# JHMRI Insectary Anopheles Rearing Protocol (Updated Nov 2016)

Ref: Chris Kizito

Mosquito rearing is done in climate controlled walk-in environmental growth chambers (GCs). The institute has seven new chambers and two old ones. Each of these GCs is fitted with automated controls that regulate climate variables (i.e., temperature and humidity). Six of the chambers are used for uninfected mosquito rearing. Chamber W4713FZ1, is used for housing *Plasmodium berghei* infected mosquitoes and other experimental cages is maintained at 19.50 C. All other chambers are maintained at 270 C except when a change is requested or needed for a specific reason. All chambers are set to maintain 80% humidity. The climate settings are monitored daily and a log is kept outside all chambers. The chambers are set at a light: dark photo period of 14:10 hours with a 1 hour ramping period. A manual over- ride switch is available in case access is needed during the dark period but it must be turned off on exiting the chamber to switch back to the automated controls. The GCs are accessed from the laboratory preparation (“Prep”) areas.

To avoid cross contamination between species, an effort in made to isolate species as much as possible. Where possible each chamber houses only one species. Do not mix up species.

In addition to the walk-in growth chambers, JHMRI has ten reach-in incubators. You may request to use any of these walk-in incubators at your own environmental settings. Always clean the incubator after use.

*Plasmodium falciparum* and Sindbis infected mosquitoes are housed in the walk-in chambers or reach-in incubators located within the more secure sub-rooms of Room W4710. Extra access required for entry.

* You must always double cage your *pf*-infected mosquitoes;
* To prevent accidental escape of infected mosquitoes do not use sucrose bottles in cages with *pf*-infected mosquitoes. Use sugar cubes or wet cotton pads;
* No live *pf*-infected mosquitoes shall be taken out of Room W4710. All work on these mosquitoes must be done inside this area.
* Use the provided freezer to kill all unused mosquitoes still in cups or cages.
* Do not dump **any** mosquitoes or their carcasses into the sink. Either freeze or properly wrap in a plastic bag before damping into the biohazard box.

The insectary is charged with ZERO TOLERANCE for loose mosquitoes at all times and in all areas to the best of our ability. Light traps are located in all areas of the insectary for trapping all flying insects including any loose mosquito. All live mosquito handling must be done inside the GCs. Mosquitoes out of the cages for experiments in the lab areas must be immobilized in 70% ethanol or on ice. It is your responsibility to look for and kill any mosquitoes you lose. Call for help if needed but do not just walk away. Clean up your work area of any dead mosquitoes and trash. All dead mosquitoes must be safely wrapped and placed in the freezer.

Entrance to the Insectaries is restricted only to authorized persons by an access card key.

**GENERAL ANOPHELENE REARING AND HANDLING.**

**MATERIALS USED**

1. Metal cages. Large size (8” x 8” x 8”) and small size (6” x 6” x 6” )
2. Small glass bottles for sucrose solution.
3. Cotton.
4. Paper towels
5. White plastic photo trays (10” x 12” x 2”)
6. Pupae aspiration systems
7. Adult picking vacuum apparatus
8. White nylon mosquito tray cover nets
9. Small plastic dishes for pupae
10. Larval food
11. 50ml beakers or plastic cups for egg collection

**REARING PROCEDURE**

1. ADULTS: Adult mosquitoes are kept in metal cages which are washed and disinfected with bleach. The base of the cage must be lined with a paper towel to absorb moisture and blood. Up to 500 adults may be kept in the large cages (8”x8”x8”) while the small cage (6”x6”x6”) can keep up to 200. Adult mosquitoes are maintained on 10% sucrose solution in small bottles or soaked cotton pads or sugar cubes placed on top of the cage. The sucrose bottles in the colony cages must be changed every week. Cages are labeled to show type of species, date those mosquitoes were pupae or date of collection. Assigned investigator cages must include the investigator’s name and date of experiment.

1. BLOOD FEEDING ADULTS FOR EGGS
 Adult colony cages are checked daily to make sure the mosquitoes are healthy and they have sucrose. It is also important that the cage has enough female mosquitoes to supply enough eggs. Adults will begin mating within 12 hrs after emergence.

 We prefer to give a fresh colony cage a blood meal for eggs at least two or three days post emergence. Clean (uninfected) mice are used as the source of the blood meal. Depending on the number of mosquitoes in the cage, two or three mice may be adequate for each cage. Top feeding is used, where the anesthetized mouse is placed on top of the mosquito cage. This eliminates the potential for mosquito escape during feeding.

Mice are anesthetized using an anesthetic solution (ketamine 20mg/ml and prochloroperazine 1mg/ml made up in 0.9% saline). About 0.15ml – 0.25ml is used depending on the size of the mouse. To avoid over bleeding the mice, feed for not more than twenty minutes. However, more time could be necessary if the mosquitoes have not fed well. The cage is labeled with the date of feeding. The mouse cage is also dated. Each mouse is allowed at least a two week period before being used to feed again. The mosquitoes will be ready to lay eggs two days after getting the blood meal.

1. COLLECTING EGGS
Two days after feeding, the mosquitoes are offered a damp filter paper over an oviposition cup to lay eggs. A 50ml plastic cup or beaker is half-filled with distilled water and lined with a cone shaped filler paper (sizes may vary). The mosquitoes will lay eggs onto this moist surface. This method makes it easier to remove the eggs and also to pre-treat the eggs with the 10% bleach before transferring to trays.

1. HATCHING THE EGGS (Day 1)
	1. Preparing the hatching trays: To clean trays, add distilled water up to 2/3 capacity. Transfer the tray to a rack or stable position. Trays should not be moved after adding food and eggs. After water has settled, add ground larval food plus two pellets of cat food. (Depending on availability the type of food used may vary but if available, Purina Indoor cat chow is currently used at JHMRI).
	2. To prevent mould, fungal or bacterial contamination, the eggs are and sterilized with 10% bleach while still in the oviposition cup. Pipette the diluted bleach over the eggs on the fitter paper. Add the bleach starting from the top and around the filter paper to ensure that the filter paper is also decontaminated. Keep the eggs in contact with the bleach for 3-4 minutes. After treatment rinse the eggs with distilled water and if possible remove any dead mosquitoes with a pair or forceps. Most of the eggs will be seen to sink in the water while rinsing.
	3. Transfer the filter paper into the hatching tray or using a wash bottle wash off the eggs from the fitter paper into the tray. Label the tray with a tape indicating species and date the tray is started. This is day one of the batch.

1. DILUTION: Day 4

Overcrowding inhibits larval growth. The young larvae are split up or diluted into smaller numbers to make as many trays as necessary. Good larval density is maintained at about 0.3 larvae/cm2 which makes it to about 300 to 350 larvae per tray in the 10”x12”x2” trays. The water level is maintained at not more than 2cm deep to save the larvae long distances to the surface to breath. A turkey buster or a plastic pipette may be used to dilute the larvae. After dilution, add a little ground larval food and two or pellets of cat food to each tray. Larval feeding is then maintained at two pellets of cat food per day (or alternative food source) as found necessary. Generally, first instars would need about 0.1 mg/larva and the later instars would need about 0.3 mg/larva. Too much food must be avoided as it causes water contamination and eventual suffocation of the larvae. A good indicator of this condition will be very cloudy and slimy water in the tray.

1. CHANGING WATER: Day 8 or 9

Water in the larval trays is changed every four to five days after dilutions; this helps to get rid of the polluted water, which normally inhibits Anophelene growth and kill the larvae. An adequate sieve (we use size 40) is used to filter out the larvae. The tray content is poured into and the larvae collected over the sieve. Rinse the tray with water and add the larvae back. Take a good look at the density in the tray again. To minimize larval mortality and maintain good growth, you may add some little ground food in addition to the pellets after changing the water. Two cat food pellets are added at this point. Add another 2 pellets the following day and one or none on the third day. Maintain the same amount of water in the tray (2cm).

1. COVERING TRAY WITH NETS: Day 9 or 10:

If adults are to be collected instead of pupae, the trays must be covered with nets after changing water or once pupae begin to appear in the trays.

1. THE PUPAE (After day 9 or 10) AND COLLECTING ADULTS:

With healthy larvae, pupation may start anytime after day 9 or 10 and eclosion will occur in 24 hours except for some species like *A. freeborni* and dirus which may take up to 48 hours.

* 1. Pupae Collection: Pupae are collected daily either by hand picking or any other available method. The pupae are then carefully washed and transferred into small plastic emergence dishes or cups which are then placed in the cage.
	**Pupae collection must be done daily with this method**.
	2. Net Covering and Adult collection: This is the main method currently used at JHMRI. The trays are covered with nets the day pupae begin to appear in the trays. Adults may be collected as needed or until up to 80-90% of the pupae have eclosed. Care must be taken to make sure the trays are completely covered to avoid adult from escaping. Growth chambers have adult collection vacuum systems where the empty cage in placed into a vacuum box and the adult mosquitoes are sucked up directly into the cage from the tray. The vacuum is set not to exceed a certain marked limit beyond which adults are easily killed. **New users must first receive training before using this system.**
	\*It is possible to accidentally set loose some mosquitoes in the process of picking up the adults. Maximum care is needed to minimize this. All areas of the insectary are provided with a bottle of 70% ethanol to knock down loose mosquitoes in case of accidental loss”.
1. DIRTY EQUIPMENT : CAGES,TRAYS, SUCROSE BOTTLES
* All **used cages** with the live mosquitoes are placed in the -200 C freezers located in the hallway and in W4710 from where they are picked up for cleaning. Please do not clean cages yourself.
* **Used sucrose bottles** must be removed from the cages before placing cage in the freezer. Remove and damp the cotton in the biohazard box, rinse the bottle and take the dirty bottles the wash room W4713.
* **All dirty/used trays** must be taken to the wash room, W4713. You may not clean the trays yourself.
* **Nets**: **Please leave all used nets inside the chambers**. DO NOT take nets to the wash room. If you used nets and they are dirty, you must bleach the net for a few minutes, rinse and hang to dry inside the chamber.
* Always clean up after yourself.