Joint FAO/IAEA Programme
Nuclear Techniques in Food and Agriculture

GUIDELINES FOR STANDARDISED MASS REARING OF *ANOPHELES* MOSQUITOES

Version 1.0

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CONTENTS

List of figures

List of tables

Disclaimer

The recommendations given in these guidelines are considered appropriate at the time of its preparation. They may be modified in the light of further knowledge gained at subsequent stages.

We presented here the equipment & material with trademarks currently used at the IPCL. This does not imply that these are endorsed or recommended by the FAO/IAEA in preference to others of a similar nature that are not mentioned. Equivalents are welcome.

1. INTRODUCTION

People contract malaria through the bite of female *Anopheles* mosquitoes infected with *Plasmodium* parasites. Malaria kills a huge amount of people worldwide every year, most of them children under the age of five and pregnant women in sub-Saharan Africa (Breman *et al*., 2001). In 2015, the number of malaria deaths had fallen to 429,000 but half of the world's population still remains at risk of becoming infected (WHO, 2016).

Anopheles arabiensis Patton, along with *An. gambiae* Giles and *An. funestus* Giles, are the primary vectors of human malaria in sub-Saharan Africa and surrounding islands (Gimnig *et al*., 2001). The distribution of *An. arabiensis* spreads along a belt from West Africa to Eastern Africa and along the eastern coast to a Southern belt (Figure 1). The other two sibling species have similar distributions which result in many areas of co-existence. However, *An. arabiensis* shows greater behavioural plasticity by exhibiting zoophilic, exophagic and exophilic behaviour (White, 1972). They prefer dry savannah-like habitats and breed in small, temporary puddles as well as in permanent rice fields and fish ponds (Sinka *et al*., 2010). They are also well adapted to urban environments.

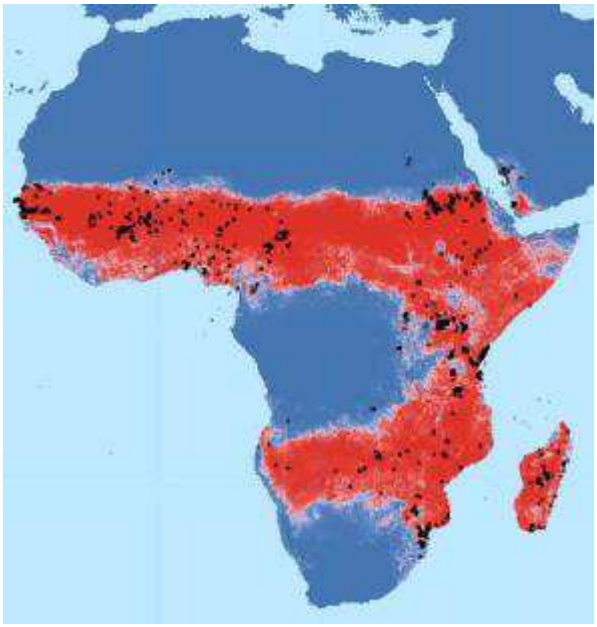

Figure 1. ***Map showing predicted probability of occurrence (red area) and confirmed occurrence points (black dots) of*** **An. arabiensis** ***in Africa*** *(Sinka et al., 2010).*

Conventional vector control strategies rely on insecticide-treated bed nets (ITNs) and synthetic pesticide DDT (dichloro-diphenyltrichloroethane) for indoor residual spraying (IRS). As *An. arabiensis* exhibits exophilic behaviour, IRS and ITNs are not as effective as they are against *An. gambiae* and *An. funestus* (Kitau *et al*., 2012). Nowadays DDT is less commonly applied due to widespread resistance in mosquitoes (Etang *et al*., 2006) and the fact that it has been banned in various countries because of its potential hazards to humans and the environment. Pyrethroid and organophosphates insecticides are used instead as they have a faster mode of action and are less toxic than other insecticides to humans. However,

due to their widespread use worldwide, resistance to pyrethroids has emerged in *An. arabiensis* and *An. gambiae* and is spreading at a fast pace (Nardini *et al*., 2013; Abilio *et al.*, 2011; Santolamazza *et al*., 2008).

Amongst alternative vector control methods that are being advocated is the use of genetic and transgenic technologies. An example of genetic control is the 'sterile insect technique' (SIT), a term that was first coined by Knipling (1959). The SIT is a species-specific and environmentally - friendly pest control method that relies on the mass-rearing of a target species, sex separation and transport and systematic release of sterile males in target areas where they compete with wild males for mating with wild females. Since the released males are sterile, their matings result in no viable offspring. Over time, the targeted population is suppressed in a sustainable and environmentally friendly way. When sufficient numbers of sterile males are systematically released over a target area for a long enough period, it is possible to drastically reduce – and in some favourable conditions even eliminate – a specific pest population.

A successful courtship between a wild female and a sterilised male is likely to cause the female to lay only sterile eggs for her entire life, since re-mating is usually prevented in female *Anopheles* (Rogers *et al*., 2009).

The SIT, as part of an area wide integrated pest management (AW-IPM) programme, has been successfully applied to eradicate the New World screwworm fly (*Cochliomyia hominivorax* Coquerel) from North and Central America (Wyss, 2000), the Mediterranean fruit fly (*Ceratitis capitata* Wiedemann) from California, Chile and some parts of Central America (Klassen and Curtis, 2005) and the tsetse fly (*Glossina austeni* Newstead) from the island of Zanzibar (Vreysen *et al*., 2000). These successful programmes demonstrate the potential of SIT.

A feasibility study conducted in the 1970's against *An. albimanus* Wiedemann in El Salvador was the first successful SIT pilot project in mosquitoes (Lofgren *et al*., 1974). It was based on the use of a genetic sexing strain that was characterised by pesticide resistant males. Eggs were treated with propoxur (o-isopropoxyphenyl methylcarbamate) which killed the females. This method did not only free up rearing space for the males but also eliminated the risk of accidental release of females (Bailey *et al*., 1980). Over 1 million chemo-sterilised males were released over a 150km^2 area in combination with larval control measures. After a 4 month trial period, the wild *An. albimanus* population had decreased by 97% (Dame *et al*., 2009).

The increasing resistance problems and the lack of new tools in the fight against malaria have revived interest on the SIT as well as molecular tools aiming to suppress or replace a vector population (RIDL, cytoplasmic incompatibility and vector-incompetence) and consequently mass-rearing of mosquitoes (Klassen and Curtis, 2005). Several reviews recently published provide summaries of the approaches currently under development for mosquito control (McGraw and O'Neill, 2013; Alphey *et al*., 2014; Burt, 2014, Gabrieli *et al*., 2014, Lees *et al*., 2015, Bourtzis *et al.,* 2016). Leading the vanguard in reviving the use of the SIT against mosquitoes was an Italian group (Bellini *et al.*, 2007) who released ca. 1000 - 15000 irradiated *Ae. albopictus* pupae per hectare per week, inducing up to 68% sterility in the target populations in 3 pilot sites of between 16 and 45 ha (Bellini *et al*., 2013). Releases continued for 5 years, and demonstrated the potential contribution of sterile males to suppressing an *Ae. albopictus* population.

Technical requirements for application of the SIT include colonisation and mass rearing (Benedict *et al*., 2009), sterilisation without imposing unacceptable fitness costs (Bakri *et al*., 2005), an efficient sex separation method (Gilles *et al*., 2014) and a technique to release large numbers of sterilised pupae or adults at the target site. A fully mechanised and standardised mass-rearing system has never previously been developed for the production of mosquitoes on a mass-scale, and this is a requirement for the upscaling of mosquito production to a sufficient scale for widespread releases. The goal is to develop and optimise a mass-rearing capacity that is efficient and reliable while maintaining a high standard output at a low cost.

In 2004 the Tropical Medicine Research Institute in Khartoum, Sudan initiated – with the support of the Food and Agriculture Organisation of the United Nations (FAO) and the International Atomic Energy Agency (IAEA) - a feasibility study on integrating the SIT against *An. arabiensis*. The FAO/IAEA Insect Pest Control Laboratory (IPCL) at Seibersdorf, Austria has designed, produced and tested a mass-rearing system for larvae and adults, as well as a system to separate larvae from pupae on a large scale (Balestrino *et al*., 2011). The larval unit reduces significantly manpower and the space needed for rearing. It consists of a rolling stainless steel tilting rack system holding 50 thermo-formed plastic trays that can rear the larvae hatching from around 4,000 eggs each (Balestrino *et al.,* 2012). The expected output of a fully loaded rack is between 100,000 – 150,000 pupae. The adult cage has a stocking capacity of up to 15,000 *An. arabiensis*, before the number of eggs produced fails to increase with an increased stocking number of adults. Once the cage is loaded with pupae, it is completely sealed and can be managed from outside with minimal risk of mosquitoes escaping. It provides mosquitoes with a constant supply of sugar and access to blood as required. Eggs are collected and the cage cleared of debris through an internal water irrigation mechanism.

Here we provide a description of all procedures required for the mass-rearing of *An. arabiensis* using these newly designed adult cage and larval rearing unit, with a step-by-step guide to establishing, up-scaling and maintaining a large *Anopheles* colony, including all stages of the mosquito's life cycle. The aim of this document is to give the user an explanatory and practical hands-on description of how to use the equipment for mass rearing purposes and provide support in the form of photos, timelines and further readings. The methods presented are those developed and used at the FAO/IAEA IPCL, and are a good starting point for standardised rearing methods. However, the FAO/IAEA is optimising the rearing systems and methods and this guideline will be updated on a regular basis.

2. COLONISATION

2.1. From an established laboratory colony

It is easier to establish a new colony from an existing laboratory colony than from the field as the mosquitoes are already adapted to the insectary settings. The donor laboratory should collect a fresh batch of *Anopheles* eggs and send it through an express courier. They must be covered with moist filter paper and cotton wool and sealed tightly to avoid desiccation. The Malaria Research and Reference Reagent Resource Center (MR4) maintains a wide range of insects that can be ordered through a catalogue system (http://www.beiresources.org/Organism/90/Mosquitoes.aspx).

Upon arrival at the recipient's laboratory, eggs should be carefully dispensed into water (using filter paper to avoid them sticking to the edge of the rearing tray). For a detailed hatching and larval rearing protocol, see Section 6 EGG STAGE and Section 7 LARVAL STAGE.

The *An. arabiensis* Dongola strain used at the FAO/IAEA IPCL was obtained from the MR4 (MRA-856), with occasional refreshing using wild material collected from the same originating site, the Dongola area in Northern Sudan, and has been in the FAO/IAEA Laboratory since 2005.

2.2. From mosquitoes collected in the field

The advantage of using field collected material is the closer resemblance of their behavioural and genetic traits to the wild population than those of older laboratory colonies.

However, rearing mosquitoes collected in the field is difficult as the conditions in the insectary differ vastly from those in nature. While the aquatic rearing might be less of a problem, the adults may find it difficult to acclimatise to the cage environment. The spatial limitations, the high density and many other confinement factors will inevitably challenge the natural behaviour of both males and females. Hence, it should not be surprising if females lay few or no eggs at first, which may be due to reduced mating activity as well as reluctance to blood feeding from an artificial blood source.

Collecting engorged females from the field

Endophilic *Anopheles* females can be collected from the internal walls of residential huts or other resting sites (*e.g.* females can sometimes be found in cattle kraals or sorghum brewing pots in South Africa) by mouth aspiration. Motorised aspirators risk harming the collected mosquitoes. The live females are transported back to the insectary where, after morphological identification (Gillies and Coetzee, 1987), they are placed into individual oviposition containers. After the females have laid a batch of eggs, the female is genotyped for species or subspecies using the method of Scott *et al*., 1993 for the *gambiae* complex or of Spillings *et al.*, 2009 for *funestus* group mosquitoes. Once the identity of the mother is confirmed, egg batches and their larvae of the same type may be pooled and reared through

to adults. For a detailed rearing protocol, see Section 3 ROUTINE MAINTENANCE OF AN *ANOPHELES*. If more eggs are needed, these females can be blood fed on animals or on an artificial feeding system to induce more ovulation and oviposition. Special precautions, in the form of careful handling and containment, should be taken to avoid being bitten by these potentially infectious mosquitoes. The same applies to the use and disposal of blood sources used to feed wild-collected females.

Collecting larvae and pupae from the field

Alternatively, larvae and pupae can be collected directly from the field for rearing to adulthood and breeding after species identification, since larval sites can be easier to sample. However, this approach is more laborious as each mosquito needs to be genotyped individually to prevent cross-contamination. Larvae and pupae are not easily visible in the dirty water of many larval sites, and pupae are particularly good at hiding in dark niches. The larvae of some mosquito species can also stay submerged for a long time. When approaching a breeding pool, endeavour to not allow one's shadow to fall on the water as this often makes the larvae submerge.

3. ROUTINE MAINTENANCE OF AN ANOPHELES LABORATORY COLONY

Anopheles arabiensis (Dongola strain) are maintained under standard and constant rearing conditions. At the FAO/IAEA IPC Laboratory, adults and larval stages are kept at 27ºC ± 1 ºC, 60% ± 10% relative humidity, 12h:12h light:dark photoperiod including 1h dusk and 1h dawn (progressive decline).

Around 200 larvae are reared under relaxed conditions of low larval density (1 larva/ 5 ml) in each standard rearing tray (40 x 30 x 8 cm) with 1 L de-ionized (or dechlorinated or reverse osmosis purified) water with 50 ml of a tuna/bovine liver/vitamin mix slurry diet given daily (as detailed in Section 7 LARVAL STAGE).

Pupae are individually picked and transferred into a small bowl (500 – 1,000 pupae) that is placed inside a suitable rearing cage. Locally-sourced or custom-made cages may be used, though the FAO/IAEA IPCL uses Bugdorm© plastic cages (EM1000 - BugDorm-1 Insect Rearing Cage (300x300x300 mm), BugDorm, Taiwan) for convenience and standardisation. The walls of this cage should be roughened slightly using coarse sand paper before use to further provide a surface to which the mosquito may grip. Failure to do so may result in increased cage mortality due to exhaustion.

A 5% sugar in water solution is supplied in a small laboratory glass bottle (250 mL) with a filter paper wick (42 Ashless, 90 mm diameter. Cat 1442-090. Whatman-GE Healthcare, Piscataway, NJ, USA) as the carbohydrate source. A black foam insulation tube (Armacell, class 1 Armaflex) is placed inside the cage to provide resting sites.

Adults emerge overnight and mature within 24 hours. Mating activity will be at its peak after 2-3 days and most females will have mated by the fourth day post-emergence. Therefore it is recommended that females are blood-fed from this point onwards.

Defibrinated bovine blood, either fresh or defrosted, is given to the females using the Hemotek membrane feeding system that regulates the blood temperature electrically (details given in Section Sugar feeding solution). If a Hemotek membrane feeding system is not available, alternative methods may be used such as placing a hot water bottle on top of the feeding plate.

Gravid females lay their eggs on the third night after blood feeding. To facilitate oviposition, an egg cup (a bowl or Petri dish containing a moist sponge with filter paper on top) is placed inside the cage. On the following day, the egg cup is removed and the eggs are transferred into a standard larval rearing tray. If it is found that the egg batches are poor, adding an oviposition stimulant, such as a few drops of algae, onto or under the filter paper may promote egg-laying. Sometimes the females prefer a simple small bowl, with a blackened base, of open water, though this may lead to increased drowning.

For more detailed colony-scale rearing guidelines, visit https://www.beiresources.org/Reagents/ProtocolsandPublications.aspx and open the 'Methods in *Anopheles* research' laboratory manual.

4. MASS-REARING EQUIPMENT FOR ANOPHELES MOSQUITOES

4.1. Mass-rearing cages setup

A large mass rearing cage for *Anopheles arabiensis* has been designed at the IPCL (Balestrino *et al*. 2014) to optimize egg production while minimizing handling procedures to avoid risk of escapes.

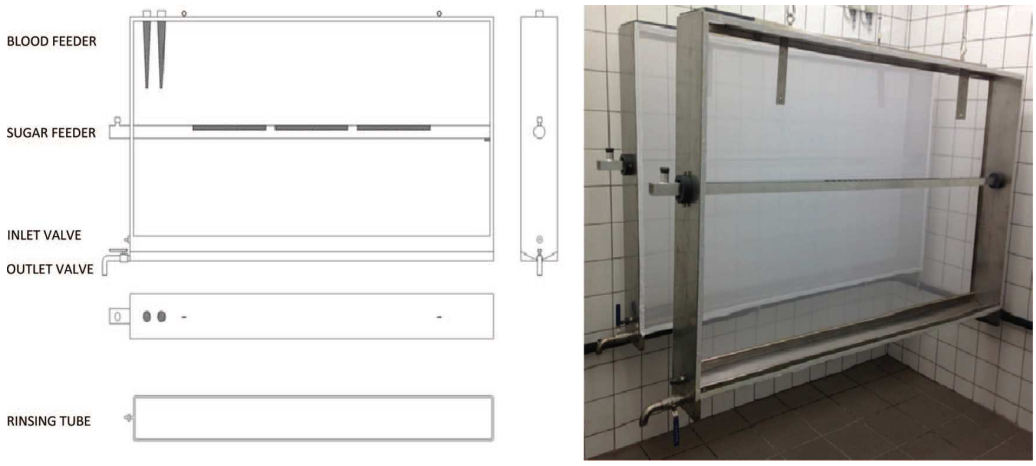

Figure 2. ***General drawings and view of the mass rearing cage for anopheles mosquitoes.***

In addition to the general description made in this article, very detailed procedures for end users in given below.

Hose pipe system

To connect the hose pipe system to the large cage, screw the grey connector (Handverbinder 21 mm, G ½" GARDENA, or similar) to the cage's open valve. Then seal the water pipe (Schlauch 19 mm, ¾" GARDENA, Nr. 18022) to the orange connector (Profi-System-Übergangsstück mit Wasserstop, 19mm ¾" GARDENA, Nr. 2814). Finally, clip the orange connector into the grey connector (Figure 3). To detach the hose pipe, simply press and pull away the orange part of the connector.

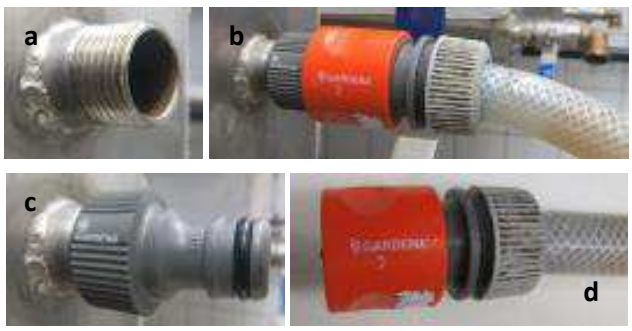

Figure 3. ***Components of the hose pipe system.*** *(a) the cage's open valve as it is when it's delivered; (b) the whole hose pipe system attached to the large cage; (c) the grey (+/male) connector fixed to the open valve; (d) the water*

Drainage system

Placed upright, with the V-shaped trough at the bottom, an open threaded pipe extends from the cage below the hose pipe attachment point. The silver stopcock is screwed to this pipe. Similarly, the bronze elbow plumbing fixture is screwed to the stopcock to serve as a movable spout, taking care to leave it loose enough to enable it to swivel either up or down (Figure 4). It is suggested to ensure that the stopcock is in the "closed" position at this point to serve against accidently spillage of pupae when the cage is loaded later.

Figure 4. ***The drainage system****. View of the water pipe system and the water outlet from inside the cage (left); the stopcock with the elbow plumbing fixture down (open position) to drain water (middle); the stopcock with the elbow plumbing fixture up (closed position) to load the cage with pupae (right).*

Hanging the cage up

Ants can destroy a cage of adults quickly. To prevent an ant infestation, and to maximise use of insectary space, the adult rearing cages have been 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 5). The black cloth shroud contained in the kit should be fitted at this point for later ease of use by passing the carabineers through the holes in the shroud. The carabineers can be hooked to two cables of appropriate length and position to suspend the cage from the roof at a convenient height.

Figure 5. ***A carabineer attached to the top of the cage****. The cage is suspended on a cable hanging from the ceiling. The black cloth shroud is draped over the cage so that the carabineers emerge from the holes and the black cloth covers the cage.*

Internal sugar feeding device

Included in the cage kit is the internal sugar feeding device (Figure 6). The tube (2.2 m long and 50 mm in diameter) should be mounted through the holes positioned halfway up the stainless steel end walls of the cage frame. The tube contains three slots which provide access for filter papers that will soak up the sugar solution and therefore should be facing upwards. The tube is sealed with a rubber plug on one end and with an upwards facing elbow plumbing fixture on the other end (Figure 6). The latter is used to fill the device with the

sugar solution. To discard the sugar solution for cleaning, remove the rubber plug from the elbow plumbing fixture and turn it down. Place a bucket underneath to collect the liquid. If necessary, tilt the cage to remove the remainder and rinse the tube with hot water.

Figure 6. ***The internal sugar feeding device****. The device spans the entire width of the mass-rearing cage. The sugar solution is poured through the open elbow plumbing fixture into the tube and can last up to 20 days.*

Net fitting

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 7). Care must be taken to ensure that it is neatly fitted and no gaps are present between the cage frame and the netting. 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 7. ***The detachable netting system using velcro® tape****. A corner of an open cage aligned with velcro® taping (left). The net is taped onto the cage by pressing it*

Blood feeding ports

The blood feeding panels are positioned on top of the cage at both ends where the blood feeding ports are located (Figure 8). They are plastic squares with a circular hole in the middle and a screw at each corner. Removing the screws allows the panel to be removed from the ports revealing a similar circular hole in the metal frame below. Position a piece of netting supplied in the kit over each of these holes so that the small holes in the four corners

of the netting align over the four screw holes. Place the plastic square over this and reinsert the screws and tighten, taking care to keep the netting taut.

Figure 8. ***The blood feeding port.*** *A membrane filled with blood can be placed on top of this net-covered panel to provide mosquitoes with blood.*

4.2. Mass-rearing trays and racks set up

Larvae are reared in a steel rack containing thermoformed ABS (acrylonitrile butadiene styrene) plastic trays as described by Balestrino *et al.* (2012) (Figure 9). The trays are made of 5 mm thick, 100 × 60 × 3 cm in size and have a flat surface with two long ridges (each 32 cm long, 2.5 cm high) running along the major axis. The ridges have been designed to give the trays extra rigidity and provide additional surface area for larvae and pupae to rest. The slots in each ridge provide a water overflow system which enables the all trays of the entire rack to be filled by adding water only to the top tray (Figure 9). Once the top tray is full, the ridges will allow the water to flow down into the tray below and so on until all the trays are full. Plastic plates are attached below each ridge to divert the overflowing water away from the next trays' ridge and prevent the water streaming straight down.

The rack structure made of stainless steel (type AISI 304) is durable and rust resistant. The distance between each shelf in the rack is 3 cm. The weight of the total structure when all trays are filled with water is around 710 kg. The rack and trays are intended to be positioned where larval culture will occur and filled with water and mosquitoes without further repositioning.

Figure 9. ***A large rearing tray (right) and rack unit (left).***

5. ADULT STAGE

5.1. Loading of the large cage

The easiest and most efficient way to load the large cage with *Anopheles* mosquitoes is at the pupal stage. The number of total pupae added to the cage should be optimised (discussed in Section Estimating the number of pupae) by monitoring survival at each developmental stage and assessing the productivity of the cage. Fill the trough at the bottom of the cage with water and spread the pupae evenly along the trough. It is recommended that wherever possible pupae are added at the same time to ensure synchronization of development. Be aware that the first emerging pupae are mostly males. Pupae produced at the second day of pupation in the mass-rearing facility (tray-rack unit) can be loaded as the sex ratio is close to 50:50. Place the net back in place and make sure the cage is completely sealed. Finally cover the cage with the black cloth shroud to create an artificial horizon that will stimulate the mosquitoes' natural mating behaviour as well as providing a resting site (Figure 10).

Figure 10. ***A large cage:*** *without the net prior to adding pupae (left), with the net partially attached after loading the pupae (middle) and fully assembled with the net and black lining (right).*

5.2. Subsequent loading of the large cage

In case it is not possible to fill the cages with the total number of pupae one time, an option is to complete the total number the following days. Before adding new pupae to a cage, first the water should be flushed from the trough to clean out any dead pupae and exuviae from the previous day (see Section Draining waste from the cages – disposal of dead adults and pupae). If pupae must be added on subsequent days, it can be done through the spout without risking the escape of adults through the open net. It is recommended that the pupae are split on each day and added to each cage evenly so that each cage is synchronised with the same adult age distribution. No more than 3 days' of pupae should be added to the cage, and care should be taken to maintain a 1:1 sex ratio, bearing in mind that male mainly pupate first. Turn the spout into an upright position and open the stopcock, 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 sufficient deionised water (or reverse osmosis purified or distilled water) to allow the pupae to spread evenly and prevent overcrowding through clustering. Close the stopcock while adding water to keep the barrier clear of pupae.

5.3. Sugar feeding solution

A 5% sugar solution is provided to the adults in the cage through the internal sugar feeding device. It contains three open slots for filter papers (25 × 20 cm) that will soak up the solution and make it available to the adult mosquitoes. A filter paper (Whatman paper, 2589 A Bogen sheets, 580 × 580 mm) is cut into three 25 × 20 cm sheets, folded along the vertical axis (25 × 10 cm). Seal each end with tape, or fold the ends, and place each sheet in a slot (**FIGURE 11**). Before loading the cage with mosquitoes, 2 litres of the 5% sugar solution is poured into the sugar feeding device which will last for the entire duration of a cage cycle (approx. 20 days).

The sugar solution (2 L) is made by adding 500 mL boiled water to 100 g of granulated sugar in a glass bottle. Stir to dissolve the sugar and top up with 1,500 mL cold water. Shake and store the bottle at 4 ºC. When needed, the solution is poured into the sugar feeding device through the open elbow plumbing fixture at the end wall (Figure 11).

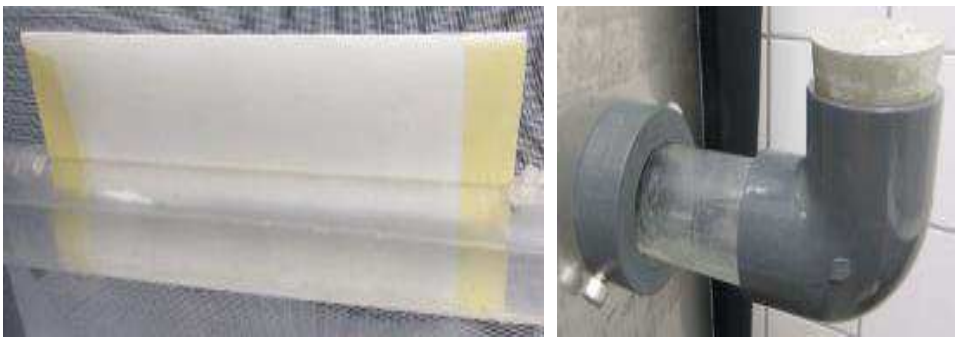

Figure 11. ***A filter paper*** *folded and inserted into the open slots with the sugar solution thereby acting as a wick to make the sugar accessible to adult mosquitoes (left); the elbow plumbing fixture allows the sugar solution to be poured into the feeding device from outside (right).*

5.4. Artificial blood feeding

Female mosquitoes are blood-fed using the Hemotek membrane feeding system (Discovery Workshops, Lancashire, United Kingdom). This method does not require any live animals and feeding can be done frequently.

Two sources of blood are routinely used at the FAO/IAEA IPCL. The preferred option is to use fresh porcine or bovine blood collected directly from a nationally authorized abattoir and used fresh. This can be stored at 5 °C for a week without spoiling. Alternatively, defibrinated bovine blood from a supplier in Slovakia is sterilized and frozen on delivery, and defrosted before use. The frozen defibrinated blood is thawed at 4 °C overnight and kept at room temperature for 30 minutes prior to feeding. If necessary, blood can be defrosted rapidly using a 40 °C water bath. Blood should be defrosted thoroughly and mixed well before use.

The Hemotek system consists of a power pack and 5 or 6 feeding units according to the model shows the 5 unit model). Each feeder is fitted with a heating and temperature control device and plugged directly into the power unit (left). Heating units with 3 m cables are available from the manufacturer allowing for greater ease of feeding. Unfortunately the voltage drop over cables longer than 3 m is too great to proper functioning of the heating unit. The original Hemotek feeding unit is supplied with small aluminum plates (3.7 cm diameter, 1.3 cm thickness, 5 mL blood reservoir). However, to increase the feeding surface area, large plates (17 cm diameter, 1 cm thickness, 50 mL blood capacity) were developed right) (Damiens *et al.,* 2013).

Figure 12. ***The hemotek membrane feeding system (discovery workshops, lancashire, united kingdom)****. The hemotek power unit with a feeder (left) and the modified aluminium plate with the much larger surface area attached to a feeder (right).*

To create a reservoir for the food, a 20 x 20 cm piece of collagen membrane (Discovery Workshops) is placed onto the flat side of the aluminum plate and sealed with the plastic ring provided (Figure 13b and c). On the rear side of the plate, unscrew either one of the two white knobs and attach a disposable Luerlock syringe (BD, Franklin Lakes,NJ, 50 mL capacity (Figure 13d).

Pour 50-70 mL blood into the syringe and let it flow into the cavity between the aluminum plate and the collagen membrane (Figure 13e). Remove the syringe and screw the white knob back into place. Swirl the blood around and check for air bubbles or leakage (Figure 13f).

Place each feeding unit with the membrane facing down into the precut openings or feeding ports on top of a large cage and allow the mosquitoes to feed for two hours. Cover the cage with a black cloth to simulate night conditions.

When feeding is completed, remove the collagen membrane from the plates over a sink. Running warm water over the plate may help loosen hardened membrane and blood. Dispose blood and collagen membrane according to guidelines. Wash the plates with warm water and detergent and/or sterilize them in an oven set at 100°C or with boiling water for 30 minutes.

Figure 13. ***Setting up the hemotek membrane feeding system***.

5.5. Draining waste from the cages – disposal of dead adults and pupae

In order to keep the cage clean, dead adults and pupae are collected daily along with the eggs from the bottom of the cage by draining the water trough.

Before opening the stopcock to collect the waste water, position a container below the stopcock and place the 50 µm sieve on top of the container's opening, with the 300 µm sieve nested in it (Figure 14). The mesh should become increasingly finer as the water passes down through them, to first collect debris and then prevent any eggs from passing into the waste water container.

Attach the hosepipe above the spout and let water flow through the internal irrigation system (Figure 14). Alternatively, water can be poured directly into the cage from outside. This can be done using a jug and splashing small amounts of water through the cage side with the larger mesh so that the trough can be cleaned methodically from one end to the other. This approach entails some water splashing around but much of it can be contained by holding a tray under the cage.

In addition, a squeeze bottle is used to spray water through the netting and flush down any debris from the walls of the trough. Dead adults will float in the water through the stopcock into the sieve (Figure 14). If the stopcock gets blocked, use the squeeze bottle to squirt water from the outside into the opening (). The debris (dead adults, pupae and exuviae) are collected in the 300 µm sieve, whilst eggs are washed through and caught by the 50 µm sieve. In this way eggs are cleaned ready for collection (Figure 14). However, if for quality

control or for research purposes you want to quantify emergence or adult survival rates, debris can be transferred into a tray for counting the number of dead pupae and adults (male and female). We recommend using a counter and the drawing of a grid onto a rearing tray for easier observation and to minimise double counting (Figure 14).

Figure 14. ***Draining waste from the cages***. From *left to right: waste water container with a sieve on top to collect debris; view inside the spout where mosquitoes may cluster and block the flow; an empty tray with grid markings and a tray filled with dead adults for counting.*

To drain all the water out of the trough, the cage can be tilted slightly but care should be taken not to spill the sugar solution in the feeder tube into the trough. Removing all water is particularly important if oviposition needs to be delayed (for synchronisation of egg collection) as otherwise gravid females may lay their eggs onto the wet surface. Once all water has been drained, quickly close the stopcock to prevent mosquitoes escaping.

Figure 15. ***Internal irrigation system***. *View of the spout outlet from inside the cage (left) and the hose pipe and stopcock from outside the cage (right).*

6. EGG STAGE

6.1. Collection of eggs

To allow gravid females to lay their eggs, use the hosepipe to fill the trough with sufficient water (but do not overfill otherwise eggs will get stuck under the internal pipe). The following day and then two times a week (Mamai *et al.,* 2017), eggs can be collected along with the dead adults as described above.

During the flushing, the dead adults will remain in the top sieve (mesh size 300 µm) while the eggs will float through and be collected in the lower sieve (mesh size 50 µm, Maïga *et al.,* 2016).

Wash the walls of the trough using a squeeze bottle, with repeated sweeping motions in the direction of the drain, to collect any eggs which remain stranded. After all water has been drained, close the stopcock and rinse the upper sieve gently with the hose to flush out any eggs attached to the dead bodies or the sieve.

The bottom sieve will have retained all eggs. Continue rinsing the lower sieve gently with water to remove any unwanted debris and to collect the eggs to one side of the sieve.

Prepare a filter paper funnel (*e.g.* coffee filter) to collect the eggs and – with a squeeze bottle – carefully flush the eggs out of the sieve and into the filter paper funnel. Ensure all eggs are washed from the wall and the seam between the sieve netting and the frame (Figure 16).

Figure 16. ***Collection of eggs from the mass-rearing cage****: (a) squeezy bottle to rinse the walls of the spout; (b) waste water container with 300 µm and 50 µm sieves on top to collect debris and eggs; (c) water flowing into the cage through the hose pipe and out through the open stopcock below; (d) 50 µm sieve containing eggs scattered around; (e) eggs gently washed down to one side of the sieve; (f) transferring eggs from the sieve to the filter paper using the squeezy bottle.*

6.2. Estimating number of eggs by weighing

In order to weigh the eggs, they need to be dried in advance. Open the filter paper containing the eggs, place it onto a dry paper towel and allow them to air dry for 3 hours.

To weigh eggs, an electronic balance with an accuracy of 0.0001 g is required. Place a 1.5 mL Eppendorf tube on the balance and tare it.

Once the eggs have dried, gently brush them onto a non-adhering tracing paper (Duerer-Hase, A4, 25 sheet, 80 g, article number 101600, LIBRO, Austria) to collect and transfer them into the Eppendorf tube (Figure 17). Record the weight (mg) of all the eggs and divide it by 0.004 (the previously calculated weight per egg, Maïga *et al.,* 2016) to get an estimated number of eggs in the Eppendorf tube.

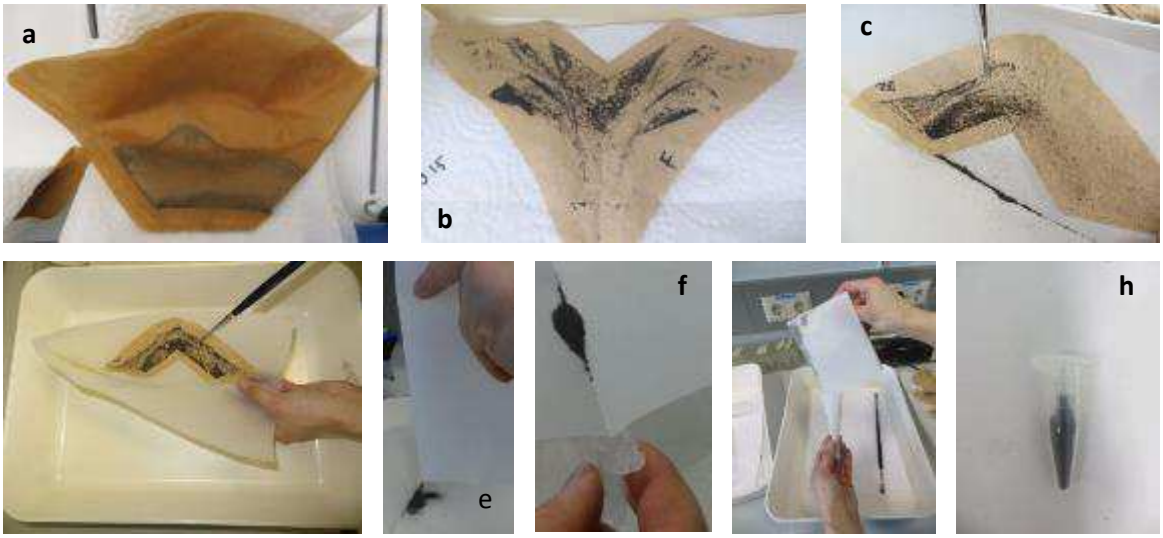

Figure 17. ***Drying and preparing eggs for quantification****: (a) collected eggs on a wet filter paper; (b) air-dry of eggs at room temperature for 3 hours; (c) brush of dried eggs from the filter paper onto a tracing paper; (d) a tray should be used as a base in case of spillage; (e) dried eggs collected onto a new tracing paper; (f) transferring dried eggs into an eppendorf tube directly; (g) transferring eggs into an eppendorf tube using a paper cone; (h) dried eggs stored inside an eppendorf tube.*

To aliquot eggs into smaller batches, weigh the amount of eggs required, *e.g.* for 4,000 eggs brush dried eggs onto a tared weigh boat until the weight reaches 16 mg (4,000 × 0.004 mg). The aliquoted egg batches can be transferred into 200 µL Eppendorf tubes and used the same day.

7. LARVAL STAGE

7.1. Transfer to large rearing trays

It is preferable that *An. arabiensis* larvae be maintained under controlled and stable conditions of temperature, humidity and lighting 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. At the FAO/IAEA IPCL *An. arabiensis* are reared in a climate-controlled room (88 m^3) maintained at 30 °C ± 1 ºC, 70% ± 10% RH with a 12h : 12h light : dark photoperiod.

A fully loaded rack can hold 50 large plastic trays each seeded with 4,000 *Anopheles* eggs (Figure 18). Therefore, each mass-rearing rack can hold up to 200,000 *Anopheles* larvae at a time. Currently, the handling and maintenance of the larval rearing (*e.g.* adding food and water) is done manually, but a system to aliquot a set volume of diet into each tray using a dosing gun is under development.

Figure 18. ***Mass-rearing rack.*** *The shelves that facilitate a sliding and tilting mechanism for the large plastic trays (left); the large plastic trays stacked in the steel rack (right) complete with water, larvae and diet slurry.*

To prepare the large trays for rearing, each tray should be filled with 4 L of water a day before adding the eggs to allow the water to reach the temperature of 27-28 °C. The subsequent day eggs (dried in the air for 3h) are added onto the surface of the water and allowed to rehydrate and hatch. To prevent eggs from sticking to the edge of the tray and drying out before they can hatch, which can reduce overall hatch rate, eggs can be added to the water inside a floating ring (Figure 18).

Figure 19. ***Overflow system.*** *Two pipelines placed over the steel rack to fill the trays with water using the overflow system.*

7.2. Feeding larvae

Larvae are fed on an artificial liquid diet that was developed at the FAO/IAEA IPCL (Damiens *et al*., 2012). The diet was designed to adequately supply all necessary components for larval growth including fatty acids, proteins, sugars and vitamins. It consists of a combination of powdered tuna meal, bovine liver powder and a vitamin mix (Table 1. *Larval diet composition (1%) for a 2 l solution.*).

Table 1. *Larval diet composition (1%) for a 2 l solution.*

	Component	**Supplier**
10 g	Tuna meal	T.C. Union Agrotech (Thailand)
10 g	Bovine liver powder	MP Biomedicals (U.S.A)
9.2 g (Optional)	Vitamin mix	Bio-Serv (U.S.A.)

Ensure the tuna meal is finely ground so that it makes a suspension in water. The dry mixture aliquots can be prepared in advance and stored at 4ºC until use. To make the liquid diet, add 2 litres of deionised water to the dry mixture and mix well. For a homogeneous mixture, the liquid diet can be processed in a commercial blender. The shelf life of the liquid diet is approximately 1 week if stored at 4 °C and up to 6 months if frozen at -20 °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 reagents in sealed containers in a fridge.

The liquid diet should be provided on a daily basis in order to achieve synchronised rearing. During first and second instar, larvae are fed 100 mL liquid diet (only the supernatant, not the solids which settle out of the suspension) per day and thereafter 150 mL (whole suspension, shaken before feeding) per day (Table 2, Figure 20). Therefore, 5 L of diet is required per larval rack for each of the first three days followed by 7.5 L on the subsequent days. Pupae will develop around 6-8 days after hatching (depending on room temperature), and need to be collected within 24 hours from sight of the first pupae to prevent emergence and escape of adults.

Table 2. *Dose recommended for 4,000 larvae in 4 l water.*

Day	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Volume (mL)	100	100	100	150	150	150	150	150

Figure 20. ***A cup filled with liquid diet (100 ml) to be dispensed into each large rearing tray***.

7.3. Collection of larvae and pupae - tray tilting

Collection of larvae and pupae is done by tilting the trays in the rack. Place a large plastic container below the collection basket to pool together all larvae and pupae of the rack that will stream down the vertical plastic wall on tilting. 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 21). 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 21. ***The stainless steel rack system*** *(left), the screw jack at the base of the rack (middle) and the trays in a tilted position (right). Pictures adapted from Balestrino et al. (2012)*

As the rack holds around 200 L (4 L x 50 trays) of water in total, the collection should be done slowly in batches of around 20 L per tilt. Pour the water from the container into a sieve to concentrate the larvae and pupae and dispense them into a small larval tray with some water. Repeat the tilting process (ca. 10 times) until all water from the trays is collected. Towards the end many larvae and pupae will be caught at the edge of the rearing trays which will need to be washed down with additional water. Collect all remaining larvae and pupae and concentrate them down to a few small rearing trays (Figure 22).

Figure 22. ***A large plastic container used to collect larvae and pupae*** *that stream down during the tilting process (left); concentrating larvae and pupae using a sieve and a water container (right); picture of a small rearing tray covered with a net to prevent any emerging adults escaping (bottom).*

8. PUPAL STAGE

8.1. Isolating pupae from larvae

Before loading a large cage with pupae, they must be separated from the remaining larvae. This can be done by transferring the larval-pupal mix into an Erlenmeyer flask (1 L). Fill the flask to somewhere below the 1 L mark and give it a firm swirl. The aim is to create a vortex which will pull the larvae down and allow the pupae to swim to the water surface for a brief moment. Top up with water while the water is still spinning which will distance the pupae from the larvae (Figure 23). As the swirl loses its momentum, the larval and pupal separation will begin to fade. At this point quickly tip the top layer (mostly pupae) into a new tray and repeat the process to extract more pupae if required. Pour the rest (mostly larvae) into a separate small rearing tray and pick any remaining pupae manually. Give sufficient liquid food to the larvae to maximise pupation overnight. Repeat the pupal extraction on the following days until enough pupae have been produced to fill the required number of large cages.

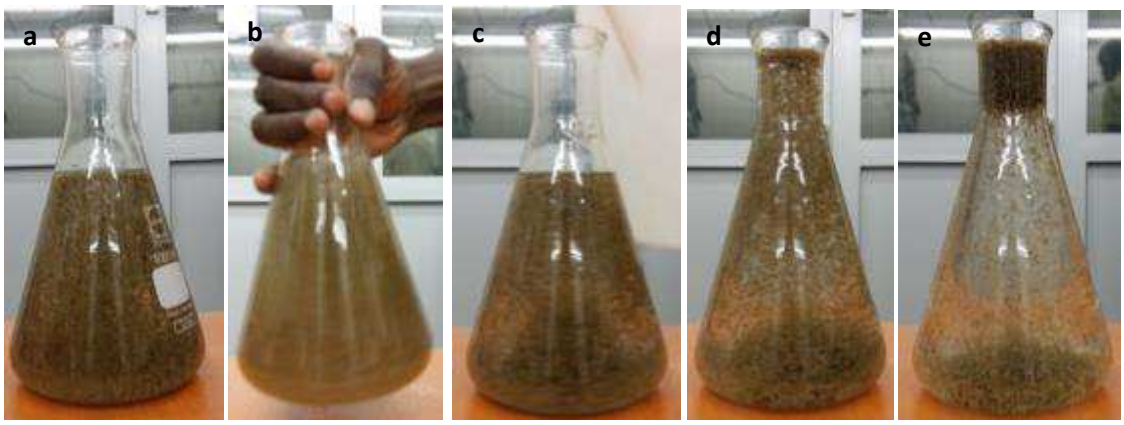

Figure 23. ***Pupal collection***. *The larval and pupal mixture can be separated by swirling an erlenmeyer flask. The vortex will collect larvae at the bottom and allow the pupae to swim to the top of the flask.*

A mass-rearing device has been developed that is able to separate *An. arabiensis* pupae from larvae based on their natural differences in buoyant density and behaviour (Balestrino *et al*., 2011). The separation is achieved through a vortex mechanism which suppresses active swimming behaviour and a cold water treatment (4 – 15 °C) that allows pupae to float and larvae to sink. However, the rate of pupal separation (1,000 individuals per minute) is not sufficient to operate on a large-scale but it proved to be a fitting prototype.

8.2. Estimating the number of pupae

Before adding pupae to a large cage, their number needs to be estimated so that each cage is filled with the correct density for optimal productivity. This can be done using a modified 50 mL conical centrifuge tube.

First cut the tube horizontally at the 10 mL marking. Then unscrew the cap and seal a fine mesh (1 × 1 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.

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 at the 10 mL marking (Figure 24). The water will flow through the mesh slowly but the pupae will remain. The number of pupae will reach approximately 5,000 when the stacked pupae reach the 35 mL marking (Figure 24).

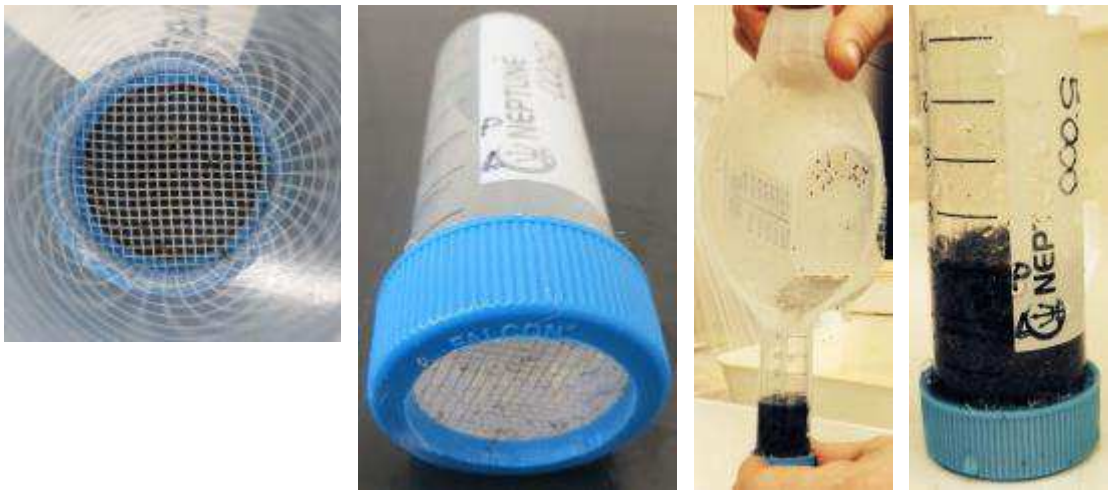

Figure 24. ***Estimating the number of pupae****. From left to right: The net (1 mm) from inside the tube; the modified 50 ml conical centrifuge tube; pupae being poured into the modified tubes; estimating the volume of total pupae.*

9. QUALITY CONTROL

An essential component of an SIT programme is to keep track of the input/output in the mass-rearing system to foresee any potential problems and minimise delays in tackling them. Therefore it is recommended to regularly monitor the mosquito life cycle at different stages.

9.1. Hatch rate

As eggs are dispensed into the rearing trays by estimation based on drying and weighting, it is a good practice to measure the actual hatch rate. As the aim is to aliquot 4,000 eggs per tray, a hatch rate of 0.8 would result in 3,200 larvae, and so the number of eggs added to each tray should be increased proportionally to compensate.

To estimate the hatch rate, simply brush 100 eggs into a tray with water (tip: add filter paper to the sides of the trays to prevent desiccation) and count the following day the total number of larvae that have hatched. Divide the number by 100 to obtain the hatch quotient and calculate how many eggs need to be transferred to the rearing tray (number larvae desired / hatch quotient = number eggs required) to obtain the true number of larvae (*e.g.* 4,000 larvae / 0.8 = 5,000 eggs required).

9.2. Pupation

The percentage of larvae that successfully pupate is not 100% due to the nature of mass-rearing. There may be several factors that play a key role *e.g.* larval food not dispatched evenly in all trays leading to overfeeding (from bacterial growth) or malnutrition and larval competition for food. Also the tilting mechanism may put additional stress on the fourth instar larvae during collection. Unfortunately this is inevitable and difficult to measure but it should be taken into account when estimating the pupal output.

9.3. Adult emergence

The number of emerged adults can be measured accurately during the initial counting of the dead pupae (as described in Section Draining waste from the cages – disposal of dead adults and pupae). The drained water will contain dead adults, empty pupal shells and dead pupae altogether. If the estimated number of pupae added to the cage is known (Section Estimating the number of pupae), the number of dead pupae can be subtracted to get an approximate number of emerged adults. This number will be important to determine the success rate of mating and blood feeding, and ultimately the fecundity after estimating the number of eggs obtained per cage.

9.4. Adult survival

As described in Section Draining waste from the cages – disposal of dead adults and pupae, counting dead adults is necessary to monitor the survival inside the cage. Newly emerged

females will require about 8 – 12 days to reach the peak of their egg production and for that reason it is critical to keep them alive for that long. The number of male and female deaths should be recorded. If males die in abundance, the mating efficiency will be low, and if females die in high numbers, then obviously the egg production will be limited. Therefore, the survival of both sexes is fundamental to maintain a good balance of mating and oviposition.

9.5. Sex ratio

The sex ratio of emerging mosquitoes should be close to 50:50. But during the aquatic stages, male larvae tend to pupate earlier than females. This may result in a bias if pupae collected over multiple days are loaded into different cages.

9.6. Mating status

A good male quality will ensure a dynamic mating activity, which is a key component in obtaining a good batch of eggs. If females are not inseminated even though they blood feed well, they will not be able to lay eggs. Therefore, it is essential to assess the mating status by periodically dissecting female spermathecae to check for insemination.

9.7. Engorged females

The number of engorged females is directly related to the total number of eggs produced by a cage. If females are inseminated but not blood feed, they will not produce any eggs. As females naturally seek blood at night, the cage is covered with a black cloth to simulate darkness. After or during blood feeding, efforts should be made to estimate the number of engorged females to see if the number of eggs produced may be linked to the number of females blood feeding.

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APPENDIX I - QUICK PROTOCOLS

Sugar feeding

Equipment and Materials

- Filter paper (Whatman 2589A, 580 x 580 mm)
- Duran® bottle (2 L)
- Granulated sugar (100 g)
- Boiling reverse osmosis (RO) water (500 ml)
- Cold RO water (1,5 L)

Procedure (before loading the cage with mosquitoes)

1. Use the filter paper to cut three sheets (25 x 20 cm) and fold them along the vertical axis (25 x 10 cm); seal the ends with tape
2. Prepare the 5% sugar solution in a Duran bottle by dissolving 100 g granulated sugar in 500 mL boiling water; add 1,5 L cold water and mix well
3. Pour the sugar solution into the opening of the sugar feeding device on the outer wall of the adult cage
4. Place the pre-cut filter paper into each slot of the sugar feeding device
5. The cage is now ready to be loaded with mosquitoes

Blood feeding

Equipment and Materials

- Hemotek membrane feeding system with modified aluminium plates
- Animal blood
- Synthetic or natural membrane (e.g. collagen)
- Disposable Luerlock syringe (BD, Franklin Lakes, NJ; 50 mL capacity)
- Disposable gloves

Procedure

1. Defrost bovine blood overnight (50 mL for each feeding unit)
2. Cut 20 x 20 cm synthetic or natural membrane
3. Remove white plastic ring from the aluminium plate
4. Attach the pre-cut membrane to the flat side of the aluminium plate
5. Seal membrane with the white plastic ring
6. Place aluminium plate with the flat side down
7. Remove one of the two white knobs on the rear side
8. Attach a disposable syringe (without the plunger) to the opening
9. Slowly pour 50 mL blood into the syringe to fill the reservoir
10. Remove the syringe and screw the white knob back on

11. Lift the aluminium plate and check for air bubbles or leakage
12. Place each feeding unit on top of a large cage to facilitate feeding
13. Cover the cage with a black cloth
14. After 2 hours remove the feeding unit
15. Rinse the feeding unit with cold water and then clean it with hot water

Waste drainage

Equipment and Materials

- Retsch® Test Sieve with steel mesh (size 300 µm)
- A 20 L plastic waste water container with same diameter of the sieve
- A wash bottle (squeeze bottle)
- A water jug (1 L)
- A hosepipe (details in Section 4.1)
- Rearing trays (with grid markings)
- A counter
- Deionised water
- A mop (to clean the floor)

Procedure

1. Place the waste water container under stopcock
2. Place the 300 µm sieve on top of waste water container
3. Open stopcock mechanism
4. Attach a hosepipe to the cage opening (above the stopcock)
5. Fill the trough with water to float the dead bodies trough the stopcock
6. Dead adults and debris will be collected in the 300 µm sieve and water will drain into the waste water container
7. Squirt water from outside into the cage to wash the walls of the trough
8. Use the water jug to pour water in batches through the netting towards the other end of the cage; the water force will push more dead adults towards the stopcock
9. If the stopcock gets blocked, use the wash bottle to squirt water into the opening
10. Carefully tilt the cage to drain any excess water from the trough; do not spill the sugar solution
11. Close the stopcock
12. Transfer debris to a rearing tray
13. Count dead mosquitoes as required (*e.g.* male and female adults and pupae)

Egg collection

Equipment and Materials

- Same as above;
- Retsch® Test Sieve with steel mesh (size 50 µm)

Procedure

1. Use the hosepipe to fill the trough with sufficient water the night before egg collection
2. On the day of egg collection, place the waste water container under the stopcock
3. Place the 50 µm sieve on top of the waste water container
4. Place the 300 µm sieve on top of the 50 µm sieve
5. Open the stopcock mechanism to collect the waste water
6. Dead adults will be collected in the upper 300 µm sieve and eggs will be collected in the lower 50 µm sieve. Water will drain into the waste water container.
7. Squirt water from outside into the cage to wash the walls of the trough
8. Carefully tilt the cage to drain any excess water from the trough. Do not spill the sugar solution
9. Close the stopcock
10. Transfer dead mosquitoes from top sieve to a rearing tray and count as required (*e.g.* male and female adults and pupae)
11. Rinse lower sieve gently with water and transfer eggs carefully into a filter paper funnel
12. Air dry eggs for 3 hours

Egg Weighing and Aliquoting

Equipment and Materials

- Electronic balance (accuracy of 0.0001 g)
- Eppendorf tubes (0.2 mL and 1.5 mL)
- Marker pen
- Water colour brushes (size 10 – 12)
- Tracing paper (Duerer-Hase, A4, 25 Blatt, 80g, Artikel Nr. 101600 LIBRO)
- Weighing boat

Procedure

1. Place an empty 1.5 mL Eppendorf tube on the balance and tare it
2. Cover the working area with tracing paper (in case of egg spillage)
3. Carefully brush the dried eggs from the filter paper onto a folded tracing paper
4. Transfer eggs from tracing paper into the 1.5 mL Eppendorf tube
5. Close the cap and place the tube on the tared electronic balance
6. Measure and record the weight
7. Divide the weight by 0.0004 g (theoretical weight per egg) to estimate the number of eggs collected in the Eppendorf tube
8. To aliquot 4,000 eggs, gently brush eggs onto a tared weighing boat until the weight hits 1.6 g (4,000 eggs x 0.0004 g per egg = 1.6 g)
9. Transfer eggs into a 0.2 mL Eppendorf tube for dispersion into a large rearing tray

Rearing Larvae in the Steel Rack

Equipment and Materials

- Steel rack
- 50 large plastic trays
- Water (min 200 L at 28-32 ºC)
- Water jugs (1 L and 4 L)
- Larval diet components (tuna meal, bovine liver powder and vitamin mix)
- Commercial blender
- Falcon™ tubes (50 mL)
- 50 plastic beakers (150 mL)

Procedure

1. Pre-fill each large plastic tray in the steel rack with 4 litres of water
2. Add 4,000 dried eggs to each plastic tray (water temperature 28-32 ºC)
3. Prepare daily fresh artificial liquid diet (add 10 g tuna meal, 10 g bovine liver and 9.2 g vitamin mix into a commercial blender and mix with 2 L water)
4. Dispense diet to each rearing tray as recommended for 4,000 larvae in 4 L

Day	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Volume (mL)	100	100	100	150	150	150	150	150	150

5. Larvae will start pupating between 5 – 9 days (depending on temperature, larval density and food availability)
6. The rearing trays should be tilted within 24 hours of sight of the first pupae

Tray Tilting

Equipment and Materials

- Steel rack
- 50 large plastic trays
- Large plastic container
- A marker pen
- Retsch® Test Sieve with steel mesh (size 300 μm)
- A 20 L plastic waste water container with same diameter of the sieve
- A wash bottle (squeeze bottle)
- A water jug
- A hosepipe or a large water storage container with a water jug (4 L)
- Small rearing trays

Procedure

1. Fill up the 20 L plastic waste water container with water
2. Pour the water into the large plastic container and mark the water level

3. Discard water and place the large plastic container below the collector basket
4. Use the screw jack to tilt the trays slightly (until the water starts floating)
5. The larvae and pupae will stream down the plastic wall onto the steel collector basket and then into the plastic container
6. Stop the flow before the water level reaches the marking
7. Place the sieve on top of the 20 L waste water container
8. Pour the water collected in the plastic container into the waste water container
9. Transfer the larvae and pupae collected in the sieve into a small rearing tray
10. Discard waste water from the 20 L waste water container
11. Place large plastic container back below the collector basket
12. Repeat the tilting process (steps 4 - 11) until all water has been drained from the large rearing trays
13. To collect the remaining larvae and pupae, rinse the trays from top to bottom using a hosepipe or a water jug (4 L) and collect as described above

Isolating Pupae from Larvae

Equipment and Materials

- Erlenmeyer glass bottle (1 L)
- Small rearing trays
- Water jug (1 L)

Procedure

1. Pour up to 1 L of the larvae/pupae mix into the Erlenmeyer glass bottle
2. Create a water vortex by swirling the bottle around and top up with water
3. The vortex will temporarily separate the larvae from the pupae
4. Quickly tip the top layer (mostly pupae) into a new tray
5. The bottom layer consists mostly of larvae and can be reared in a new tray
6. Add sufficient liquid food to the larvae to maximise pupation overnight

Estimating Number of Pupae

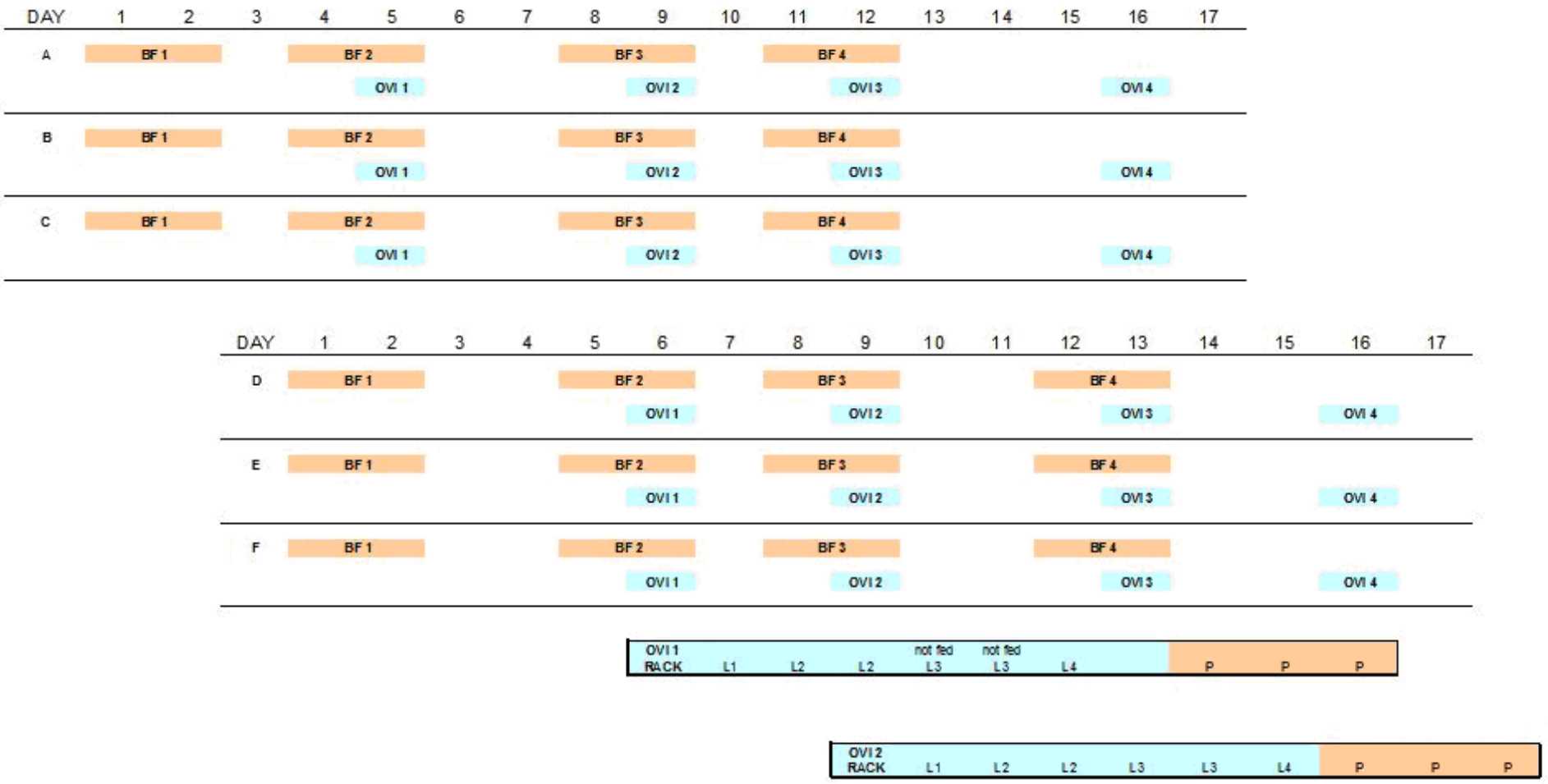
Equipment and Materials

- Modified 50 mL Falcon™ tube

Procedure

1. Hold modified tube upside down and add pupae slowly through the opening
2. The water will flow through but the pupae will remain stacked at the bottom
3. The number of pupae reaches approximately 5,000 when the stack reaches the 35 mL marking

APPENDIX II - Time Schedule for a full cycle of *Anopheles arabiensis* mass-rearing based on insectary data (FAO/IAEA).



APPENDIX III – Special Equipment and Materials with Specifications

Item	Specifications	Example of supplier and/or distributor
Bugdorm© plastic cages	(300 mmx300x300)	(EM1000 - BugDorm-1 Insect Rearing Cage, BugDorm, Taiwan)
filter paper wick	(42 Ashless, 90 mm diameter.	Cat 1442-090. Whatman-GE Healthcare (Piscataway, NJ, USA)
black foam insulation tube		Armacell, class 1 Armaflex
Hemotek		Discovery Workshops, Lancashire, United Kingdom
grey connector 21 mm, G ½"		Handverbinder GARDENA, or similar
orange connector 19 mm ¾"		Profi-System-Übergangsstück mit Wasserstop, GARDENA, Nr. 2814
Velcro®		
filter paper	Whatman paper, 580 × 580 mm	2589 A Bogen sheets
20 × 20 cm piece collagen membrane		Discovery Workshops
Luerlock syringe	50 mL~~l~~ capacity	BD, Franklin Lakes, NJ, US
filter paper funnel (e.g. coffee filter)		
tracing paper	A4, 25 sheet, 80g, article	Duerer-Hase, number 101600, LIBRO, Austria
Tuna meal		T.C. Union Agrotech, Thailand
Bovine liver powder		MP Biomedicals (U.S.A)
Vitamin mix		Bio-Serv (U.S.A.)
Retsch® Test Sieve	with steel mesh (size: 50 µm and 300 µm)	
Electronic balance	accuracy of 0.0001 g	

FalconTM tubes		