Joint FAO/IAEA Programme
Nuclear Techniques in Food and Agriculture

GUIDELINES FOR ROUTINE COLONY MAINTENANCE OF *AEDES* MOSQUITO SPECIES

Version 1.0

Food and Agriculture Organization of the United Nations
International Atomic Energy Agency
Vienna, 2017

Edited by:

Hamidou Maïga, Hanano Yamada, Bimbile-Somda N. Severin, Danilo de O. Carvalho, Wadaka Mamai, Rafael Argilés Herrero, Konstantinos Bourtzis and Jeremy Bouyer
Insect Pest Control Section, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture

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CONTENTS

1- INTRODUCTION

This document aims to provide a description of procedures required for *Ae. aegypti* and *Ae. albopictus* colony routine rearing. This guide is a summary of necessary steps such as optimizing climatic conditions in the insectary, egg hatching, larval rearing, pupal and larval sorting, sugar and blood feeding, egg collection, handling and storage, used at the FAO/IAEA Insect Pest Control Laboratory (IPCL) to build and maintain a lab colony. This is a temporary guide and will be continuously updated.

2- COLONY REARING ROOM

Controlling the rearing environment is essential for all insect rearing. *Aedes aegypti* and *Ae. albopictus* are maintained in 30 x 30 x 30 cm cages in an insectary deprived of natural light, under standard and constant rearing conditions 26ºC ± 1ºC, and 70±5% relative humidity, 12h:12h light:dark photoperiod including dusk (1hour) dusk and dawn (1hour) transitional periods. Lighting at larval stages is known to drive adult behavior and is particularly important for mating and oviposition.

Biosafety issues need to be addressed and escape of any mosquito regardless of strain must be strictly avoided. Guidance on biosafety matters can be found in several documents, such as the Arthropod Containment Guidelines (see American Committee of Medical Entomology in selected References).

Furniture made of rust-proof metal, plastic or fiber glass are recommended, and should be equipped with wheels where possible to allow for regular cleaning underneath and around the furniture. Rearing Cages are kept on shelves (Figure 1).

General cleanliness and sterility in the insectary should be promoted to reduce infections, pests and infestations of other potentially harmful insects such as predatory ants, roaches and book lice (*Psocoptera*) that can destroy egg stocks. Dust and scales shed by adult mosquitoes can also cause serious respiratory symptoms and should be removed as often as possible.

Figure 1. Shelves with cages in rearing room at the IPCL, Seibersdorf. Cages are labelled with strain name, origin, date, rearing generation.

3- EGG HATCHING

Mature eggs *(see EGG DRYING/EMBRYO DEVELOPMENT AND EGG STORAGE)* of 2 to 3 weeks are hatched by submerging in hatch solution media overnight (700 ml deionized water containing 0.25 g Nutrient Broth (CM0001, Oxoid, Hampshire, England) and 0.05 g Brewer's Yeast).

Eggs can also be hatched using cooled boiled distilled water in jars (jam jars, or other sealable containers) (Figure 2A) filled with water (~3/4). Jars must be tightly closed for both methods to deoxygenate the water and promote good hatching. Care should be taken to move jars as little as possible to prevent oxygen production. Alternatively, eggs can be hatched by adding 1-2 ml of larval diet to distilled water in a sealed container kept for 2-3 days under laboratory conditions before adding the egg papers (Yamada, pers. com) (see Figure 2B).

For a routine rearing, the number of eggs to be hatched should be decided according to the number of cages that are desired: assuming that approximately 70-80% of the eggs will result in adults or more if good rearing practices are maintained. The following table shows the quantity of ingredient and volume of distilled water needed for hatching solution preparation (See recipe, Table 1 Hatching solution recipe**)**.

As using hatching solution media is more costly that using boiled cooled water, it will be more advisable to hatch egg with boiled cooled water with or without additional larval diet (Figure 2B), or pre-prepared distilled water plus larval diet.

It is important that after hatching, jars, containers and egg papers are either treated with boiled water or kept in the freezer (for at least 3 days), before washing and/or disposal. It is also important when working with different strains or species to use/change gloves to avoid any contamination.

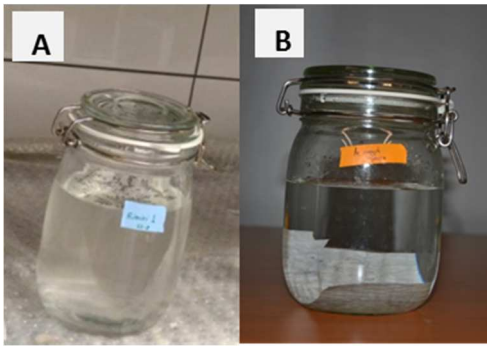

Figure 2. Jam jar filled with hatching solution media overnight (A). Egg papers can also be hatched without any prior brushing (B).

In Figure 2, jam jars are filled with hatching solution media overnight (700 mL deionized water containing 0.25 g nutrient broth and 0.05 g brewer's yeast). Up to 10, 000 loose eggs can be hatched in each jar (A). Egg papers can also be hatched without any prior brushing (B).

4- LARVAL REARING

Between 1,500 and 2,000 first-instar larvae (L1) are reared per plastic tray (40 × 30 × 8 cm) (Figure 3) containing 1-1.5 liter of distilled or deionized water (lowering the larval rearing density will decrease selection pressures and will result in larger, healthier pupae and adults).

Larvae are fed on a 4 % liquid diet that was developed at the IPCL in Seibersdorf, Austria (Puggioli et al. 2013). The diet is designed to adequately supply all necessary components for optimal larval growth and includes powdered tuna meal, bovine liver powder and yeast (see recipe in Table 2). The daily feeding regime is as follows: Day 1 and 2: 8 mL; Day 3 and 4: 16 mL; Day 4-until pupation: 32 mL.

Larval trays (Figure 3 A-B) are covered with a transparent Plexiglas to avoid evaporation and thus evaporative cooling and to keep larval water with constant temperature. It also prevents contamination by other strains or insects. Locally made netting (Figure 3 C) can be used to cover trays when there is a risk of adult emergence during the weekends and holidays.

Reduced rearing duration and improved body size can be achieved with 500-1,000 L1 per tray. In this case, larval diet quantity should be adjusted accordingly. Larval development and pupation synchronization can be also achieved with low densities per tray and with room temperature above 27ºC, and eggs from a narrow window collection.

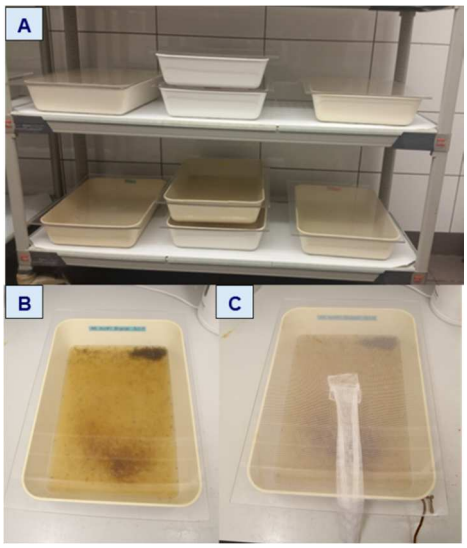

Figure 3. Larval trays covered with a transparent Plexiglas (A-B). Locally made netting can be used to cover trays (C)

5- SORTING LARVAE AND PUPAE

In standard laboratory rearing at smaller numbers, pupae can be manually separated from larvae by removing individuals with a plastic pipette (Figure 4 A). If the opening of the pipette is too small it can be snipped at the end to accommodate larger pupae. Pupae (1,000 – 2,000) are separated and transferred into 10 x 10 x 8 cm (long:large:high) bowl (Figure 4 B) inside a rearing cage (30× 30 × 30 cm). Smaller bowls might be used for small number of pupae to collect.

Figure 4. Pipette (A) used for pupae collection. Pupae are collected by hand and transferred into 10 x 10 x 8 cm bowl (B).

Locally-sourced or custom-made cages may be used, though the IPCL uses Bugdorm plastic cages (EM1000 - BugDorm-1 Insect Rearing Cage, Taiwan) (see above section 2- *COLONY REARING ROOM* for convenience and standardization).

For routine colony maintenance a ratio of 1:1 (female: male) can be used though a higher female ratio produces higher egg yield. A ratio of 3:1 can be used to upscale the colony. Up to 1,000 males and 3,000 females can be maintained in a 30 x 30 x 30 cm BugDorm cages (Carvalho et al. 2014).

The size difference between male and female *Aedes aegypti* or *Ae. albopictus* can be used to facilitate the separation of the sexes during the pupal stage. Different methods can be used either by submerging pupae under a series of sieves with decreasing aperture, or by using a mechanical device designed and manufactured for the separation of the developmental stages, sexes, and species of mosquitoes (Focks, 1980).

Pupation duration is about 48 hours (depending on the larval rearing room temperature) and so caution should be taken to separate pupae and larvae before emergence starts.

6- SUGAR FEEDING

A 5-10% sucrose solution is supplied to the adult through a feeder as the carbohydrate source. The sucrose solution is poured in a plastic tube (e.g. Falcon 20 or 50 ml capacity) covered with a polyester technical fabric (100-150 µm) and secured with a holed cap (Figure 5 A). This feeder is then hung upside down in the adult cage (Figure 5 B) allowing the mosquitoes to feed through the fabric.

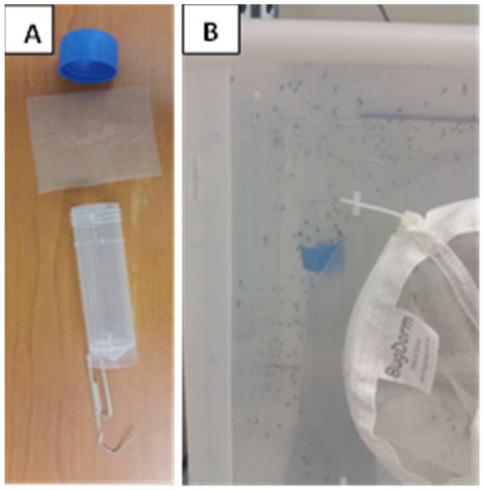

Figure 5. Plastic tube covered with a polyester technical fabric (mesh openings 100-150 µm) and secured with a holed cap (A) and hung upside down in the adult cage (B).

7- BLOOD FEEDING

Defribrinated fresh bovine (alternatively porcine or other blood) is provided to female mosquitoes (from 4 to 5 days post-emergence) two times per week using the Hemotek (Discovery Workshop 516A , Burley Road, Acrington, Lancs, BB5 6J2, England) (Figure 6 A) membrane feeding system that regulates the blood temperature.

To create a reservoir for the blood, a 20 × 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 6 B and C). On the rear side of the plate, unscrew either one of the two white knobs and attach a disposable Luer lock syringe (BD, Franklin Lakes, NJ; 50 ml capacity) (Figure 6 D).

Pour 50-70 ml blood into the syringe and let it flow into the cavity between the aluminum plate and the collagen membrane (Figure 6 E). Remove the syringe and screw the white knob back into place. Swirl the blood around and check for air bubbles or leakage (Figure 6 F).

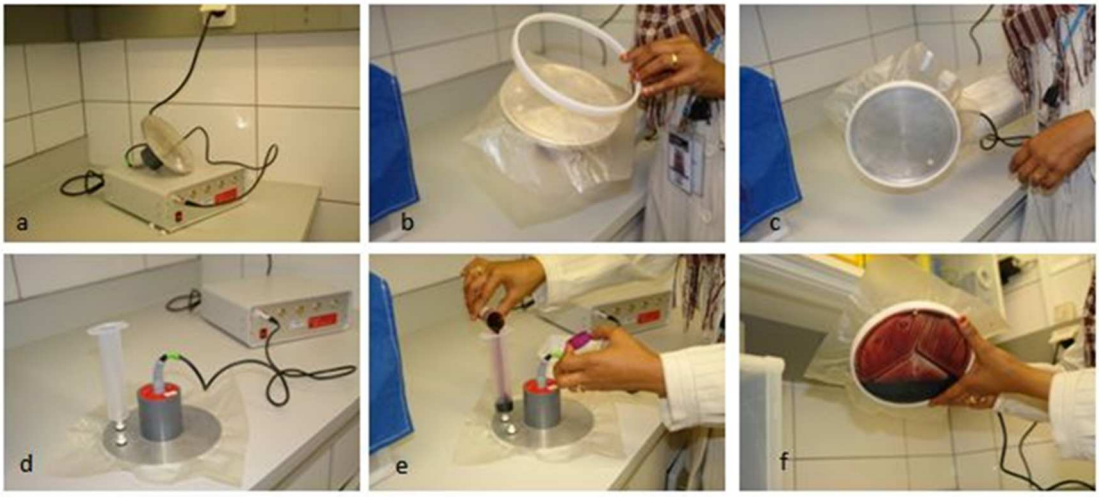

Figure 6. Setting up the Hemotek membrane feeding system.

If a Hemotek membrane feeding system is not available, alternative methods may be used such as placing a hot water bottle or a warmed bean bag (Carvalho et al. 2014) on top of the feeding plate

A collagen sausage casing (Edicas 23NC, FIBRAN S.A., Girona, Spain) filled with defribrinated fresh bovine blood heated in a warm water bath at 38-42° C (for 10 min) can be used for blood feeding on top of or in the cage (Figure 7). The sausage can be reheated and re-used for multiple cages. This method is commonly used for *Aedes* species blood feeding at the IPCL.

Sausage casings should not be too full in order to provide more surface area for mosquito feeding. The size of the sausage can be adjusted depending on rearing cage size and adult density.

Figure 7. A blood collagen sausage casing filled with bovine blood and placed on top of or in the cage.

8- EGG COLLECTION

Females are allowed to oviposit in a cylindrical black plastic cup (such as a plant pot approximately 12 cm high and 8 cm wide) filled 1/3 with deionized water and lined with germination paper (Sartorius Stedim Biotech GmbH, Göttingen, Germany) (Figure 8 A). Oviposition containers can be of various sizes depending on adult cage density. Dark cup color and addition of larval rearing water can promote oviposition in reluctant and newly colonized strains (Sucharit &Tumrasvin 1981). During oviposition, it is important that no other open water sources are present in the cage (Figure 8 B) which will deter oviposition in the egg cups.

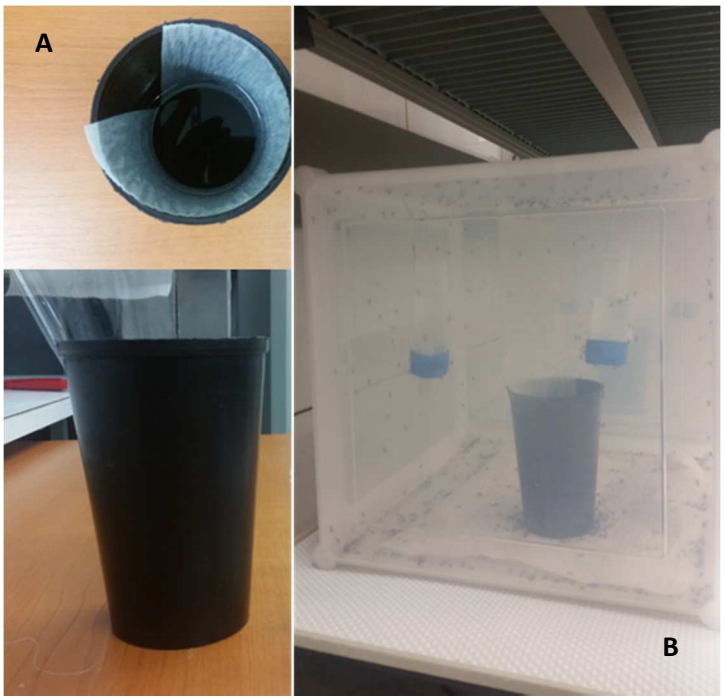

Figure 8. Oviposition cup with germination paper lining the inside, filled with distilled or deionized water (A). No other open water sources are present in the cage (B).

Oviposition will begin on the third day after blood feeding and may continue for 1 or 2 more days with progressively fewer eggs being laid (Figure 9). The same oviposition paper can be left in the cage for the duration of oviposition before collection. However, if left too long, premature hatching may take place resulting in a loss of larvae.

Figure 9. Egg paper showing the clustering of eggs near the surface line of the water.

9- EGG DRYING/EMBRYO DEVELOPMENT AND EGG STORAGE

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, eggs must be kept in a moist environment (80 -100% RH) for a period of 36-72 hours. 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 can then be placed into lidded (but not air-tight) containers Figure 10) for the embryonation period, during which the papers can dry gradually and will stay moist enough for development, but not so moist as to induce hatching. Larval rearing trays with the Plexiglas covers can be used for drying in insectary conditions, if no appropriate container can be found.

Figure 10. Containers with lose fitting lids lined with paper towels to keep egg papers moist for embryonation period (left). Containers are covered but not air-tight (right).

After 3 days of slow drying in the covered container, if storage is the aim, the lid of the container can be left slightly open to allow more air circulation and drying for 10 to 14 days. Once dried, the papers can then be either immediately hatched or dried further for storage in Ziploc bags (Figure 11) for up to 10 weeks in laboratory conditions (see 2.Colony rearing room section above) (Zheng et al. 2015a). Storing eggs in water produced an 85 % hatch rate after 5 months in both *Ae. aegypti* and *Ae. albopictus* species (Zheng et al. 2015b).

When hatching stored eggs, be sure to use older eggs first. If the eggs are allowed to dry too much during storage (more than 24 weeks) (Zheng et al. 2015b), the embryo within will die and the egg will collapse in on itself. During storage, papers should be checked occasionally for mold, parasites (book lice) and collapsed eggs and any affected papers should be used (hatched) or removed. If a serious problem with mold occurs, the storage containers should be thoroughly cleaned using bleach, and can be left slightly opened to encourage air circulation.

Figure 11. Ziploc bags (left) are used to store dry eggs and prevent infestation. Bags are then transferred in black boxes (center) stored up to 10 weeks (right).

10-RECIPES

a. Hatching solution recipe

Weigh 0.25 g of nutrient broth. Complete weight to 0.3 g by adding 0.05 g of yeast. Put the quantity of weighed ingredients into a bottle. Mix with 100 mL of water to dissolve. Fill the bottle up to 700 mL. Prepare needed volume so that no storage will be needed.

Table 1 Hatching solution recipe

item	Quantity
Brewer's yeast	0.05g
Nutrient Broth	0.25g
Water	700mL

b. Preparation of 4% *Aedes* larval diet solution

Weigh each of diet ingredients according to needed quantity of food. Put the quantity of weighed food into a bottle. Add water to dissolve larval food. Fill the bottle up to the volume needed. Label the bottle with preparation date, and initials of person who prepared the diet solution.

Table 2 *Aedes* larval diet solution

Diet stock	100 mL	500 mL	1 000 mL	2 000 mL
Tuna meal (g)	2	10	20	40
Bovine liver powder (g)	1.4	7	14	28
Brewer's yeast (g)	0.6	3	6	12
Total (g)	4	20	40	80

c. Sugar solution (10%)

Weigh 100 g sugar, poor into a bottle and mix with 100 mL of boiled distilled water to dissolve sugar. Fill with cold distilled water up to 1 L. Quantity of sugar and water volume can be adjusted accordingly if lower quantity of sugar solution is needed. Label bottle with preparation date, initials of person making sugar solution.

5% sugar solutions are also sufficient and tend to remain more fluid for a longer duration and do not congest sugar feeders as quickly.

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12-ANNEX

Example of rearing schedule

Suggested 3 week rearing schedule starting on a Monday:

Adults cycle:	**Immature stages:**
Week 1:	
Monday: Collect blood from your regular abattoir/blood source. Blood feed a subset of the colony (2 cages (test vs. control) of 25-50 females each) and check for any mortality the following day. **(Caution should be taken if female mortality is >10%)**.	Prepare hatch solution and leave until Wednesday.
Tuesday: Blood feed the colony	
Wednesday: No attention required	Hatch eggs and pan L1 larvae in rearing pans and add larval diet
Thursday: Blood feed the colony and add oviposition cups to the cages.	Feed larvae
Friday: Check sugar feeders.	Feed larvae
Saturday/Sunday: No attention required	Feed larvae
Week 2:	
Monday: Collect blood from your regular abattoir/blood source. Blood feed a subset of the colony (2 cages (test vs. control) of 25-50 females each) and check for any mortality the following day. **(Caution should be taken if female mortality is >10%)**. Collect egg papers from the oviposition cups and replace with new papers. (These papers will have 2 batches of eggs from the 2 blood meals from	Feed larvae and check for any pupation. Collect pupae if present and place them into a new cage labelled with today's date, strain name, and generation # (Prepare hatch solution and leave until Wednesday).

the previous week). Let the eggs slowly dry over 3-4 days, then dry the papers completely and store for 10-14 days before hatching.	
Tuesday: Blood feed the colony	Feed remaining larvae.
Wednesday: No attention required	Collect remaining pupae and larvae. (Hatch eggs and pan next L1 larvae in rearing pans and add larval diet and restart larval rearing cycle)
Thursday: Blood feed the colony	Pupae emergence. Enter the adult rearing cycle with first blood feeding on following Tuesday.
Friday: Check sugar feeders.	
Saturday/Sunday: No attention required	
Week 3:	
Monday: Collect blood from your regular abattoir/blood source. Blood feed a subset of the colony (2 cages (test vs. control) of 25-50 females each) and check for any mortality the following day. **(Caution should be taken if female mortality is >10%)**. Collect egg papers from the oviposition cups and replace with new papers. (These papers will have 2 batches of eggs from the 2 blood meals from the previous week). Let the eggs slowly dry over 3-4 days, then dry the papers completely and store for 10-14 days before hatching.	
Tuesday: Blood feed the colony	(Blood feeding #1)
Wednesday: No attention required	(Hatch eggs and pan next L1 larvae in rearing pans and add larval diet and restart larval rearing

	cycle)
Thursday: Blood feed the colony	
Friday: Check sugar feeders.	
Saturday/Sunday: No attention required	
End of cycle:	
Monday: Recover egg papers and close cage/cohort. Wash cages and sugar feeders, and freeze oviposition cups to kill any loose eggs. Let the eggs slowly dry over 3-4 days, then dry the papers completely and store for 10-14 days before hatching.	

NB: If different strains are being reared in the same insectary, hatching of morphologically similar strains can start alternating weeks to avoid confusion and cross contamination.