

Standard Operating Procedures:
Rearing Aedes aegypti for the
HITSS and Box Laboratory Assays

Training Manual
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Developed by Tarra Foggie and Nicole Achee

Disclaimer: This manual is intended to provide general guidelines only for rearing *Aedes aegypti* for behavioral evaluation using the HITTS and Box Laboratory Assays. For more information and specific guidance on rearing *Aedes aegypti* for behavioral evaluation please contact Nicole L. Achee at nachee@usuhs.mil.

TABLE OF CONTENTS

I.	<u>REARING SCHEDULE</u>	3
II.	<u>ASSAY INSECTARY ACTIVITIES</u>	
	a. Making Sugar Solution	4
	b. Moisten Sugar Pads	4
	i. Sugar pads in Gallon Buckets	4
	ii. Sugar pads on Pint Cartons	4
	c. Hatching Eggs	5-6
	d. Separating Larvae	6
	e. Feeding Larvae	7
	f. Refreshing Larval Water	7
	g. Picking/Sorting Pupae	8-9
	h. Sorting Females for Assays	9-10
	i. Starving Females for Assays	10
III.	<u>COLONY INSECTARY ACTIVITIES</u>	
	a. Making Sugar Pads	11
	b. Starving for Blood Feeding	11
	c. Blood Feeding	12
	i. Preparing the blood	12
	ii. Feeding adult buckets	12-13
	iii. Cleaning membrane feeders	14
	j. Egg Harvesting	15
	k. Replacing Oviposition Cups	16
	l. Hatching Eggs	16
	m. Separating Larvae	16
	n. Feeding Larvae	16
	o. Refreshing Larval Water	16
	p. Picking Pupae	16
	q. Setting Up New Adult Buckets	16
IV.	<u>APPENDICES</u>	
	a. Appendix I: Larval Eclosion Datasheet	17
	b. Appendix II: Egg Harvest Datasheet	18

***Aedes aegypti* ASSAY REARING SCHEDULE:**

Activity	Monday	Tuesday	Wednesday	Thursday	Friday
Make Sugar Solution	When needed				
Moisten Sugar Pads	X Wed-Fri Gallons	X Thur-Fri Assay/ New Gallons	X Fri. Assay/ New Gallons	X New Gallons	X New Gallons
Hatch Eggs			X (see assay calendar)	X (as needed)	
Separate Larvae				X	X (as needed)
Feed Larvae	X* Pellet (As needed/cup)		X CHOW (All)	X CHOW (All)	X PELLET (All)
Refresh Larval Water	X	X			
Pick/Sort Pupae	X	X	X	X	X
Sort Females for Assays	X				
Starve Females for Assays	X (For Tues)	X (For Wed's)	X (For Thu's)	X (For Fri's)	
Supply Inventory	X				

***Aedes aegypti* (THAI STRAIN) COLONY REARING SCHEDULE:**

Activity	Monday	Tuesday	Wednesday	Thursday	Friday
Make Sugar Pads		X			X
Blood Feeding		X			X
Starve for Blood Feeding	X			X	
Egg Harvest	X				X
Replace Oviposition Cups		X			X
Hatch Eggs			X 2,000 eggs		
Separate Larvae				X 40 cups	
Feed Larvae	X* PELLET (As needed/cup)		X CHOW (All)	X CHOW (All)	X PELLET (All)
Refresh Larval Water	X	X			
Pick/Sort Pupae	X	X	X	X	X
Set Up New Adult Buckets		X 250/ gallon	X 250/gallon	X 250/gallon	X 250/gallon

ASSAY INSECTARY ACTIVITY DESCRIPTIONS

Make Sugar Solution: Make a 10% sugar solution in a designated glass container using tap water (500ml) and nectar food (50 g) (Figure 1 A and B). Mix well to ensure sugar has completely dissolved. Replace lid and store sugar solution at 4°C for up to two weeks. The container should be washed with 10% diluted bleach prior to mixing new solution.

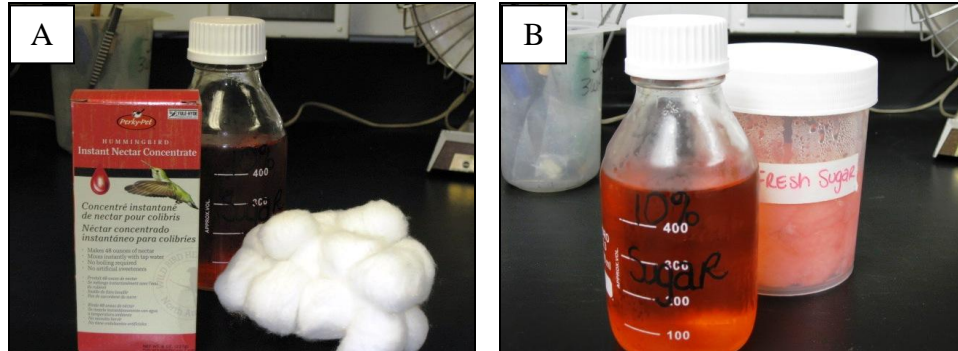


Fig. 1A and B: Materials used to make sugar solution and mixed 10% Sugar Solution Container with prepped cotton pads

Moisten Sugar Pads:

I. Sugar Pads in Gallon Buckets: Remoisten sugar pads in individual buckets with fresh sugar water using a squeeze bottle (Figure 2 A and B). **Monday throw away all sugar pads from gallon buckets containing assay mosquito populations.**

II. Sugar Pads on Pint Cartons: Remoisten sugar pads by lightly adding sugar water using a squeeze bottle (Figure 2C). The sugar pads should be moist to the touch but not dripping.



Fig. 2A-C: Remoisten sugar pads in gallon buckets using a squeeze bottle (A and B). Similar methods are used to remoisten pads on pint cartons making sure solution does not drip from the cotton into the carton (C).

ASSAY INSECTARY ACTIVITY DESCRIPTIONS

Hatch Eggs: Hatch the necessary number of eggs needed to fulfill larval requirements for assay testing. To do this, cut a dried oviposition egg paper (**eggs must be at least 2 weeks old**) into 1-inch pieces making sure to cut away excess paper that does not contain eggs prior to placing into hatching cup (Figure 3A-C). Label each cup with “egg harvest system”: Strain, Date Harvested (DH) and Hatch Date (H): ex. Thai F4 DH: 12/1/04 H: 1/1/05 (Figure 3D).

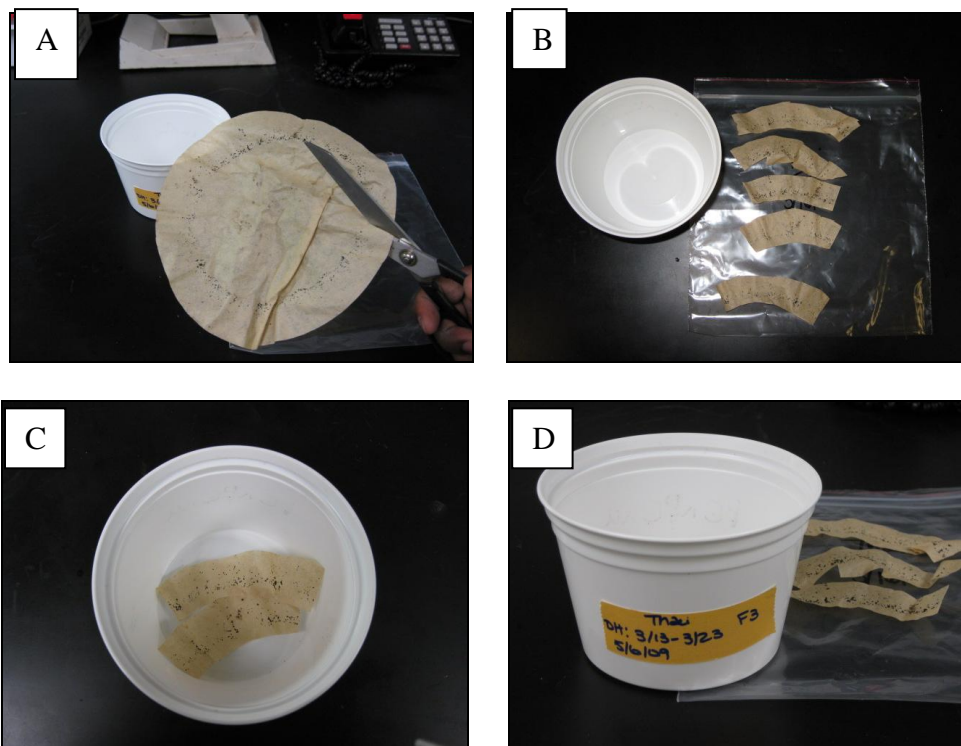


Fig. 3A-D: Cut a dried oviposition egg paper containing the number of eggs required for assays (A) into 1-inch pieces (B) before placing into a labeled hatching cup (C and D).

Next, fill the hatching cup with ~450 ml of tap water (first beveled line from bottom) ensuring the egg paper is fully submerged under water. Then place under vacuum for 15-30 min (Figure 4A-C). Vacuum hatching will synchronize the rearing process.



Fig. 4A-C: Fill the rearing cup containing egg strips with ~450 ml tap water(A) and ensure that the paper is fully submerged (B) before placing under vacuum for 15-30 min.(C).

ASSAY INSECTARY ACTIVITY DESCRIPTIONS

Hatch Eggs (cont): After eggs have vacuum-hatched, place one scoop (using orange-tagged pipette) of finely crushed fish pellets into the hatch cup (Figure 5A-C). On the first day after hatch, transfer egg papers into a new hatch cup (properly labeled and filled with water) and leave overnight to allow for delayed hatching (Figure 5D and E).

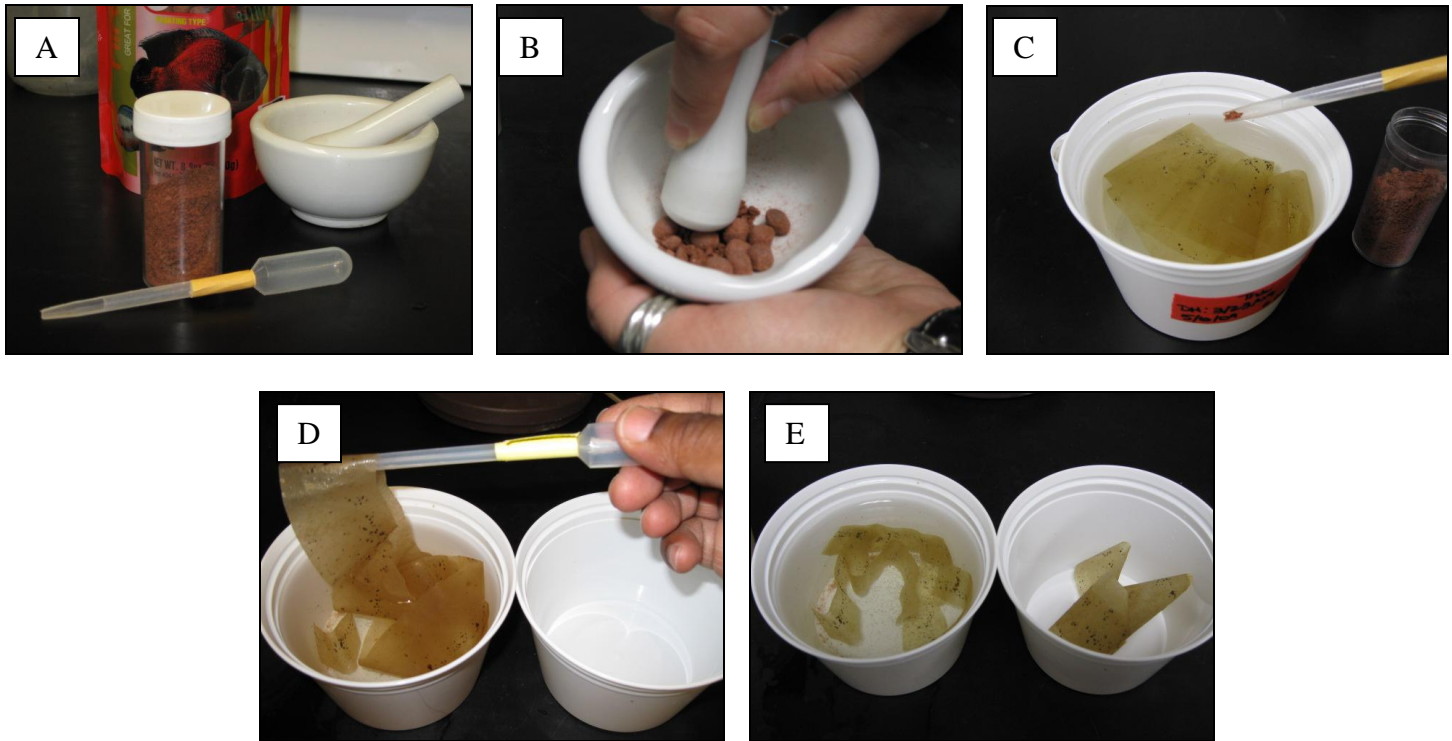


Fig. 5A-E: Feeding larvae after hatching requires crushed fish food pellets and a pipette that is modified to deliver a small amount of food (A). After hatching, place one scoop of ground fish food (B) into the rearing cup (C) and ensure that the food is distributed throughout. On the first day post-hatch transfer egg strips (D) into new hatch cups (properly labeled and filled with water) and leave overnight for delayed hatching (E).

Separate Larvae: The first day after egg hatch sort groups of 50 larvae into individual larval rearing cups and fill each cup with ~450 ml of tap water (first beveled line from bottom). Place sorted cups onto trays and label according to egg harvest system from egg hatch (can use the label on hatching cups) ex. Thai F4 DH: 12/1/04 H: 1/1/05 (Figure 6 A-C). Record the final number of larvae sorted onto the Larval Eclosion datasheet to estimate % egg hatch (Appendix I).

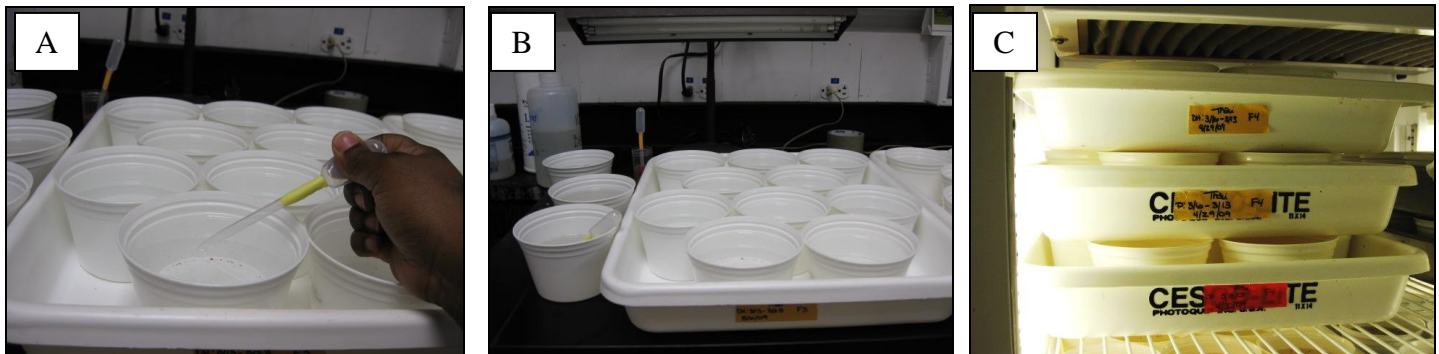


Fig. 6A-C: The first day after hatching, larvae are sorted into individual rearing cups in groups of 50 (A). The cups are then placed into properly labeled trays (B). After sorting, all larvae are fed (See Feed Larvae) then placed into incubators (C).

ASSAY INSECTARY ACTIVITY DESCRIPTIONS

Feed Larvae: After sorting larvae into groups of 50, add a scoop (using orange-tagged pipette) of finely crushed fish pellets into all larval cups. On Day 3 and 6 post-hatch, place one chow pellet into each larval cup (Figure 7 A and B). ***If > 5-10 pupae/ cup on Monday do not feed larvae**

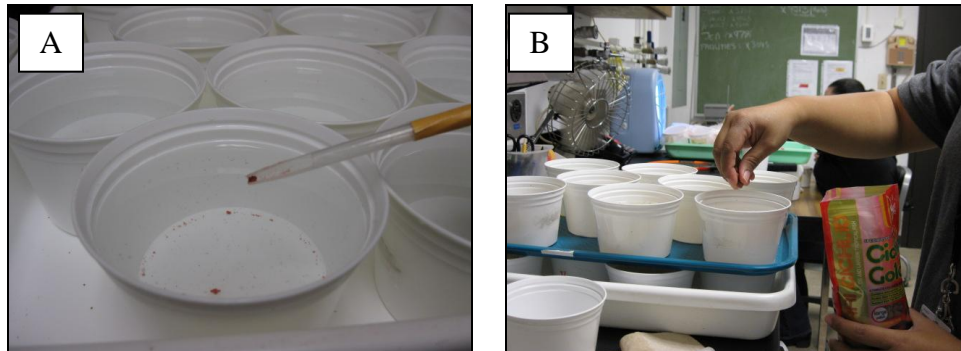


Fig. 7A and B: One scoop of finely crushed fish food pellets is added to each larval cup the first two days post-hatch (A). On Day 3 and 6 post hatch one whole pellet is placed into each cup (B).

Refresh Larval Water: Drain water from all larval cups until ~1 inch remains at the bottom making sure not to lose any larvae. Refill to ~450 ml using tap water (first beveled line from bottom) (Figure 8 A and B).

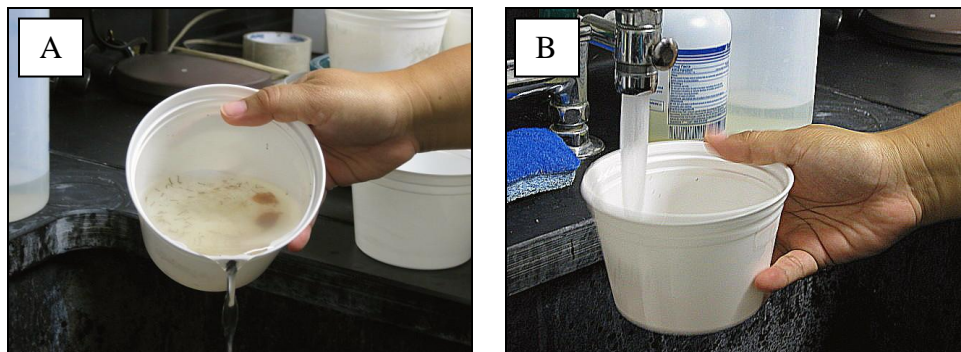


Fig. 8A and B: Water is drained from larval cups until ~ 1 inch of liquid remains (A) then refill with ~ 450 ml tap water (B).

ASSAY INSECTARY ACTIVITY DESCRIPTIONS

Pick/Sort Pupae: Before sorting pupae, remove larvae from rearing cups using a large-mouthed plastic pipette and separate female/male pupae by size either manually or using a mechanical separator (Figure 9 A-D).

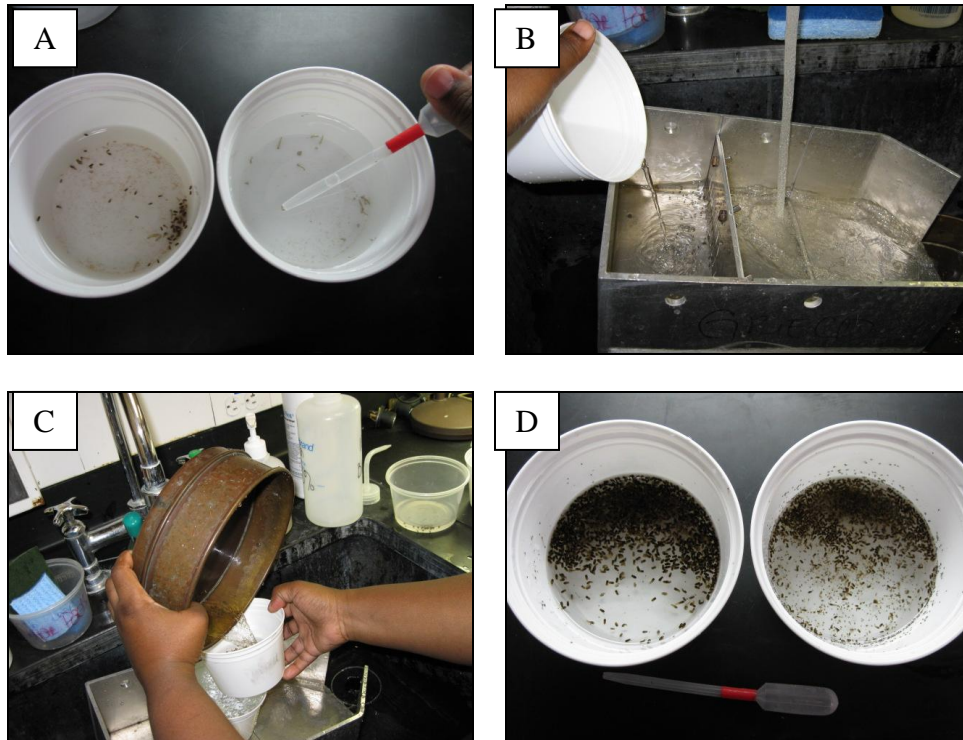


Fig. 9A-D: Before sorting pupae, larvae are removed from rearing cups using a pipette (A) then sorted into groups of males and females either manually or by mechanical separation. Water is continuously poured into the mechanical sorter with male pupae passing through the comb and collecting in a sieve (C). After 10 cups, the male pupae are poured from the sieve and female pupae are then flushed into a separate sieve. The end result is two cups with either male or female pupae (D).

Once separated from males, sort female pupae into groups of 250 using a pupae estimator and place each group into individual larval rearing cups (Figure 10 A-C).

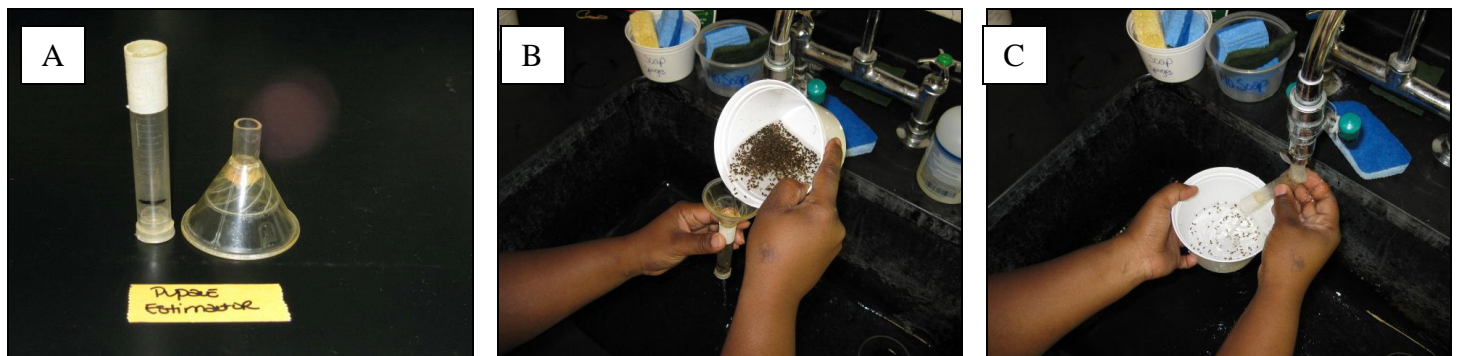


Fig. 10A-C: Pupae estimator used to sort female pupae into groups of 250 (A). Sorted from males, female pupae are poured into the pupae estimator (B) then flushed out of the estimator (C) into individual rearing cups.

ASSAY INSECTARY ACTIVITY DESCRIPTIONS

Pick/Sort Pupae (cont): Once the female pupae have been sorted into groups of 250, each group is placed into individual gallon buckets labeled with strain, pupation date, female count and generation: EX: Thai P: 8/31/04 200ct. F4. (Figure 11A and B). Place two sugar pads on a small petri dish then INSERT petri dish into each bucket. (Figure 11C). Cover with netting and store in the incubator. Kill male pupae by pouring bleach into the cup. ***Male pupae can be saved for colony material if needed.**



Fig. 11A-C: Groups of 250 female pupae (A) are placed into labeled gallon buckets containing a petri dish with sugar pads (B) then covered with netting and stored in an incubator (C).

Sort Females for Assays: Mechanically aspirate females from 1-gallon buckets and group into required modified pint cartons (10's or 20's) according to the assay schedule for that week (*see Assay Scheduling description*) (Figure 12 A-B).

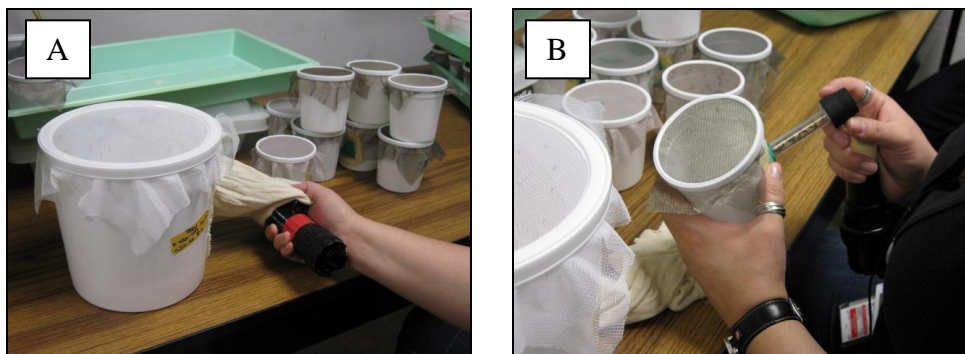


Fig. 12A and B: Mechanical aspiration of females from gallon bucket (A) into pint cartons (B) sorted into groups of 10's or 20's as needed for weekly assays.

ASSAY INSECTARY ACTIVITY DESCRIPTIONS

Sort Females for Assays (cont): Group pint cartons by daily requirements and place into green transporting trays (Figure 13 A-B). Place a cotton pad soaked in 10% sugar solution on top of each pint carton. Properly label all transporting trays with the age and generation of mosquitoes, day the pint cartons will be used and the number of 10's and 20's cartons (EX: For Thursday / 6 Day Old (DO) F4/ 7(10's) then store in incubators.

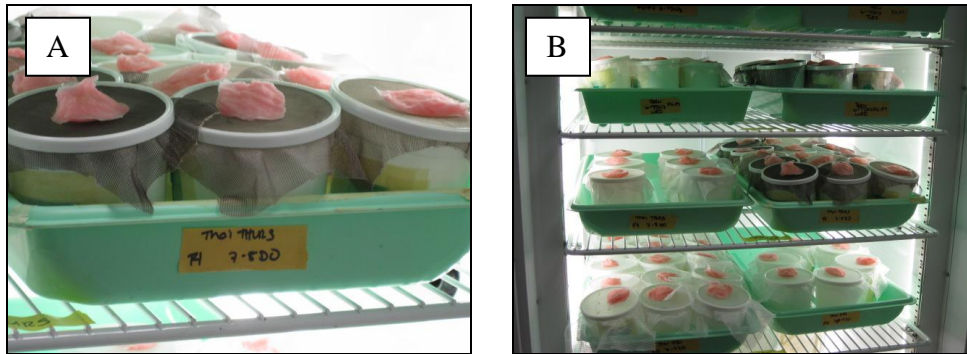


Fig. 13A and B: Properly labeled transporting trays containing pint cartons of grouped mosquitoes (10's or 20's) for assays with sugar pads (A). Grouped mosquitoes are then kept in the incubator until time of assay (B).

Starve Females for Assays: Remove sugar pads from designated pint cartons. Store some of the sugar pads in the refrigerator and use these pads for the 1- gallon buckets holding pupae picked the same week. **Females should be starved 24 hr prior to use in assay test system** (Figure 14 A-C).

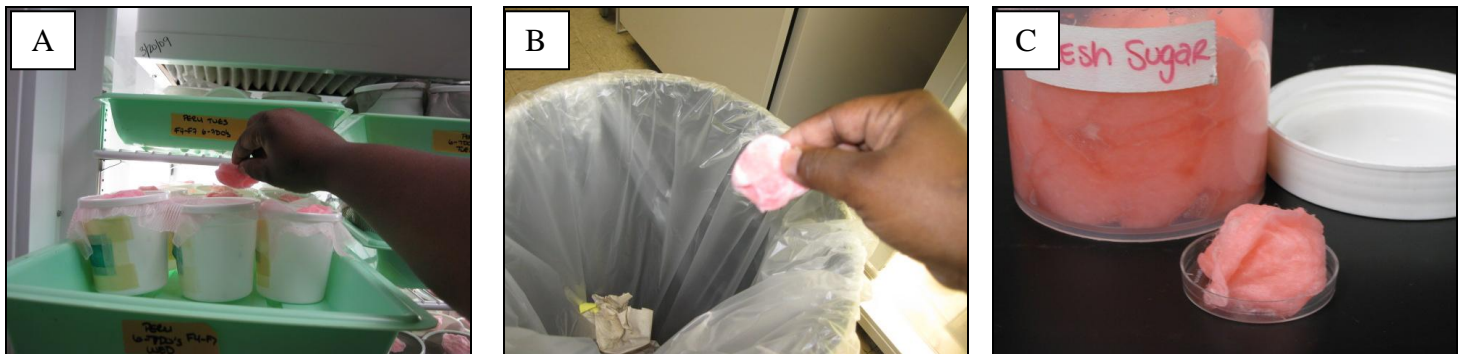


Fig. 14A-C: Remove sugar pads from pint cartons 24 hrs prior to assay (A) and properly dispose of them in the trash(if signs of mold) (B) or store for use in 1-gallon buckets that same week (C).

COLONY INSECTARY ACTIVITY DESCRIPTIONS

Make Sugar Pads: Moisten two cotton balls with fresh 10% sugar solution and place into a small petri dish. Insert one petri dish into each 1-gallon plastic bucket holding adult mosquitoes. Sugar pads should be moist to touch (Figure 1 A and B).

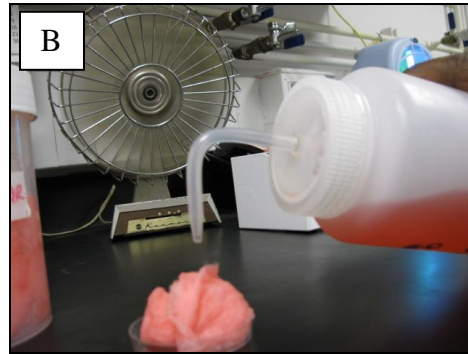


Fig. 1A and B: Sugar pads for adult gallon buckets are placed into a petri dish (A) then moistened using a squeeze bottle (B).

Starve for Blood Feeding: Remove sugar pads from adult feeding containers. Throw away cotton pads and clean petri dish with soap and tap water or soak in 10% bleach as needed (Figure 2 A and B).



Fig. 2A and B: Once the sugar pads are removed from gallon buckets, they are disposed of in the trash (A) and the petri dish cleaned with soap and water or 10% bleach solution (B).

COLONY INSECTARY ACTIVITY DESCRIPTIONS

Blood Feeding:

I. Preparing the Blood: Wearing gloves and a lab coat, mix blood thoroughly then pour into a 50cc plastic tube (Figure 3 A and B). **Use 5 ml of blood for each adult bucket.** Measure out ATP. **The ratio of ATP: blood should follow: 0.025g ATP/10 ml blood.** After weighing, pour the ATP into the 50cc plastic tube and shake vigorously until dissolved.

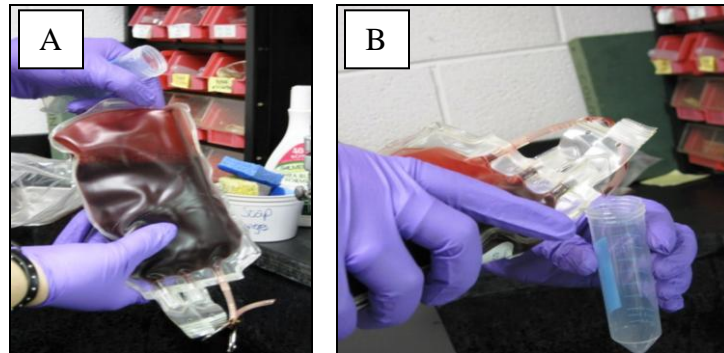


Fig. 3A and B: Mix blood (A) before pouring 30ml into a 50cc plastic tube (B). Combine with ATP as described.

II. Feeding Adult Buckets: Attach a 3 x 3 in. square piece of synthetic membrane tightly across the bottom of each glass feeder using a rubber band. **Make sure the “bumpy” part of the membrane is on the outside and covers all bottom edges of the glass feeders** (Figure 5 A and B).

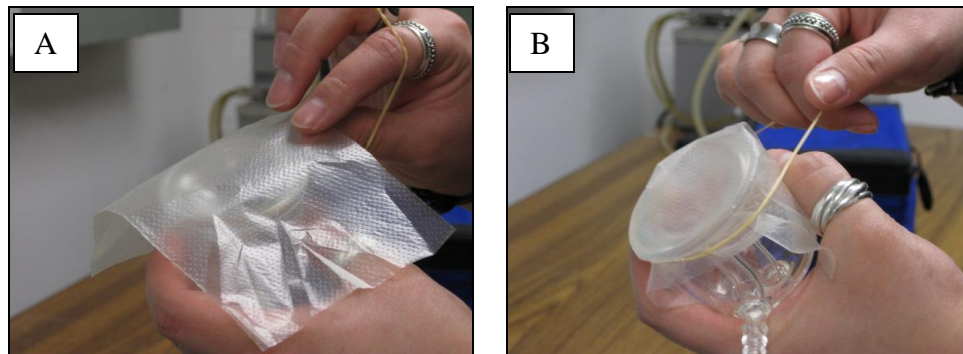


Fig. 5A and B: Carefully attach pre-cut synthetic membrane “bumpy” side up (A) around feeder and fix using a rubber band (B).

Place one feeder on top of each adult bucket then attach tubing from a circulating water bath to the feeders (Figure 6 A and B). If feeding more than one bucket be sure to connect all glass feeders together.

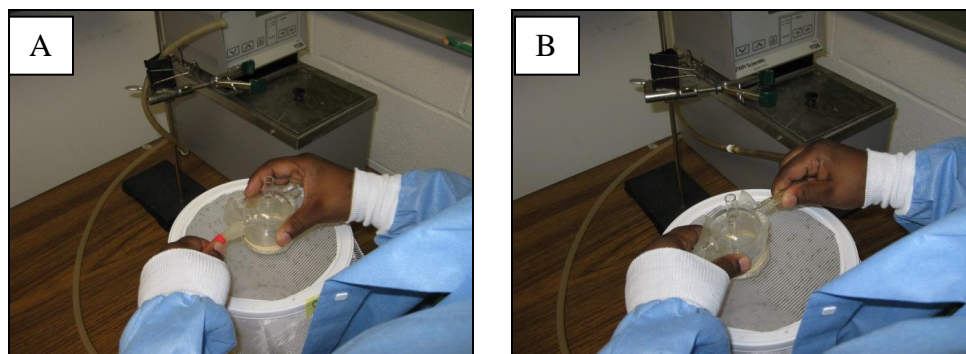


Fig. 6A and B: Place one feeder on top of each bucket (A) and attach tubing from the circulating water bath (B).

COLONY INSECTARY ACTIVITY DESCRIPTIONS

II. Feeding Adult Buckets (cont):

Once all feeders have been connected to the tubing, turn the water bath on and place the 50cc plastic tube of blood into the water bath chamber to warm for at least 15 min (Figure 7 A and B). **The plastic vial should be warm to the touch prior to placing the blood in the glass feeders.** Once the blood is warmed, add 5 ml of blood into each glass feeder using a pipette (wear gloves and a lab coat) (Figure 7C).



Fig. 7A-C: Once tubing is connected to all feeders, turn the water bath on (A) then place the 50cc plastic tube containing blood in the water chamber to warm (B). Once the blood is warm, add 5ml to each feeder (C).

When all feeders are filled, cover the adult buckets with a dark plastic sheet (Figure 8A and B). **Allow females to feed for ~ 1 to 1 ½ hour.** Clean the 50 cc tube, its cap and the pipette using a 10% bleach solution and rinse thoroughly with water. Leave the 50cc tube uncovered to dry.

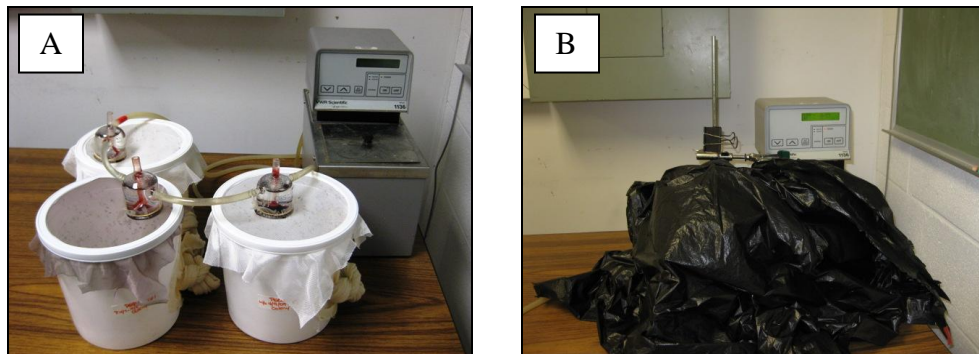


Fig. 8A and B: After all feeders are filled with blood (A), cover the buckets with a dark plastic sheet (B).

Once females are engorged (enlarged abdomens), turn off the circulating water bath, remove tubing from the glass feeders and place feeders into a washing pan (Figure 9A-C). **Make sure to drain each feeder and tubing section prior to disconnecting.**



Fig. 10A-C: After feeding, turn water bath off and detach intake tubing from feeders (A), drain water from system (B) and place feeders in a cleaning pan (C).

COLONY INSECTARY ACTIVITY DESCRIPTIONS

III. Cleaning Membrane Feeders:

Wearing gloves and a lab coat fill a pan with 10% bleach solution then carefully remove membrane pieces from each glass feeder and allow excess blood to drain into the solution (Figure 11A-C). Allow the blood to soak for ~15 min (or until solution is no longer colored) then flush with tap water. Soak the used membrane in the same bleach solution then dispose of according to Biohazard Waste protocols.



Fig. 11A-C: Make a 10% bleach solution in a cleaning pan (A) then remove membrane from feeders (B) and drain excess blood into the cleaning pan (C).

Once all membrane pieces have been removed, gently clean glass feeders in fresh 10% diluted bleach solution using a scouring pad, rinse thoroughly with tap water then place over paper towels to dry (Figure 12A and B).

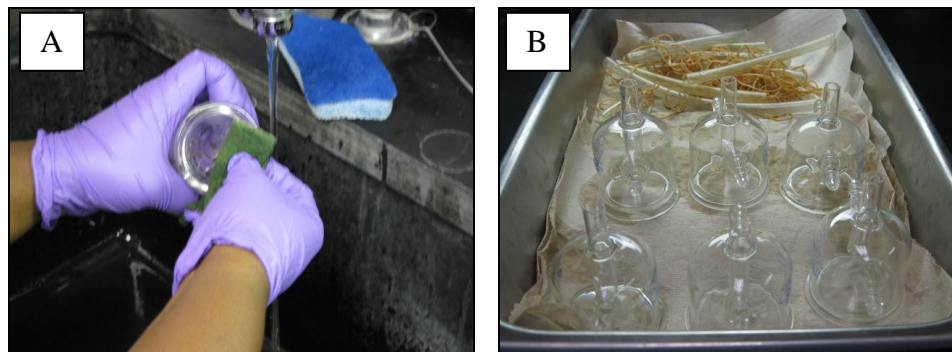


Fig. 12: After draining blood from feeder clean thoroughly with 10% bleach solution and rinse with tap water (A). Once cleaned, glass feeders and tubing are placed on paper towels to dry (B).

COLONY INSECTARY ACTIVITY DESCRIPTIONS

Egg Harvest: Remove oviposition cups from adult colony buckets, drain excess water and remove dead adults using forceps (Figure 13A-C). Extract the filter paper and place egg-side up on a clean surface in an incubator to allow water to evaporate (no less than 1 hour) (Figure 13D).

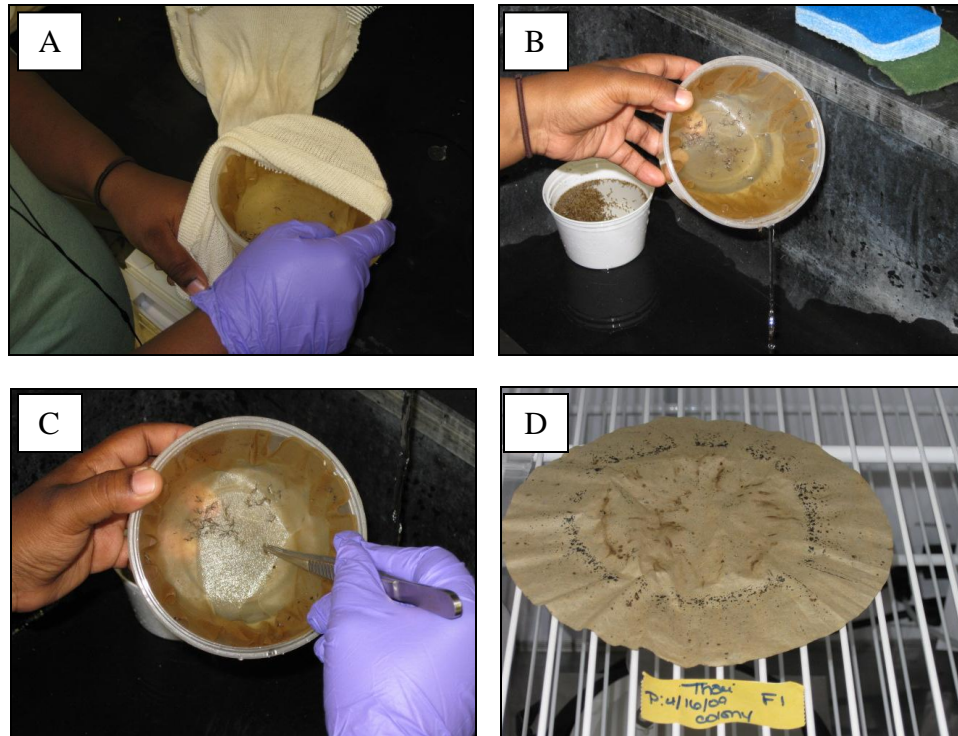


Fig. 13A-D: Remove oviposition cups from colony buckets (A), drain off the water (B), and remove dead mosquitoes (C) before placing in the incubator to dry (D).

After drying, estimate the number of eggs on each filter paper and record on the Egg Harvest datasheet (Appendix II) then fold coffee filters in half, with eggs towards the center, and place into individual ziplock bags labeled with “egg harvest system” and egg amount: EX: Thai DH: 10-8-04 / F4 ~8,000. Where DH=date of harvest; F4=generation of eggs; Strain= Thai (Figure 14A- C).

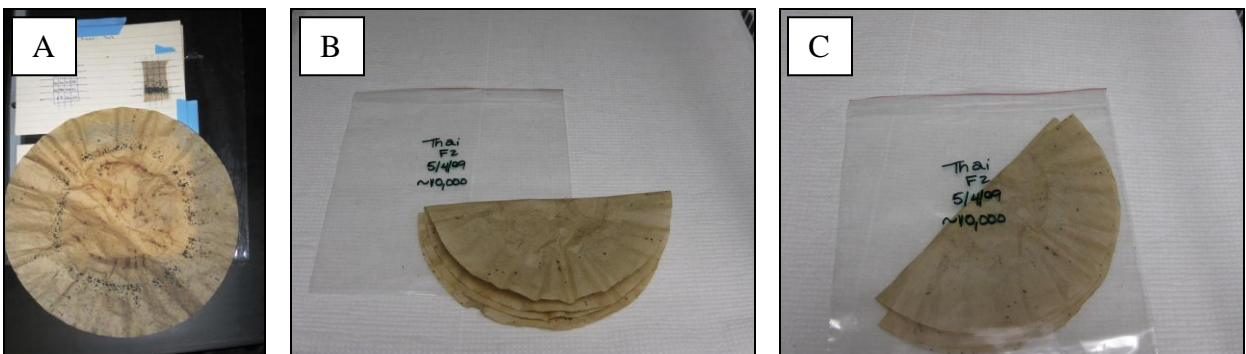


Fig. 14A-C: After drying, the number of eggs on each filter paper are estimated using a reference guide (A). Egg papers are then folded in half (B) and stored in labeled ziplock bags (C).

COLONY INSECTARY ACTIVITY DESCRIPTIONS

Replace Oviposition Cups: Place a new coffee filter inside an oviposition cup (450 ml plastic cup) and fill half way with tap water. Make sure to push coffee filter completely inside cup so that it DOES NOT hang over the sides. Place one oviposition cup into each 1-gallon bucket containing adult colony mosquitoes (Figure 15A-C).

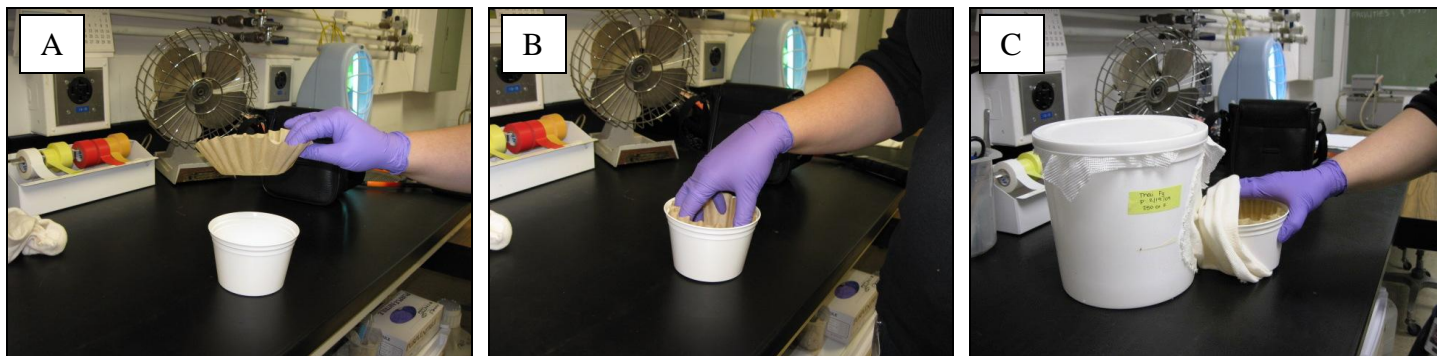


Fig. 15A-C: Oviposition cups are prepared by placing a coffee filter inside a larval rearing cup(A) ensuring it does not hang over the sides of the cup (B). The cup is then filled halfway with tap water then placed inside gallon buckets (C).

Hatch Eggs: See “Hatch Eggs” in assay activity description (Pages 5-6).

Separate Larvae: See “Separate larvae” in assay activity description (Page 6).

Feed Larvae: See “Feed larvae” in assay activity description (Page 7).

Refresh Larval Water: See “Refresh Larval Water” in assay activity description (Page 7).

Pick Pupae: Remove pupae from larval rearing cups using a plastic pipette and place into a new rearing cup containing ~100 ml of tap water. Place **both male and female** pupae into designated 1-gallon plastic buckets for colony adults and label **with strain, date of pupation and adult generation**. Place two cotton balls with fresh %10 sugar solution onto a small petri dish and insert into each 1- gallon bucket.

Set Up New Adult Cages: Freeze unproductive colony adult mosquitoes for 24 hr overnight then remove from 1-gallon buckets and dispose according to Biohazard Waste protocols. Allow netting to soak in a 10% bleach solution and clean the inside of buckets with soapy water (Figure 16A-C). Buckets can be immediately re-used once dry.



Fig. 16A-C: After removing dead mosquitoes from old colony buckets, soak netting in a 10% bleach solution (A) then clean the inside of the buckets using soapy water (B). Buckets can be reused immediately after drying C).

APPENDIX I

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APPENDIX II

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