Chapter 1: *Aedes* Laboratory Biology and Culture

1.1 Behavior and Physiology of Aedines in the Laboratory, Adapted from (Clements 1992)

Introduction

Aedes species of mosquito are not only important vectors of Yellow fever, Dengue, Chikungunya and Zika viruses, but some are also easily cultured and robust laboratory model species. Their behavior and physiology are important to understand in making decisions in the insectary and can affect choices of food, blood, egging, your choice of insectary supplies, insectary space demanded and much more. Additionally, understanding more about the differences between stocks can be used to give clues of possible contamination when you are keeping multiple strains or species. These tips can also be practical in understanding why mosquitoes are not thriving or behaving as predicted.

Eggs

Culex, *Aedes*, and *Anopheles* eggs are laid in different patterns; observing the patterns when collecting eggs can be one way of catching a cross-genus contamination event early. *Aedes* typically lay their eggs on a surface, unattached to one another and without floats, and on a substrate above the water (**Figure 1.1.1** and **1.1.2**).

Aedes eggs survive drying well (**Figure 1.1.2**) though the amount of time they can be kept dry prior to hatching varies with the species and conditions of storage. Some species and/or strains can be kept dry as much as six months prior to hatching while others require moisture to remain viable.

Figure 1.1.1. *Aedes albopictus* eggs 48 hours post oviposition on seed germination paper.

Figure 1.1.2. *Aedes aegypti* eggs 2 weeks post oviposition stored under insectary conditions.

Larval Feeding

In the wild, mosquito larvae survive in a large variety of habitats. The food types in these habitats are similar to that in the insectary in that they contain microorganisms, detritus (particulate organic matter), biofilm, and other organic matter such as dead invertebrates. A major source of nutrients for mosquito larvae comes from plant material that has been already degraded by fungi or bacteria. These can be substituted with various commercially available products such as dog, cat and fish food as well as individual ingredients such as liver powder and yeast. In the absence of other nutrient sources, *Aedes* species can consume other larvae in the tray.

In *Aedes*, **collecting-gathering** is a more common method of feeding, which involves first disturbing materials that have settled or are attached to surfaces to cause them to resuspend and then ingesting them from the resuspension mixture. Other methods of feeding include **scraping** (removal and ingestion of the biofilm and protists on the surface of submerged plants and other surfaces), **shredding** (biting off small fragments of plants or dead matter), and **predation** (eating other insects). Much of the differences seen in feeding preferences can be attributed to differences in mouthparts and head structures (**Figures 1.1.3** – **1.1.5**). More detailed information of the various structures can be found in Clements' The Biology of Mosquitoes.

Figure 1.1.3 Representative *Aedes* head and mouthparts.

Figure 1.1.4 Representative *Anopheles* head and mouthparts.

Figure 1.1.5 Representative *Culex* head and mouthparts.

Aedes larvae typically feed throughout the water column (**Figure 1.1.6**) whereas *Anopheles* prefer feeding on the surface or in shallow water (~ 1 cm).

Figure 1.1.6 *Culex* (pictured) and *Aedes* larvae feed throughout the water column.

Figure 1.1.7. *Aedes aegypti* larvae and pupae. Note how the larvae and pupae tend to cluster together.

The size of the particle that larvae can ingest increases with the size and age of the larva. Factors such as size and age should be taken into consideration when determining which larval food to use. Also, as larvae grow, the amount of food they will eat increases by as much as 5 times what they ate in the first instar.

Growth and Development

Intrinsic Effects

Mosquito larvae have four stages. The body size changes continually while the head capsule increases (mainly) only at molts i.e. saltatorially while the thorax and abdomen increase continuously. Thus, the instar is best determined by the head capsule size (Timmermann and Briegel 1993). Performing some measurements on the head capsule of your species to determine the range of values that could be observed in any stage is a good idea if working with an exact stage is important for your research project. A series of photographs of stages of larval growth over time, and regular observations for exuviae (molted cuticle) might make it easier for staging to be apparent by eye until you become familiar with your particular stocks and strains.

Generally, males develop faster and are smaller as adults than females. Males also typically spend less time in the pupal stage before emerging than females (Haddow et al. 1959; de Meillon et al. 1967). The degree of sexual size dimorphism varies between stage and species. Aedines are typically sexually dimorphic, therefore smaller pupae will be males and larger pupae will be females (**Figure 1.1.7**). This difference is very distinct in *Aedes aegypti* but considerable overlap exists between males and females of *Aedes albopictus.* Size variation also is dependent on nutrition availability for larval states; maximum size differentiation will be more readily achieved with pupae that have received sufficient nutrients as larvae.

Figure 1.1.7. *Aedes aegypti* pupae: two males (right) one female (left). Size disparities are apparent.

Extrinsic effects

Temperature

Temperature is the most important and easily controlled extrinsic factor affecting growth rates of larvae. The effect of temperature on the growth of mosquito larvae has been studied extensively. For each species, there is a temperature range in which development can occur. Within this range, growth and development vary dramatically with the temperature fluctuations. For this reason, it is important to control temperature to achieve predictable culture. Typical larval water temperatures are in the range of 26-28°C.

Nutrition

The amount of available food significantly affects larval growth. Underfeeding can cause as much delay as overfeeding but will likely be evident later, especially in the adult stage. Because the adult size is determined by the larval size, control of the amount of food is important. A good rule of thumb is to feed larvae only as much as they eat during a typical work day. As they grow, the larvae may need to be fed more than once per day.

Larval Density

Achieving the right density is very important in growth and development. The most common problems associated with over-crowding are: longer development time, reduced pupation and eclosion, and a decrease in pupal

weight. See Chapter 1.3.2 Culture section for more on proper density for aedines. Good concentrations of *Aedes* are about 1 larva/ml so if one expects to rear different amounts of strains, adjusting the number of larvae reared in trays, or acquiring several sizes of containers is expected.

Effect of Larval Health on Adults

Adults from larvae that were crowded are typically smaller and less fecund. The ultimate size of an adult mosquito will be based on genetics in combination with the environmental conditions experienced through development. Studies have shown that larvae that are reared in crowded conditions have reduced weight at emergence, take smaller blood meals and lay fewer eggs. Poor larval conditions cannot be totally overcome by good diet or care in later stages, therefore careful attention to larval conditions throughout development determines high overall quality of production.

Environmental effects on rhythms

Studies show a link between environmental factors and ecdysis. These studies are limited to certain species and conditions; however, the evidence supports the shifting of ecdysis under temperature changes, light/dark cycles, and larval stress such as salinity. For example, researchers found that in continual darkness, with variable temperature cycles, the larval-pupal ecdysis was more likely to occur during the warm phase.

The time of day/night that ecdysis will occur is species-dependent. It is thought that the trigger to molt is switched on and off based on a daily rhythmic activity cycle of 24-hour intervals that is exhibited by many organisms, or a circadian rhythm. This is not universal among all mosquitoes. Examples of some found not to have such a rhythm are *An. quadrimaculatus* (Nayar and Sauerman 1970) and *Ae. aegypti* (Haddow et al. 1959). If your insectary has problems with ecdysis being temporally irregular or extended over several days, experiment with your light cycle conditions. Light and temperature are the key factors. Typical conditions are 12:12 h light and dark, with some mechanism to provide a gradual sunset/sunrise transition.

Adult feeding

Plant Juices

Both adult male and female mosquitoes will drink plant juices as an energy source (see review in (Foster 1995)). Plant sugar is the major food resource for mosquitoes. In the wild, the most common source is floral nectar, but other sources exist such as damaged fruit or vegetative tissue. With these different meals, the mosquito would be receiving largely sucrose, fructose, or glucose, depending on the source. Other sources such as maltose or melibiose are seldom found in mosquitoes. Amino acids needed for ovary development can be found in some nectar, but the concentrations are not high enough to replace a blood meal. Natural sugar sources can have a wide range of amounts of sugar from none to 50% w/v, though 20-50% w/v is the normal range. Mosquitoes have been seen ingesting crystallized sucrose by liquefying it with saliva.

Blood

Only females take blood meals. Blood is the essential resource for protein and for most species is essential for egg development. For some species, blood also provides a source of energy since blood fed females have been shown to survive longer than females given only water. It is very common for a mosquito to take as much as 2-4 times their weight of blood in a single meal. Via diuresis, females can also excrete clear to reddish fluid while blood-feeding in order to concentrate the protein as much as 2-fold.

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1.2 Considerations for housing Aedes aegypti *and* Ae. albopictus *in the continental United States*

Aedine mosquitoes are important as vectors of several viral pathogens including West Nile Virus (WNV), Dengue Virus (DEN), Chikungunya Virus (CHIK), and the now emerging Zika Virus (ZIK). Historically, *Ae. aegypti* ranged throughout most of the Southern United States, however their range was reduced to the extreme southeast during the yellow fever eradication program (Eisen and Moore 2013). After the yellow fever eradication program ended, *Ae. aegypti* began to move back into its former native territory. Range expansion was hampered by the introduction of the non-native *Ae. albopictus* which, during initial phases of invasion, was able to out-compete *Ae. aegypti*. However, *Ae. aegypti* have adapted and have begun to move back into the former range and more importantly it is appearing in areas not previously considered to be conducive to its growth and survival (Porse et al 2015). During the last century, *Ae. albopictus* invaded and subsequently swept across the eastern United States in a rapid manner. Like *Ae. aegypti*, *Ae. albopictus* has mainly been found in the eastern part of the United States with occasional importation into ports in the west.

Both species are critically important vectors exquisitely adapted for living in association with people, qualities that make them excellent candidates for invasion. As with all vectors care should be taken to contain these species in non-native ranges..

Figure 1.2.1: The most recent estimate on the range of *Ae. aegypti* in the United States. Graph courtesy of the Centers of Disease Control and Prevention

Figure 1.2.2: The most recent estimated range of *Ae. albopictus* in the United States. Graph courtesy of the Centers for Disease Control and Prevention.

References

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1.3 Aedes *Culture*

1.3.1 *Aedes* Eggs

MR4 Staff

Introduction

For colony maintenance, blood feeding females may only be done once in their lifetime, between 3 and 7 days post-emergence. After subsequent feedings, aedines will lay eggs again, which is helpful for having eggs to store for future use and having plenty of experimental material. After blood-feeding, allow 2-3 days for embryo development prior to hatching. Certain species may take more time, so you may have to modify your schedule according to the needs of your particular colony.

Among the water types we have used, we have observed no effect on oviposition. While there are almost certainly measurable differences that would be important in mass rearing facilities, an excess of eggs beyond what is required for stock keeping is almost always obtained, and efficiency is not an issue.

Collecting Aedes *eggs*

Most eggs are laid at night, and egg dishes are typically removed either the following day, or up to two days later. Some species, such as *Ae. albopictus*, must have their egg dishes removed promptly otherwise larvae will hatch on the moist filter paper (**Figure 1.3.1.1**). In contrast, an oviposition dish can be left in a colony of, *Ae. aegypti* for several days to increase the number of eggs collected. There are several ways to collect eggs. A good general practice is to fill a cup with clean water to about 1 cm depth and line the edges with seed germination paper such that the lower edge of the paper is submerged (**Figure 1.3.1.3**). The filter paper prevents the females from ovipositing on the moist plastic sides of the cup. Ovipositional substrate should be removed, the egg sheet dried to appropriate moistness, and all adult carcasses removed to prevent infections in the new larva pans. Adults can be removed individually with forceps or rinsed off the paper by washing the egg papers gently with distilled water.

Egging and Egg Storage

1. Fill a 500ml cup with 100ml of water.
2. Line the edges of the cup to the brim with seed germination paper; make sure the bottom edge is in contact with the water to prevent the paper from drying (**Figure 1.3.1.3**).
3. Remove cup 1 – 3 days later (**Figure 1.3.1.4**). For *Ae. albopictus* it is recommended that the cup is removed the next day to prevent larvae from hatching and perishing on the moist germination paper.
4. Drain off excess water and remove dead mosquitoes with fine tip forceps.
5. For *Ae. albopictus*, leave the cup, with the lid slightly ajar, in the insectary to allow the egging papers to dry rapidly. For *Ae. aegypti* leave the cup with the lid closed for two days. If *Ae. aegypti* are dried too soon, the eggs will collapse and no larvae will hatch (**Figure 1.3.1.5**). Then open the lid slightly and allow the egging papers to dry within the insectary.
6. Once dried, examine the eggs under a dissecting microscope to ensure they are not collapsed, then place egging papers into a plastic zip-top bag and store (**Figure 1.3.1.5**). **OPTIONAL** For *Ae. albopictus*, a slightly moist cotton ball can be added to keep humidity levels high enough so eggs do not desiccate. Make sure to place the egging date on the outside of the bag.
7. Eggs properly dried and stored, even without the addition of a moist cotton ball, can be hatched with high success rates for up to two months. After two months the rate of return decreases with less than 50% hatching after six months of storage.

Figure 1.3.1.1. Poorly dried *Ae. albopictus* eggs. Note that a majority of the larvae have hatched and perished.

Figure 1.3.1.2. Properly dried *Ae. aegypti* eggs. Note that the eggs appear full and have a crystaline exterior.

Figure 1.3.1.3. Assembled oviposition cup for *Ae. aegypti and Ae. albopictus*. The sides are lined with seed germination paper.

Figure 1.3.1.4. An oviposition dish after 24 hours. Note the line of eggs at the bottom indicating how deep the water was in the cup when placed into the cage.

Figure 1.3.1.5. These *Ae. aegypti* eggs were dried to soon after removal from the cage. Note the concave appearance.

Surface Sterilizing Eggs (adapted from Dadd and Sneller 1977)

This technique was developed as part of an aseptic rearing protocol and is best used on eggs stored for longer than one month (Dadd and Sneller 1977). Eggs that are younger than one month may hatch during the sterilization process and perish due to the harsh nature of the sterilizing solution.

1. Brush eggs from filter paper into a small embryo collection cup (**Figure 1.3.1.6**) with a mesh bottom using a stiff paintbrush.
2. Place the collection cup into a 70-85% ethanol solution for 1 minute (**Figure 1.3.1.7**)
3. Before transferring to the next cup, remove all excess ethanol by placing the collection cup onto a paper towel.
4. Place the collection cup into a 9cm Petri dish containing the surface sterilizing solution: 9ml Roccal ®, 5ml bleach, and 36ml water. Soak the embryos for no more than 3 minutes.
5. Remove the cup and gently rinse with sterile water before placing the collection cup into a sterile water bath. Repeat this 2 times (**Figure 1.3.1.8**).
6. Rinse the eggs into a clean 9cm Petri dish and cover with sterile water. They are now ready to be hatched using the *Long Term Storage Hatching Protocol.*

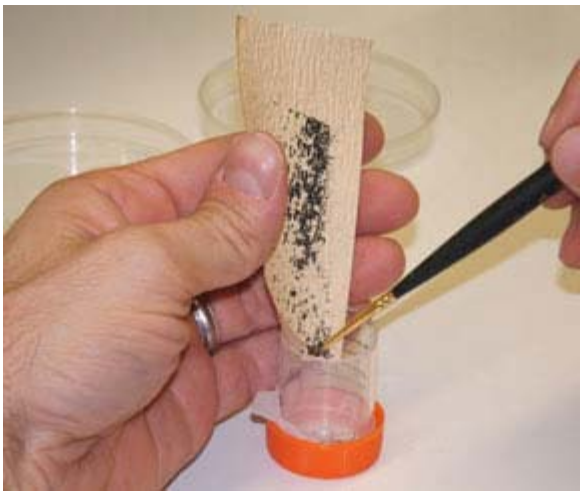

Figure 1.3.1.6. Brushing eggs into a modified 50ml Falcon tube. The top of the tube cap has been removed so a 40 micron mesh can be placed over the opening and held in place with the remaining cap ring.

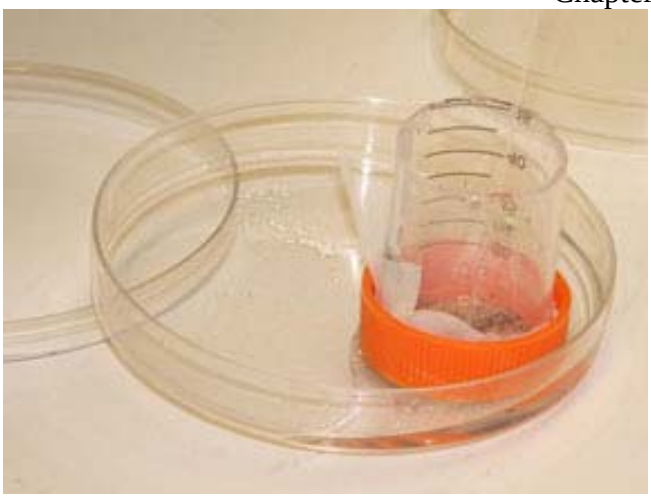

Figure. 1.3.1.7. Soaking the eggs. The soaking dish is slightly elevated by placing the top of the dish under one end.

Figure 1.3.1.8. Rinse with sterile water for a prolonged period of time to ensure all of the sterilizing solution has been removed.

Egg Hatching Protocol

From short term storage (adapted from Duman-Scheel 2010)

This method is for eggs stored longer than 3 days, but less than 1 month.

1. Fill 500ml cup with 375ml water.
2. Add 5ml liver powder slurry.
3. Cut a piece of egg paper containing approximately 300 embryos and place into the dish
4. After 1-2 days, transfer larvae with a pipette to a larger rearing pan.

From long term storage

This method is for eggs stored from 1 – 3 months. Note that eggs stored longer than 3 months will have low to negligible hatch rates and therefore eggs should not be stored longer than this period (Morlan et al 1963).

1. Place a large strip of eggs into a 9 cm petri dish (1000 eggs or more) (**Figure 1.3.1.6**).
2. Add small amount of water to barely cover the egging paper
3. Place into a vacuum (without heat) and set to 15-20 inches of mercury for 15 minutes.
4. Release vacuum valve and let the eggs rest in the oven for 30 minutes to 1 hour.
5. Check to see if eggs hatched; if so transfer egging papers and larvae into a larger rearing pan.

From long-term storage (*Ae. albopictus* – Nuris Acosta, Carlos Esquivel, and Dr. Peter Piermarini)

This method was developed in Dr. Piermarini's laboratory to overcome the issue of low hatching rates in *Ae. albopictus* eggs stored for over three months. Perform the initial hatch as described above; if low-hatch rates are seen, then try the following.

1. Remove the original egging papers and allow to fully dry under insectary conditions.
2. Place a large strip of eggs into a 9 cm petri dish (1000 eggs or more) (**Figure 1.3.1.6**).
3. Add small amount of water to barely cover the egging paper
4. Place into a vacuum (without heat) and set to 15-20 inches of mercury for 15 minutes.
5. Release vacuum valve and let the eggs rest in the oven for 2-3 hours.
6. Check to see if eggs hatched; if so transfer egging papers and larvae into a larger rearing pan.

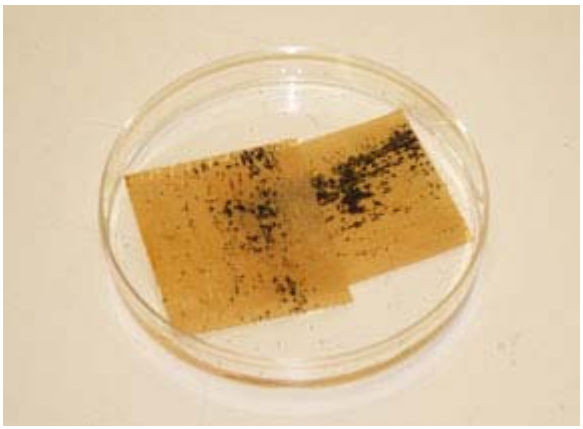

Figure 1.3.1.6. Strips of seed germination paper with adhered *Aedes* embryos. The amount of water is kept to a minimum to encourage hatching.

References

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1.3.2 *Aedes* Larval Culture

MR4 Staff

Introduction

Of the species *Aedes* most often in culture, these are generally considered less demanding to culture than are Anopheles species. Conversely, as is the case with anophelines, the number of eggs laid per female is in fairly direct proportion to their adult weight. Adult size is ultimately determined by larval size and larval culture. So, it is logical that larger larvae yield females that produce higher numbers of eggs – a desirable outcome in most laboratory cultures. Consistent use of successful practices for egg hatching and larval culture, developed and proven in a lab, should be adhered to faithfully.

Larval diets and preparation

The MR4 laboratory uses finely ground Koi Staple Diet from Drs. Foster and Smith, though TetraMin flake food is a widely used alternative. A 1% liver powder slurry has also been used by many laboratories that specialize in rearing *Aedes* (Duman-Scheel et al 2010). For large scale production, where cost may be a greater consideration, inexpensive and readily available diets such as Farex baby food, hog chow, and dog chow have also been used. Caution should be taken when using liver powder and various chow diets as the high fat content can lead to scumming on the surface of the water.

Koi pellets and analogous pellet or flake diets can be prepared in a grinding mill or blender and sifted through a 250 micron sieve (**Figure 1.3.2.1**). When fed as a powder, these diets will remain temporarily on the surface. Such finely ground food is suitable for feeding L1s and L2s. If you sift the ground diet, the larger particles of food that did not go through the sieve can be saved for feeding L3s and L4s. If possible, larval food should be stored at -20^{o}C until ready for use to prevent microbial growth. When feeding, disperse dry food evenly across the top of the water (**Figure 1.3.2.2**). It may be dispensed from a salt shaker, some other simple improvised device like a 50ml Falcon tube with holes in the cap, or shaken from a tiny weighing spoon.

Many labs prefer to use diets resuspended in water. When using such media, sufficient quantities for one week should be prepared and discarded after one week to prevent fouling or microbial contamination. During the week, store at 4°C until needed. Aliquots of pre-measured, dried liver powder can be stored in vials at -20°C and removed and hydrated as needed. If feeding with a slurry, pipette diet into two or three different areas of the pan to ensure complete coverage.

Liver powder slurry can be used in all stages of larval feeding, however if dried fish food is the diet of choice, L1 larvae should be feed 0.02% w/v bakers or brewer's yeast (final concentration). The high nutritional content, small size and inexpensive nature of the latter diet make them a good choice for feeding early instars.

Different types of food may superficially appear suitable for larval feeding, but to ensure high quality, measure survival from egg hatch to eclosion at least once with any new food source before implementing routine use. Larval culture affects adult longevity and fecundity in the long term (see **Methods in Anopheles Research**, **Section 2.3: Modifying Fecundity, Longevity, and Size**), so using the best larval food available will ultimately save you time.

Figure 1.3.2.1. Preparing food. Use a general laboratory grinder (pictured here), a grinding mill or a household coffee grinder, or mortar and pestle. To ensure food is small enough for the earliest stages, use a sieve with holes no larger than 250 microns in size.

Figure 1.3.2.2. Check pans daily and assess for feeding and density. Adjust the density by splitting or thinning to about 200-300 L2 larvae in a typical 9" x 12" tray.

Larval Density

It has been shown experimentally that emergence rates diminish as density in the pan increases (Timmermann and Briegel 1993, Macia 2009). High larval density has also found to distort sex ratios by favoring males over females in *Ae. aegypti* (Macia 2009). Loss of larval vigor irreversibly restricts adult health. so attention to this factor cannot be over-emphasized. Larval crowding stresses larvae and increases the chance of some larval infections.

A reasonable density for most L3-4 mosquitoes is 0.5 - 1 larva per ml. When it is impractical to estimate the exact density in the early (L1-2) stages, larvae are usually cultured at a high density. For this reason, the MR4 rearing schedule presented below is designed for thinning progressively in stages. Note that water depth is not as important in aedines as it is for anophelines; however a depth of no more than 2cm of water has been shown to be optimal for larval survival (Briegel 2003). Also note, *Aedes* larvae and pupae tend to cluster together in the corners or along the sides of the pan making estimations difficult (**Figure 1.3.2.3**), if enough food is provided even densities of 2 larvae per ml will yield healthy adults.

Figure 1.3.2.3: Clustering of immature stages of *Aedes aegypti*. The separation of pupae manually can be difficult so using a small bulb pipettor, gently aspirate up a small number of immatures and then return them to a clear area of the pan. Once they are back in the water the pupae will typically move out and are easier to remove.

Larval Feeding

At a constant temperature and given an appropriate amount of diet, the time from hatch to pupation should be predictable within one day from generation to generation. If pupation is delayed more than a day or two, any of the following could be responsible: temperature is too low, inadequate food was given at some stage, the density was too high, or excessive food was given in the early stages. Poor culture results in disparate developmental rates leading to pupation over the course of several days. This results in a great deal of extra work for the technicians. Ideally, almost all pupae form within a 2 to 3 day period.

The pans should be examined daily to ensure that the larvae are developing as expected and the density is appropriate. If you notice great differences in sizes of larvae between or within one tray of a cohort at any time, you likely have them too crowded and/or they are underfed. Not surprisingly, the amount of food provided daily must increase as the larvae develop and as their density increases. However, there is a limit to the amount of food and larvae you can place in one tray, so we recommend adhering initially to the density guidelines mentioned above and modifying only the amount of diet.

Underfed trays will contain larvae that die, are slow-growing or are variable in development rate.

Overfeeding is common and is indicated by numerous observations that precede larval death.

1. *Foul smell*. If you smell a foul odor when you remove the cover, you're feeding too much. A healthy organic odor is normal. However, what is considered healthy is admittedly dependent on personal aesthetics!
2. *Excessive turbidity*. Yellowish to greenish-colored water is fine and often appears in later stages of rearing (referred to as *gelbstoff*). However, if the water is turbid, feed less or not at all until the water clarifies. If turbidity persists, filtering the larvae out from the old culture water and at least a partial water change may be necessary. Greater turbidity is tolerable during the L3 and L4 stages whereas L1s and L2s are more sensitive. You will develop judgment regarding how much turbidity is appropriate.
3. *Excessive surfactants*. When the water in the pan is agitated, bubbles that form should burst rapidly. If they persist, bacterioneuston has formed an excessive surface microlayer which is not healthy for larvae. Check for bubbles by sloshing the water gently. Larvae exposed to water with high levels of surfactants often do not survive and re-feeding of the adult stock may be necessary. If bubbles persist, filtering the larvae out from the old culture water or dragging a tissue over the surface and at least a partial water change are recommended. If this is observed routinely, the culture conditions must be changed.

More signs of poor larval health related to density and feeding rates

The slowest growing larvae and those cultured in turbid water often develop melanotic nodules in the abdomen and thorax, or black patches on the cuticle. These are both bad signs, and the individuals that have these should be discarded - they seldom survive. See the section on minimizing infections for photos of infected larvae. Sub-optimal larval rearing conditions can also result in missing setae or those that are covered with black film (probably fungus). This can often be observed in the slowest-growing larvae even under good conditions, but if the condition is prevalent, a change in your methods is warranted.

Pupae that are not curled into the typical 'comma' shape but have a horizontally extended abdomen will not emerge. If you observe this among the first-forming pupae, it may not be too late to rescue the remaining larvae by changing the culture conditions.

The metamorphic transitions are the most sensitive stages to the effects of poor larval health. This can be observed as failure of larva to pupate or pupae to emerge as adults. One should observe >95% of adults emerging from the pupal stage under good conditions. The effects of poor larval/pupal conditions are often evident in a short adult life span, and males are especially sensitive to this effect.

Larval Rearing Protocol, MR4.

Day 1: Hatch eggs according to the long term egg hatching protocol listed above.

Day 2: Check eggs for hatching. Add a small volume of dried fish food (small pinch).

Day 3: Split and thin pans as needed. An appropriate number of larvae per 9 in X 12 in pan should not exceed 300. Feed each pan with ½ tsp of fish food.

Day 4: Check larvae, if water is clear add 3-4 koi food pellets (250 mg).

Day 5: Thin larvae as needed. Feed accordingly. A regular pan with approx. 300 larvae should get about 5-8 pellets (300-500mg).

Day 6: Check larvae-feed if necessary (500-750mg of koi pellets).

Day 7: Feed larvae as needed (500-1000mg). Pupae may start to appear on this day; they typically do not emerge for over 24 hours and can be left in the pan until the next day to reduce time spent separating pupae from larvae.

Day 8: Pick pupae. Feed larvae as needed (no more than 4 pellets – 250mg). Amount of food should decrease once pupation is observed to avoid excessive fouling of pans. Using a pupa picking device, carefully remove pupae from pan and place in an 8 oz. plastic cup with clean water. For every stock, make a cage or cup for pupae to emerge into. Write stock name and date of pupation in appropriate tape color and using the appropriate color ink. Provide cage/cup with sugar water pad or vial.

Day 13-15: Blood-feed adults

Larval Rearing Protocol (adapted from Duman-Scheel et al. 2011)

Day 1: Place a strip of approximately 300 eggs (less than 1 month old) into a 500ml cup containing 375ml of water and 5ml of liver powder slurry.

Day 2-3: Transfer larvae to a larger container and add 15ml of liver powder slurry.

Day 4-7: Check every other day to see if larvae need feeding, if the water is clear add more liver powder slurry. If larvae tend to only feed in one area of the pan, add an additional 5ml of liver powder slurry to the pan.

Day 8: Pick pupae. Feed larvae as needed. Amount of food should decrease once pupation is observed to avoid excessive fouling of pans. Using a pupa picking device, carefully remove pupae from pan and place in an 8oz. plastic cup with clean water. For every stock, make an emergence cage or cup for pupae. Write stock name and date of pupation in appropriate tape color and using the appropriate color ink. Provide cage/cup with sugar water pad or vial.

Day 13-15: Blood-feed adults

Day	Stage	Amount of diet
1	Hatching L1	60 mg ground koi diet
2	L1/L2	4 koi pellets (250 mg)
3	L2	6 koi pellets (400 mg)
4	L2/3	6-8 koi pellets (400-500 mg)
5	L3	8 koi pellets (500 mg)
6	L3/4	10 koi pellets (750 mg)
7	L4	8 koi pellets (500 mg)
8	L4/pupae	4 koi pellets (250 mg)

Table 1.3.2.3. Approximate food amounts to be fed to the L1-L4 larvae per day assuming L3 and L4 densities of 1 larva/ml. The table assumes younger larvae are at a higher density – at least 2 X.

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Maciá A (2009) Effects of larval crowding on development time, survival and weight at metamorphosis in *Aedes aegypti* (Diptera: Culicidae). Rev Soc Entomol Argent 68: 107-114.

Sneller VP and Dadd RH (1977) Requirement for sugar in a chemically defined diet for larval *Aedes aegypti* (Diptera: Culicidae). J Med Entomol 14: 387-392.

2.1 Infecting *Aedes aegypti* with *Brugia pahangi*, *Brugia malayi* or *Dirofilaria immitis*.

Introduction

This document describes the procedures used to infect *Aedes aegypti* with *Brugia pahangi*, *B. malayi* or *Dirofilaria immitis*.

Materials

- Sugar
- Blood collected from infected animal
- Blood collection tubes with Heparin
- Membrane feeding apparatus
- Nylon mesh material (cut to 12” x 12” squares)

Procedure

1. Allow approximately 600 female pupae to emerge as adults into a one-gallon carton. Three to five-day-old mosquitoes are used for producing infective larvae.
2. The sugar source is removed from the cartons before the infective blood meal.
3. Blood is collected from an infected animal and heparinized, and a microfilarial count is made. If the blood contains too many microfilariae, it is diluted with normal heparinized blood. The best results are obtained using the following concentrations of microfilariae in the membrane feeder. If the counts are lower than these values, low numbers of infective larvae will be produced.

Brugia malayi	80-100 mf/20µl
Brugia pahangi	100-200 mf/20µl
Dirofilaria immitis	70-90 mf/20µl

4. Mosquitoes are allowed to feed on infected blood maintained at approximately 37°C in a membrane feeding apparatus. This usually requires 1-2 hours. With *D. immitis*, it is important that fresh blood (collected within one hour of infection) be used for infecting each carton of mosquitoes.
5. Mosquitoes may also be infected by feeding on an infected animal (for details, see other SOPs).
6. After infection, a fresh sugar source and cotton containing water are placed on the nylon screen. Water is added to the cotton as needed, and the cotton is replaced every 5-7 days.
7. The cartons are covered securely with another piece of nylon screen as a precautionary measure.
8. Infective larvae of *B. pahangi, B. malayi,* and *D. immitis* are collected no earlier than 11, 14, and 16 days, respectively, after the infective blood meal.

2.2 Collecting Infective Larvae of *Brugia pahangi, B. malayi, Dirofilaria immitis,* and *Dirofilaria repens* From Infected Mosquitoes

This documents the procedures used in collecting infective larvae of *Brugia pahangi, Brugia malayi*, *Dirofilaria immitis*, *Dirofilaria repens,* and other mosquito-borne filariae from infected mosquitoes.

Materials

- Mortar and pestle
- Hank's Balanced Salt Solution (with Pen/Strep)
- 20 x 100 mm Petri dishes
- Glossy cardstock
- Pasteur pipet or micropipette
- 150 mesh sieve
- Microwave or hot water bath (37°C)

CAUTION: Hank's Balanced Salt Solution may cause irritation of the skin and eyes. No toxicity expected from inhalation. May cause nausea or vomiting if ingested. Read the detailed instructions pertaining to the reagents before use (MSDS sheet).Gloves should be worn.

Procedure

1. Remove sugar source and water-soaked cotton from the cartons.

2. Remove dead mosquitoes by aspiration from the carton (this may be performed as early as 24 to 48 hours prior).

3. Place cotton containing ether on the nylon screen and then place the carton in a plastic bag for several minutes. This procedure must be done under an explosion-proof hood. Alternatively, the mosquito cartons may be placed in a freezer for approximately one minute.

4. After all mosquitoes are immobilized remove the top of the carton. The infected mosquitoes (maximum of 2,250 mosquitoes) may be transferred to a piece of glossy paper (or similar smooth material). Replace the screened lid of the carton in case infected mosquitoes remain in the carton.

5. Quickly put the infected mosquitoes into a mortar without solution and gently crush them. Add 2 to 3 ml of chilled (refrigerated) Hanks' balanced salt solution (HBSS, pH 7.0) containing pen-strep (final concentration= 0.4 units penicillin/ml, 0.4 mcg streptomycin/ml, PS) to the mortar and gently crush the mosquitoes again.

6. The crushed mosquitoes on the pestle and in the mortar are then rinsed with the chilled HBSS-PS onto a 150 mesh sieve contained in a plastic petri dish.

7. The sieve containing the crushed mosquitoes is gently, but quickly, agitated to remove scales, eggs, and debris body parts (e.g., legs) of the mosquitoes. This procedure is repeated 3 to 4 times, using several dishes of fresh, chilled HBSS-PS. As approximately 10% of the larvae may remain in these washings; it is advisable to keep these dishes until one has collected a sufficient number of larvae, i.e., it may be necessary to use all of the larvae from the washings to obtain a sufficient number to work with.

8. The sieve is then removed to fresh, warm 37°C (microwave on high level for a minimum of 90 seconds and a maximum of 105 seconds) HBSS-PS to allow the larvae to migrate out of the mosquitoes; the sieve is transferred to Petri dishes containing fresh HBSS-PS every 15 to 30 minutes. (Note: most of the *Brugia* larvae will have migrated out of the mosquitoes within about 2 hours. Larvae of *D. immitis* are less active, therefore, it is necessary to allow 1 to 2 more hours for collecting these larvae.)

9. When inoculating *Brugia* larvae intraperitoneally into jirds, the solution must be quite clean, i.e., free of debris and not "cloudy," to avoid peritonitis and possibly death of the jirds. Thus, the solution should be relatively "clean" before inoculation of larvae either SC or IP, but particularly before IP injections.

Chapter 3.1: Forced Salivation of Infected *Aedes aegypti* and *Culex quinquefasciatus* into capillary tubes containing Immersion oil for Detection of Mosquito-borne Virus.

Smartt, Chelsea T., Shin, Dongyoung, and Chen, Tse-Yu

Introduction

This document describes the procedure used to collect saliva from virus-infected *Aedes aegypti* and *Culex quinquefasciatus.*

Materials

- Capillary tubes (75 mm, 70 µL capacity)
- Immersion oil
- Forceps
- 1.5ml tubes
- BA-1 diluent (M-199 salts, 1% bovine serum albumin, 350mg/L sodium bicarbonate, 100U/ml penicillin, 100mg/L streptomycin, and 1mg/L Fungizone in 0.05M Tris, pH 7.6)
- Salivation Devise: The device is constructed using a 25.5 cm x 17.5 cm piece of plexiglass. Laboratory bench paper is placed under the plexiglass to create a white background and secured to a test tube rack using tape. Autoclave tape is placed around the plexiglass sticky-side up in order to secure the mosquitoes.

Procedure

1. Fill the capillary tubes with 0.01 ml immersion oil, using micropipette.
2. Immobilized the infected mosquitoes with cold (i.e. 1 minutes -20°C).
3. While anesthetized and without damaging the mosquito body, remove the wings and legs and place live mosquito bodies onto a salivation device by lightly attaching the bodies to the sticky side of the autoclave tape.
4. Place the capillary tube over the mosquito's proboscis and allow the female to salivate for approximately 45 minutes.
5. After 45 minutes, collect the mosquito bodies into 1.5ml tubes containing 1 ml BA-1 diluent (1 body/tube) and store them at -80°C for subsequent use.
6. Saliva from each mosquito is ejected from the capillary tube into a 1.5ml tubes containing 1 ml of BA-1 (1 tube /mosquito) by inserting a pipette tip into the end of the capillary tube and expelling air into the capillary tube. Store the tubes at -80°C for subsequent use.
7. The stored samples can now be used for subsequent processing, i.e. RNA extraction, virus isolation etc.

3.2 *Aedes* species specific diagnostic PCR assay (Wesson et al 1992)

Adapted by MR4 Staff

Introduction

The ability to discriminate between *Aedes aegypti* and *Ae. albopictus* using morphological characteristics may be compromised if distinctive coloration is worn away or if specimen damage occurs. At the molecular level, the multi-copy variable second internal transcribed region (ITS2) of ribosomal DNA can be used to discriminate between these two species. Because this assay is based on sequence variations within the region, it is possible that PCR amplification of specimens other than *Ae. aegypti* and *Ae. albopictus* may yield similar results.

PCR to discriminate between various aedines (Wesson et al 1992)

Prepare PCR Master Mix for 1 25µl PCR reactions.[1] Add reagents in the order presented.

96	48	1	Reagent
1590 µl	795 µl	15.9 µl	Distilled H_2O
500 µl	250 µl	5.0 µl	5X PCR buffer
100 µl	50 µl	1.0 µl	dNTP (2mM concentration)
50 µl	25 µl	0.5 µl	CP-P1A (10 pmol/µl) - F [GTGGATCCTGTGAACTGCAGGACACATG]
50 µl	25 µl	0.5 µl	CP-P1B (10 pmol/µl) - R [GTGTCGACATGCTTAAATTTAGGGGGTA]
100 µl	50 µl	1.0 µl	$MgCl_2$ (25 mM)
10 µl	5 µl	0.1 µl	Promega GoTaq DNA polymerase (5U/ µl)
2.4 ml	**1.2 ml**	**24 µl**	**Total (To each 24 µl reaction add 1 µl template DNA)**

Table 3.2.1. F and R indicate forward and reverse orientation.

PCR cycle conditions
94°C/5min x 1 cycle
(95°C/1min, 54°C/30sec, 72°C/1min) x 35 cycles
72°C/5min x 1 cycle
4°C hold

Run samples on a 1.5 % agarose gel stained with EtBr or other intercalating agent such as SYBR Green or Gel Red.

Primers create fragments of 600 *Ae. albopictus* and 365 *Ae. aegypti* (**Figure 3.2.1**)

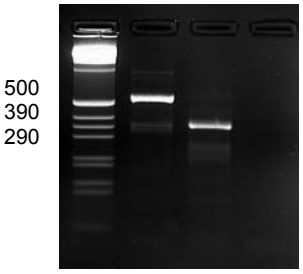
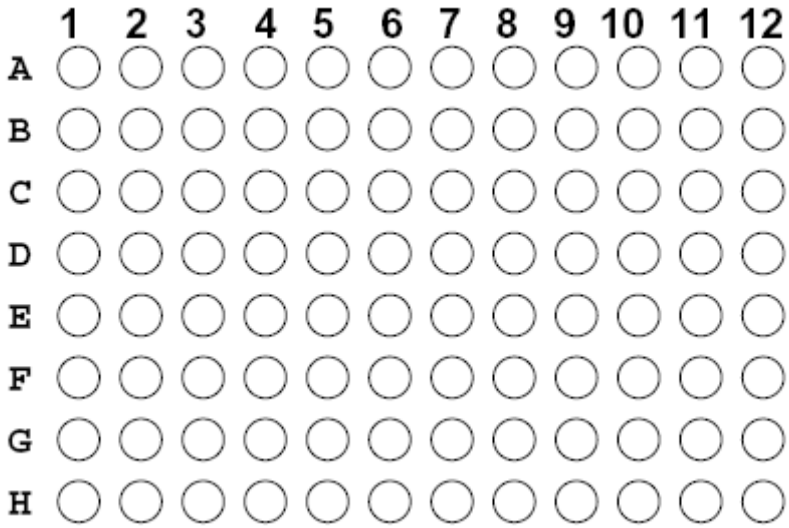


Figure 3.2.1. Lane 1 1kb ladder, Lane 2 *Ae. albopictus*, Lane 3 *Ae. aegypti*, Lane 4 blank control

[1] Amounts for larger master mixes have been adjusted upwards to be sufficient for 50 and 100 reactions to compensate for imprecise measurements.

96 well PCR sample preparation template



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3.3 Mitochondrial DNA PCR Assays for *Aedes* Mosquitoes

Introduction

Mitochondrial DNA (mtDNA) is one of the most commonly studied regions in insect systematics due to its high rate of homoplasmy (Caterino et al 2000). Within the mtDNA there are several segments of which only a few are routinely examined in aedines: cytochrome c oxidase I (COI), cytochrome c oxidase II (COII), cytochrome b, and NADH ubiquinone oxidoreductase.

Cytochrome c oxidase I (COI)

Table 3.3.1: Prepare PCR Master Mix for 1, 48, or 96 25µl PCR reactions. Add reagents in the order presented.

96	48	1	Reagent
1587.5 µl	793.75 µl	15.875 µl	Distilled H_2O
500 µl	250 µl	5 µl	5X PCR buffer
100 µl	50 µl	1 µl	dNTP (2mM concentration)
100 µl	50 µl	1 µl	$MgCl_2$ (25 mM concentration)
100 µl	50 µl	1 µl	CI-J-1632 (10pmol/µl)
100 µl	50 µl	1 µl	CI-N-2191 (10pmol/µl)
12.5 µl	6.25 µl	.125 µl	GoTaq DNA polymerase (5U/ µl)
2.5 ml	**1.25 ml**	**25 µl**	**Total (to each 25 µl reaction add 1 µl template DNA)**

PCR cycle conditions

95°C/5min x 1 cycle
(97°C/30s, 40°C/45s, 72°C/60s) x 35 cycles
72°C/5min x 1 cycle
10°C hold

Run samples on a 1.5% agarose gel stained with an intercalating agent such as EtBr, load 10µl of sample. You will expect and approximately 597 bp product (**Figure 3.3.1.**).

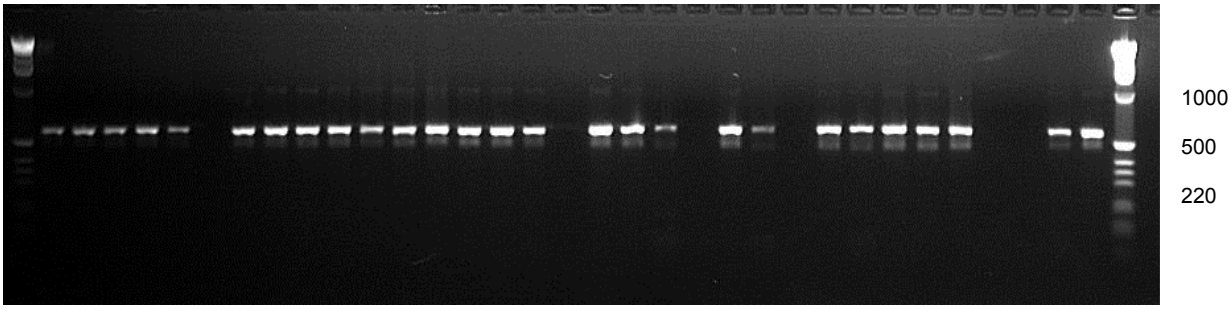


Table 3.3.1: Lane 1, 1kb ladder, lanes 2-17 *Ae. aegypti* LVP-IB12, lanes 18-29, *Ae. aegypti* Line 12, lane 30, 1kb ladder.

NADH ubiquinone oxidoreductase, subunit 5

Table 3.3.2: Prepare PCR Master Mix for 1, 48, or 96 25µl PCR reactions. Add reagents in the order presented.

96	48	1	Reagent
1587.5 µl	793.75 µl	15.875 µl	Distilled H_2O
500 µl	250 µl	5 µl	5X PCR buffer
100 µl	50 µl	1 µl	dNTP (2mM concentration)
100 µl	50 µl	1 µl	$MgCl_2$ (25 mM concentration)
100 µl	50 µl	1 µl	ND5F (10pmol/µl)
100 µl	50 µl	1 µl	ND5R (10pmol/µl)
12.5 µl	6.25 µl	.125 µl	GoTaq DNA polymerase (5U/ µl)
2.5 ml	**1.25 ml**	**25 µl**	**Total (to each 25 µl reaction add 1 µl template DNA)**

PCR cycle conditions

98°C/2min x 1 cycle
(95°C/30s, 45°C/30s, 72°C/45s) x 5 cycles
(95°C/30s, 46°C/45s, 72°C/45s) x 28 cycles
72°C/5min x 1 cycle
10°C hold

Run samples on a 1.5% agarose gel stained with an intercalating agent such as EtBr, load 10µl of sample.

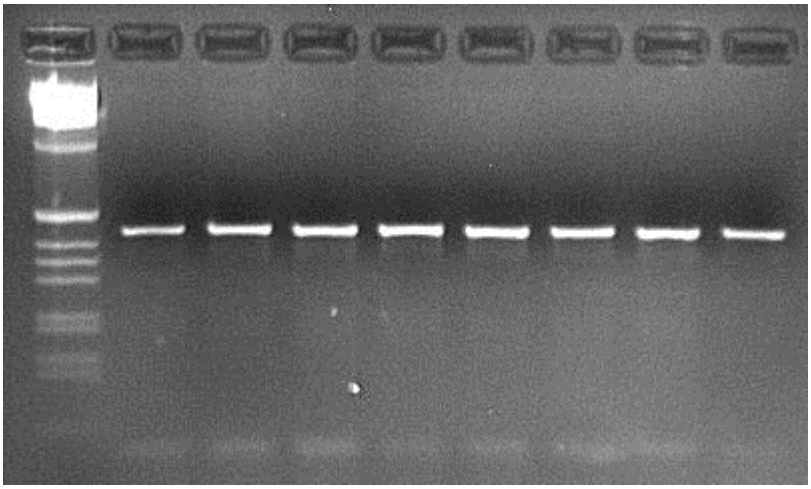
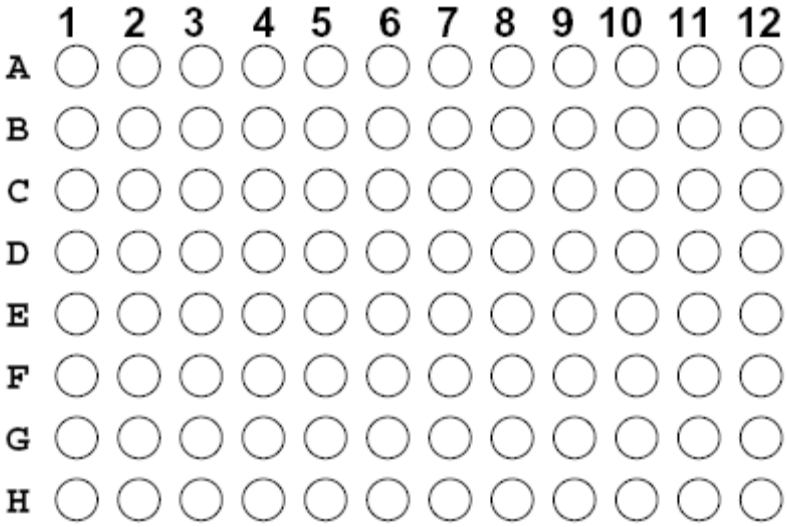


Figure 3.3.2: Lane 1, 1kb ladder, lanes 2-9, *Ae. aegypti* LVP-IB12.

96 well PCR sample preparation template



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