Standard Operating Procedure for maintaining *Aedes aegypti* and *Aedes albopictus* colony mosquitoes

Effective Date: 27 October 2022

SOP #: CAE-2022

PacMOSSI
Pacific Mosquito Surveillance Strengthening for Impact

Scope

This SOP describes the steps involved for rearing *Aedes aegypti* or *Aedes albopictus* mosquitoes.

Overview

Description: This protocol begins with egg laying and lists the steps throughout the mosquito life cycle to maintain a colony through multiple generations. The mosquito specimens could be used for testing product efficacy or answering other research questions. Parts of this protocol may need to be followed to rear field collected specimens for insecticide resistance testing.

Target species: *Aedes aegypti* and *Aedes albopictus*, adaptations may be required if rearing other *Aedes* species.

Advantage: Rearing mosquitoes in colony provides simple access to specimens that are standardized by species, age and sex.

Disadvantage: Requires basic insectary facilities.

Materials

For personal protection:

- ◯ Laboratory coat
- ◯ Nitrile gloves
- ◯ Safety glasses (when handling bleach, alcohol and blood)

For egg collection and hatching:

- ◯ Plastic container (for oviposition cup, ≈ 50-200 ml or 7 cm diameter)
- ◯ Ovistrip made from filter paper (Whatman, number 903) or cotton

For larval trays:

- ◯ White plastic trays (≈ 6 x 30 x 20 cm (h x w x d) or shallow bowls (≈ 32 cm diameter)
- ◯ Water (rain water or de-chlorinated tap water)
- ◯ Squirt (wash) bottle
- ◯ Ground fish food (e.g. TetraMin® Tropical Flakes)
- ◯ 50 ml plastic tube
- ◯ Plastic calibrated pipette (1 – 3 ml)
- ◯ Nets to cover larval trays (cut to size of bowl or tray)

For pupal cups:

- ◯ Plastic container (for pupal cup, ≈ 50-200 ml or 7 cm diameter)
- ◯ Sieve (100 micron)

Emergence cages:

- ◯ Adult mosquito cages (30 x 30 x 30 cm or larger)

Blood feeding:

- ◯ Water-jacketed glass membrane feeders
- ◯ Rubber hoses
- ◯ Mini aquarium pump
- ◯ Water bath
- ◯ Sterile, disposable 3 ml pipettes
- ◯ Human blood from a safe source
- ◯ Parafilm membrane
- ◯ Rubber bands

Sugar feeding:

- ◯ Squeeze bottle containing 10% glucose solution
- ◯ Cotton wool or filter paper
- ◯ Petri dish or glass flask

Cleaning cages:

- ◯ Aspirator (mouth or battery operated)
- ◯ 10% bleach
- ◯ Paper towels

General:

- ◯ Permanent marker e.g. Sharpie
- ◯ Scissors
- ◯ Towels
- ◯ Fine forceps
- ◯ Thermo-hygrometer

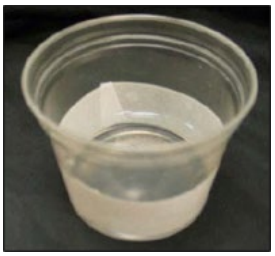
Laboratory setting

Photoperiod:
For laboratories with day/night timers, set the timer to 12 hours light and 11 hours dark with 30 minutes dusk and 30 minutes dawn. Following power outages, check and reset the timer.

Temperature/humidity:
Rearing temperature for larvae and adults should be 27°C ± 2°C with a relative humidity of 70% ± 5% in rooms holding adults. A damp towel placed on top of cages with adult mosquitoes can be used to maintain or elevate humidity.

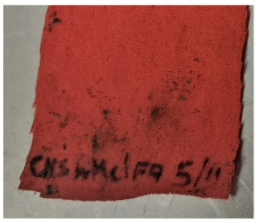
Egg collection

1. Line a plastic container (oviposition cup) with an oviposition strip made from filter paper or cotton.
    a. For filter paper, it should be ≈ 8 cm wide and at least as long as the circumference of the egg collection cup.
    b. For cotton ovistrips (measuring 5 x 10 cm), it can be pegged to the side of the container.
    c. Add water to the plastic container so that the ovistrip is half submerged.
    d. Ensure that the ovistrip is labelled with colony information and date.



Left image: MR4 Methods in *Anopheles* Research

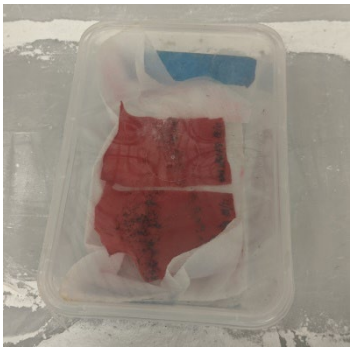
2. Place the oviposition container inside the adult mosquito cage two days following blood feeding of adult mosquitoes (when egg laying will begin).
    a. The mosquitoes will lay eggs on the oviposition strip just above the water line.



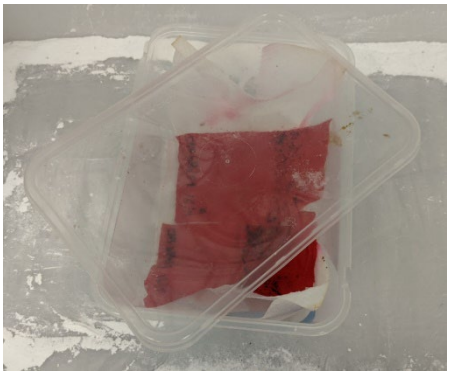
Left image: MR4 Methods in *Anopheles* Research

3. After 2 to 3 days, carefully remove the oviposition container from the adult cage.
    a. Remove the ovistrip from the container.
    b. Use forceps to remove any dead mosquitoes from the ovistrip.

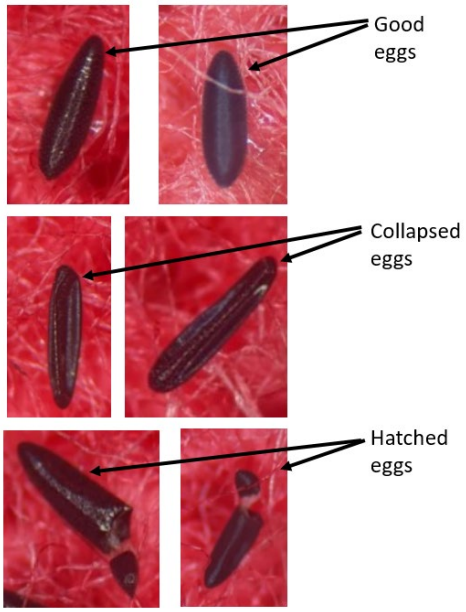
4. Place moist ovistrip inside take-away container lined with paper towel and close the lid.
    a. This time period allows the embryos to develop while still moist, termed condition the eggs



5. After 48 hours, undo the lid and leave it offset on top of the container to dry out the ovistrips.



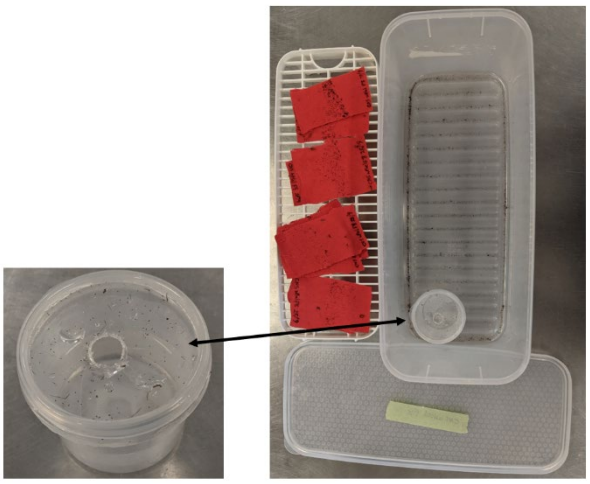
6. After another 48 hours, check the condition of the eggs using a dissecting microscope.
    a. If the eggs have collapsed (look like a deflated football) then they are less likely to hatch. This is a good indication that the ovistrips were stored in conditions that were too dry.
    b. If the eggs are missing their tops and a very small white larvae is visible then they have hatched which means that the strips were too wet.



7. **Now that the eggs have been properly conditioned they can either be:**
    a. Stored for months in a dry sealed container
    b. Hatched and reared for different purposes

8. To store the eggs, place the dried ovistrips inside a storage container on a rack.

9. To regulate the humidity in the storage containers, mix as much salt as you can into a small container with water (saturate the water with salt). Then place this container, with a small hole in the lid, in the bottom of the egg storage container.
    a. Close the lid tightly on the container and label it using a sharpie and tape.



Egg hatching

1. Estimate the number of eggs you want to hatch. This can be difficult as they are often very dense and some eggs may be laid on top of others. It helps to estimate the number of eggs in a smaller section of the ovistrip then multiple this by the number of those sections in total on the strip.

2. Measure out the required amount of dechlorinated water for hatching.
    a. In this photo, a bucket is used and the dotted line is used to indicate the level of 4L of water.

3. Yeast can be added to deoxygenate the water, which increases the hatch rate for *Aedes aegypti* and *Aedes albopictus*.
    a. Measure the yeast. For 4L of water use 0.7 g of yeast.
    b. Too much yeast will turn the water milky and increase the death rate of the hatching larvae.

4. Add both the eggs and the water with yeast to a larval tray for hatching.
    a. Many thousands of larvae can be hatched in one larval tray. Note that they will drown if they are unable to reach the surface if it is already blocked by other larvae.
    b. Label the larval tray.

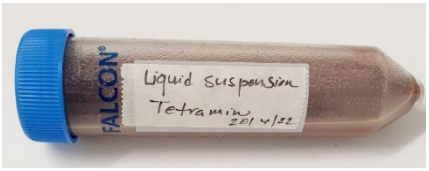

5. Leave the larvae to hatch for 24 hours.
    a. Make sure the larval tray is stored at the recommended temperature (27°C ± 2°C).

Rearing immatures

1. On the first day after hatching, split the larvae into consistent densities among a number of larval trays to prevent over-crowding.
    a. A standard ratio is around 150 larvae per litre of water.
    b. To pipette a high number of larvae in each attempt, gently swirl the water by stirring with a pipette so that the larvae get pulled into the middle of the tray in one clump (using a round tray is useful here). Only swirl larvae for short periods of time (<1 minute) as they may drown if kept underwater for too long.

2. Store the larvae trays securely in the insectary space at the recommended temperature.
    b. If trays are stacked, then sufficient space between them to allow light to reach all trays.
    c. Cover trays with netting to prevent any lose adult female mosquitoes ovipositioning in the trays.

3. Prepare larval food. Larvae can be fed ground fish food either directly or in a suspension. The suspension helps the food to distribute through the water column, which is where *Aedes* larvae feed.
    d. Grind fish food (such as TetraMin® Tropical Flakes) using a coffee grinder.
    e. Using dry food. Store in an airtight container in the fridge. One suggestion is to use two lids, with a square cut out of the lower lid. When serving, each spoonful can be leveled by sliding against the edge of the square.
    f. Using a wet suspension. Prepare the 10% (w/v) suspension in rainwater in a 50 ml tube (the ratio is 1 g of ground fish food for each 10 ml of water). Shake the tube vigorously to suspend the powder. Store in the fridge.



4. **Feed the larvae. Do not overfeed the larvae, as bacterial growth or scum may develop from uneaten food.**

   g. **Dry ground fish food. Sprinkle evenly on the water surface to avoid large clumps. Feed** a pinch of food (**300** mg or 1/16th tsp) **per 500 larvae, this is sufficient food for 4 days. Then on the 4th day after hatching, feed 700** mg (1/8th tsp) **of food to the late instar larvae.**

   h. **Wet suspension. Use a calibrated pipette to transfer the food. The feeding rates are outlined in the below table.**

5. **At 6 days after hatching, start to check for pupae.**

   i. **Check on the size and number of larvae. At this stage, the larvae should be L4 in size. There may also be some males which have already turned into pupae (circled in red).**

6. **At 7 days after hatching, pupae will need to be picked. By this stage, must immatures should be at the pupae stage. Pupae can be picked using a pipette, irrigation syringe or mesh spoon.**

   a. **One method is to swirl the water to draw the pupae into the middle of the round tray and draw them out with the large irrigation syringe, or mesh spoon.**

   j. **Ensure that there is enough water so that they can swim freely.**

Right image: Papua New Guinea Institute of Medical Research

An overview of the usual schedule for rearing immatures is presented here:

Day	Expected mosquito stage	Food per tray (200 - 400 larvae)
0 (hatch day)	Eggs	Yeast or 0.5 ml
1	I instar	0.5 ml
2	I instar	0.5 ml
3	I and II instar	1 ml
4	II instar	1 ml
5	II and III instar	1.5 ml
6	III and IV instar	2 ml
7	IV instar and pupae	Pick pupae and feed 2 ml
8	Mostly pupae, few IV instar larvae	Pick pupae and feed 1 ml
9	Mostly pupae	Pick all remaining pupae

Setting up adult cages

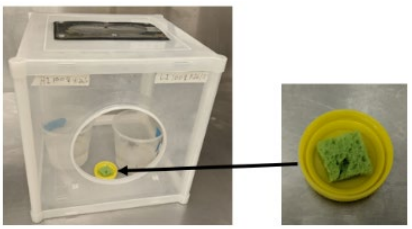
1. Construct the adult cages.
    a. Commercial cages are usually 30 x 30 x 30 cm, but can also be constructed from local materials.
    b. Label the cages with the date and other relevant information.


Right image: Papua New Guinea Institute of Medical Research

2. Place the pupal cups into the bottom of the cage (without mesh or a lid on the top).
    a. Up to 1,200 pupae can be added to each cage.
    b. Remove the pupal cup after 3 days when the pupae have emerged.

3. Provide a sugar source for the adults that emerge, usually a 10% sugar solution (made by mixing 1 g sugar per 10 ml of water). There are different methods for providing sugar:
    a. A sugar wick can be made from a glass jar containing the sugar solution.
    b. Cotton wool soaked in the sugar solution can be placed directly on top of the cage, and is usually covered with a petri dish (to minimize drying out).
    c. A small sponge soaked in the sugar solution sitting in a small container, can be placed directly into the cage.





Top right image: Papua New Guinea Institute of Medical Research

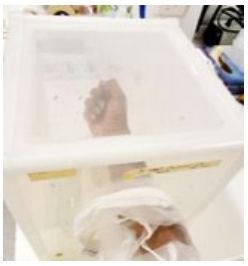
4. Store the adult mosquitoes at the recommended temperature (27°C ± 2°C) and humidity (70% ± 5%), in a safe location.
    a. A damp towel can be placed on top of the cages to keep the humidity high.
    b. Always ensure that the entrance sock is fitted securely and closed properly.

Blood feeding

1. To maintain the colony over multiple generations, the females must be blood fed to produce eggs. Blood feeding is usually performed 6 days after setting up the cage, when the females are at least 3 days old and most likely to blood feed. This can be achieve by directly feeding on humans or by membrane feeding, as follows:

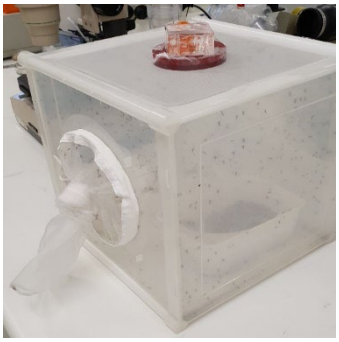
Directly feeding on humans.

a. When using humans to directly blood feed, but the mosquitoes and the person should be free of pathogens.
b. The volunteer exposes a limb (leg or arm) to the mosquitoes by resting it directly on the outside of the cage, or placing inside for 10 minutes per cage.
c. Ethics approval should be sought.



Membrane feeding.

d. Pour 20 ml of defibrinated or heparinized animal blood into a petri dish or water-jacketed glass membrane feeder. Cover with parafilm.
e. Ensure blood is free from pathogens.
f. The blood feeder is placed on top of an adult cage with the parafilm surface facing down towards the mosquitoes.
g. The blood is kept warm (37 to 40°C) by using a water bath to circulate heated water past the blood, or placing a heated gel pack or small cell culture flask filled with hot water (≈50°C) on top of the petri dish with blood.



Bottom image: Timinao et al. 2021. Parasites and Vectors

2. After blood feed, place an oviposition cup in the bottom of the cage, and then repeat the rearing cycle as needed to maintain the colony.

Additional notes:

- When opening and closing adult cages, always be careful to prevent mosquitoes from escaping.

- If the eggs are collected, stored and hatched properly you should expect a hatch rate better than 80% (at least 4 out of every 5 eggs laid should hatch).

- Maintain all of the mosquito stages (eggs, larvae and adults) at the recommended temperature (27°C ± 2°C) in a space that is safe from other animals interfering with them.

- Mosquitoes that are 3 – 5 days old can be used for insecticide resistance testing (if F0 or F1) or for monitoring product efficacy (if it is known that they are not resistant to the insecticides to be tested).

- Dispose of all solid and liquid waste according to your laboratory guidelines.

- Prevent eggs and larvae escaping through the sink to sieving (100 micron) liquid waste or by killing an immatures by pouring boiling water over eggs and larvae.

- To destroy any unwanted adult mosquitoes, place the entire adult cage in a freezer for an hour. Then dismantle and clean before reuse.

- All equipment, including sponges are washed using a very weak bleach solution.

- Ensure that the mosquitoes are kept away from heat sources such as direct sun

- Ensure that the mosquitoes are kept in a location that is free from ants. Ant traps can be set around the legs of tables, for example table legs can be placed into a shallow dish containing water or talcum powder that the ants cannot cross.

Safety

Contracting mosquito-borne disease if performing human blood feeding

There is a potential for mosquito-borne diseases to be transmitted if an infected person blood-feeds a colony prior to another person. Ensure that you consider and control such risks adequately and in adherence with the policies of your institution.

Having a sensitivity if performing human blood feeding

Some people may be highly sensitive to blood-feeding colonies. Use soothing creams or antihistamines after blood feeding to minimise any reactions.

References

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Benedict MQ. (2010) MR4 Methods in *Anopheles* Research. 2 edition. CDC Atlanta GA, USA.

Timinao L, Vinit R, Katusele M, Schofield L, Burkot TR, Karl S. Optimization of the feeding rate of *Anopheles farauti* s.s. colony mosquitoes in direct membrane feeding assays. Parasit Vectors. 2021;14(1): 356.

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