}$ C. In the lab, oviposition papers are collected and gently cleaned by spraying with a wash bottle with deionized water to remove any debris which could be potential sources for mold development when dried. The papers shall then be placed into lidded (but not air-tight) containers for the embryonation period, during which the papers can dry gradually but will stay moist enough (maintaining the relative humidity inside the container at 80-100% RH) for development, but not so moist as to induce hatching. After 3 days of slow drying in the covered container, the lid of the container can be left slightly open to allow more air circulation and drying for one to two weeks. Larval rearing trays with the Plexiglas covers can be used for drying in insectary conditions (if no appropriate

container can be found). Egg filter papers can then be transferred into plastic Ziploc bags and kept in a plastic box for storage or stored in a climatic chamber when possible under optimum relative humidity (80 ± 5 % RH) and temperature ($25 \pm 1^{\circ}$ C). Caution should be taken to consider differences between species related to the optimum storage conditions.



Figure 21. Different container types for egg drying and storage method.

5.2 Egg estimation

To weigh eggs, an electronic balance with an accuracy of 0.0001 g is required. Eppendorf tubes are used and need to be tarred before use. Eggs are gently brushed (using a medium-bristled brush) onto a non-adhering tracing paper (Duerer-Hase, A4, 25 sheet, 80g, article number 101600, LIBRO, Austria) or by using aluminum foil sheet to collect and transfer them into the Eppendorf tubes (Figure 22c). To reduce workload, eggs can more easily be weighed using a 10×10 cm of aluminum foil or small containers. When eggs are not clean, the brushing process is performed through a 224 or 300 µm sieve (Retsch®, Haan, Germany) to remove debris of adult mosquito body parts such as wings, legs, abdomen and heads.

For egg estimation different equations of the relationship between weight and quantity of eggs (Zheng et al. 2015b) are used according to the species: *Ae. aegypti* (y = 0.0088 x - 0.3324) and *Ae. albopictus* (y = 0.007 x + 3.0134), where x=number of eggs and y= egg weight (mg).

As some possible variations in egg weight according to species, strains and age of eggs are not to exclude, it is recommended that each lab establishes its own equation. For example, the equations described above were established using not older than 15 day-old eggs post-oviposition (Zheng et al. 2015b).



Figure 22. Egg estimation process including brushing eggs by using a medium-bristled brush (a) on aluminum sheet (b) before transferring to Eppendorf tubes (c) for weighing using electronic balance (d)

6. Relevant and cited references

- Balestrino, F., Benedict, M.Q. and Gilles, J.R.L. (2012). A new larval tray and rack system for improved mosquito mass rearing. J. Med. Entomol. 49 (3): 595 605.
- Balestrino, F., Puggioli, A., Gilles, J.R.L. and Bellini, R. (2014a). Validation of a new larval rearing unit for *Aedes albopictus* (Diptera: Culicidae) Mass Rearing. PLoS ONE 9(3): e91914. doi:10.1371/journal.pone.0091914.
- Balestrino, F., Puggioli, A., Bellini, R., Petric, D. and Gilles, J.R.L. (2014b). Mass production cage for *Aedes albopictus* (Diptera: Culicidae). J. Med. Entomol. 51 (1): 155 163.
- Bellini, R., Puggioli, A., Balestrino, F., Carrieri, M., Urbanelli, S. (2018). Exploring protandry and pupal size selection for *Aedes albopictus* sex separation. Parasit Vectors. 11 (Suppl 2):650.
- Bond, J.G., Ramírez-Osorio, A., Marina, C.F., Fernández-Salas, I., Liedo, P., Dor, A., Williams, T. (2017). Efficiency of two larval diets for mass-rearing of the mosquito *Aedes aegypti*. Plos One. 12(11):e0187420.
- Carvalho, D.O., Nimmo, D., Naish, N., McKemey, A.R., Gray, P., Wilke, A.B.B., Marrelli, M.T., Virginio, J.F., Alphey, L., Capurro, M.L. (2014). Mass Production of Genetically Modified Aedes aegypti for Field Releases in Brazil. J. Vis. Exp. (83), e3579.
- Damiens, D., Benedict, M. Q., Wille, M. and Gilles, G.R.L. (2012). An inexpensive and effective larval diet for *Anopheles arabiensis* (Diptera: Culicidae): Eat like a horse, a bird, or a fish? J. Med. Entomol. 49 (5): 1001-1011.
- Damiens, D., Soliban, S. M., Balestrino, F., Alsir, R., Vreysen, M. J. B. and Gilles, J.R.L. (2013). Different blood and sugar feeding regimes affect the productivity of *Anopheles arabiensis* colonies (Diptera: Culicidae). J. Med. Entomol. 50 (2): 336 – 343.
- (FAO/IAEA.) Food and Agriculture Organization of the United Nations/International Atomic Energy Agency. (2017). Guidelines for routine colony maintenance of *Aedes* mosquito species. Version.1.0.
- Focks, D.A. (1980). An improved separator for the developmental stages, sexes and species of mosquito (Diptera: Culicidae). J. Med. Entomol. 17, 567-568.
- Hien, D.S. (1975). Biology of *Aedes aegypti* (L., 1762) and *Aedes albopictus* (Skuse 1895) (Diptera, Culcidae). II. Effect of environmental conditions on the hatching of larvae. Acta Parasitologica Polonica 23:537-552.
- Maiga, H., Mamai, W., Bimbilé Somda, N.S., Konczal, A., Wallner, T., Herranz, G.S., Herrero, R.A., Yamada, H., Bouyer, J. Reducing the cost and assessing the performance of the adult massrearing cage for the dengue, chikungunya, yellow fever and Zika vector, *Aedes aegypti* (Linnaeus). PLoS Negl Trop Dis. 2019 Sep 25;13(9):e0007775.
- Puggioli, A., Balestrino, F., Damiens, D., Lees, R.S., Soliban, S.M., Madakacherry, O., Dindo, M.L., Bellini, R., and Gilles, J.R.L. (2013). Efficiency of three diets for larval development in mass rearing *Aedes albopictus* (Diptera:Culicidae). J. Med. Entomol. 50 (4): 819-825.
- Zhang, D., Zhang, M., Wu, Y., Gilles, J.R.L., Yamada, H., Wu, Z., Xi, Z., Zheng, X. (2017). Establishment of a medium-scale mosquito facility: optimization of the larval mass-rearing unit for *Aedes albopictus* (Diptera: Culicidae). Parasit Vectors. 10:569.
- Zheng, M.L., Zhang, D.J., Damiens, D.D., Lees, R.S., Gilles, J.R. (2015a). Standard operating procedures for standardized mass rearing of the dengue and chikungunya vectors Aedes aegypti and Aedes albopictus (Diptera: Culicidae) II Egg storage and hatching. Parasit Vectors. 8:348.

- Zheng, M.L., Zhang, D.J., Damiens, D.D., Yamada, H. and Gilles, J.R.L. (2015b). Standard operating procedures for standardized mass rearing of the dengue and chikungunya vectors *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) - I - Egg quantification. Parasit Vectors. 8: 42.

7. Appendix

a- Example of schedule of Aedes aegypti mass-rearing larval and adult stages

Larval stage:

Day -1: Monday

- Estimate roughly the total number of larvae to be reared considering each mass-rearing tray will be filled with between 18, 000L1/tray.
- Brush eggs and weigh equivalent egg number needed.
- Take from these loose eggs 3 samples of 100 150 eggs.
- Put each sample in 50mL centrifuge tube (VWR, UK) filled with 40mL of boiled cooled osmosis water with 2mL of 4% larval FAO/IAEA diet. Use 40mL of hatching solution instead for *Ae. albopictus* egg hatching.
- Prepare jam jars filled with 0.7L of boiled osmosis water and store at laboratory temperature

Day 0: Tuesday

- Check the egg hatch rate of 100 eggs from each of 3 samples (under stereomicroscope by counting hatched and unhatched eggs) to predict the needed number of eggs for the 18, 000 L1.
- Estimate the required egg numbers using an equation (Weight (mg) = $(0.0088 \times \text{Number of counted} \text{ eggs}) 0.3324$) for *Ae. aegypti*
- Put each egg batch into a jam jars filled with 0.7L of boiled and subsequently cooled (deoxygenated) osmosis water by opening to add the eggs and 10 mL of 4% larval FAO/IAEA diet, before closing the jars again to avoid re-oxygenation. Use 0.7L of hatching solution instead for *Ae. albopictus* egg hatching.
- Prepare larval diet for the next larval feeding day and store at 4° C.

Day 1: Wednesday

Sieve the content of hatching jars and transfer to mass-rearing trays previously filled with 5L of deionized or distilled water. Add 100 mL of larval food to each tray.

Day 2: Thursday

Add 200 mL of larval food to add to each tray

Day 3: Friday

Add 400 mL of larval food to add to each tray (200 ml in the morning and 200 ml in the afternoon)

Day 4: Saturday

No feeding is needed

Day 5: Sunday

No feeding is needed. Pupation starts (room at temperature about $28\pm 1^{\circ}$ C).

Pupal/Adult stage:

Day 6-9: Monday-Thursday: Pupal loading into the mass-rearing cage

- Tilt the trays to collect larvae and pupae about 36 hours after the first pupae appear (to maximise the number of pupae while avoiding the emergence of adults and separate using mechanical sexing tools.
- Pour the larvae back to the rearing trays and feed with 50mL larval diet (if more than one tilting is needed).
- Estimate the number of pupae by sex volumetrically using tubes marked for each sex.
- Load cages with estimated number of pupae Load 13,333 female pupae and 4,444 male pupae at once (if enough pupae are produced) in the cage allowing stocking the cage with 12,000 and 4, 000 female

and male adults respectively, based on an emergence rate and survival of 90% before the first blood feeding was performed. The setting up of the mass-rearing cages by providing 10% sugar solution to adults in the cage through the internal sugar feeding cup and by fixing the mesh netting to all cages are preferentially done on Day 3.

- Follow the schedule below for further tasks such as blood feeding (BF), inserting egg papers (EP) and egg collection (EC), starting with the first day of loading pupae up to the 6th egg collection, covering a total active period of 37 days. Only days with tasks (PL, BF, EF and EC) are shown (Table 1).

Day	6	7	8	9	14	16	20	23	27	30	34	37
Pupal loading	PL1	PL2	PL3	PL4								
Blood feeding					BF1	BF2	BF3	BF4	BF5	BF6	BF7	
Egg papers						EP1	EP2	EP3	EP4	EP5	EP6	
Egg paper removal							EC1	EC2	EC3	EC4	EC5	EC6

Table 1. Example of rearing schedule

Pupal loading (PL) over 4 days (or less dependent on the pupae production), 7 blood feeding days (BF), 6 egg paper days (EP) and 6 egg paper removal days (named egg collection days) (EC)

b- Quick protocols

Blood feeding

- Fill sausage casing with 150mL of pig fresh blood
- Warm sausages in water bath (42°C) for 10min, feed for 30min and put back sausage into water bath for next 10min. Repeat the feeding 4 times (2 hours) per blood feeding day.
- Insert sausage into mesh socks on top of the cage

Egg collection

- Put egg papers into containers on the 3rd day after blood feeding starts
- Remove and replace egg papers 2 times per week (Monday and Thursday)
- Keep eggs for maturation and drying for one to two weeks before egg brushing/quantification
- Collect eggs over 2-3 weeks.

Survival (Adult index)

- Draw six of 10×10 cm squares (three on each cage side) in the diagonal way, using a fine marker
- Count in each square number of male and female mosquitoes. Care should be taken to do so at the same time every day.
- Start checking adult index 2 days after the pupal loading.

Hatching solution preparation

- Weigh 0.25 g of nutrient broth (Sigma-Aldrich).
- Complete weight to 0.3 g by adding 0.05 g of yeast.
- Put the quantity of weighed ingredients into a bottle.
- Mix with 0.1L of water to dissolve.
- Fill the bottle up to 0.7L. Prepare needed volume so that no storage would be needed.