Vosshall Laboratory Standard Operating Procedures for rearing *Aedes aegypti* Wild-Type Colonies and Individual Strains

Laboratory of Neurogenetics and Behavior, The Rockefeller University, 1230 York Avenue, Box 63, New York, NY 10065, USA

This protocol standardizes mass rearing of *Aedes aegypti* wild-type laboratory strains such as Orlando (ORL) and Liverpool (LVP-IB12) for maximal synchronization, uniformity, and fitness. By beginning cultures with large numbers of eggs and thinning at the larval stage, genetic bottlenecks are avoided.

Supplies needed

Item	Brand/Vendor	Cat #	Notes
Larval pan-large	Atlanta Fixture	14CW	50.8 x 30.5 x 10.2cm
Larval pan-small	Atlanta Fixture	24W	27.9 x 22.9 x 10.16cm
Adult cage-large (Bug Dorm)	BioQuip	1452	30 x 30 x 30cm
Adult cage-small (collapsible)	BioQuip	1450A	20.3 x 20.3 x 20.3cm
Tetramin fish food	Pet Mountain	16152	
Sugar feeder containers	Fisher Scientific	02911944	
Sugar feeder wicks	Richmond Dental	201205	
Sucrose	Amazon Sugar	B00060N5OW	
Plastic egg storage containers L	Rubbermaid/Amazon	7J76	41.9 x 29.2 x 8.9cm
Plastic egg storage containers S	Rubbermaid/Amazon	B00GJ840DA	23.9 X 23.9 x 8.1cm
Transfer Pipette Disposable	Fisher	137119D	
Whirl-Pak bag/egg storage	Nasco	B01065WA	
Whatman Filter paper circles	VWR	28450-048	5.5D
Pupal Cups (small)	VWR	8900-662	4 oz
Pupa Cups (medium)	VWR	8900-664	8 oz
Pupal Cups (large)	VWR	8900-668	32 oz
Cotton balls	Fisher Scientific	7886	
Cotton in roll	Stockroom	939000	
Tubular stockinette	VWR	56612-664	Case of 6
Tetramin food (small)	Pet Mountain	16106	7 oz
Tetramin food (large)	Pet Mountain	16152	10 oz
Xylazine	Fluka	46995	
Ketamine	Stockroom item, but must be picked up from Christopher Keogh in Purchasing	243000	*DEA Controlled Substances form needed to pick it up, signed by Leslie. Form must be filled out properly, and empty Ketamine vial

			must be turned in to Purchasing.
Needles 26 gauge	Stockroom	1219000	
Syringe 1ml	Stockroom	1869000	
Dishwashing Soap (Cascade)	Amazon	B002IXHQ9K	
Netting Washing Soap	Amazon	Ecos	
Autoclave bags	Fisher Scientific	01-826E	Biohazard disposable
Syringe with needle attached	VWR	BD329412	
Zip Ties	Tiewrap.com	350253	
Replacement sleeves (BugDorm cages)	Bio Quip	1452A	
Funnels for sucrose	Fisher Scientific	4256-0234	
Porcelain Mortar	Fisher Scientific	S337621CR	
Porcelain Pestle	Fisher Scientific	S337621CRM	
Forceps Dumont #5	Fine Science Tools	11254-20	
Kill tank filters	McMaster-Carr	98315k58	

Locations in laboratory where work is carried out

Smith S422A Insectary (Harris Environmental Room) 28^{o}C, 80% RH

Set to L:D 14:10

The light cycle settings are NOT changed when the time changes in the spring/fall of each year. The following lights on/lights off schedules vary according to the local time and are indicated by a sign posted on the door of the insectary. The sign should be switched the first Monday after the time changes in the spring and fall.

Eastern Standard Time (spring-fall)

Lights On: 7 A.M.

Lights Off: 9 P.M.

Eastern Daylight Savings Time (fall-spring)

Lights On: 6 A.M.

Lights Off: 8 P.M.

If you need to turn the lights on briefly for any URGENT need during the dark cycle, you need to use the override switch located in the control panel and indicated by a sign. IMPORTANT: turn the lights back off after you are done so that 14:10 light cycle is not disrupted.

Smith 422B Procedure Room

Smith S425 Tropical Room (Percival Environmental Room) 25^{o}C, 80% RH

The light cycle settings are NOT changed when the time changes in the spring/fall of each year. The following lights on/lights off schedules vary according to the local time and are indicated by a sign posted on the door of the Tropical Room. The sign should be switched the first Monday after the time changes in the spring and fall.

Eastern Standard Time (spring-fall)

Lights On: 7 A.M.

Lights Off: 9 P.M.

Eastern Daylight Savings Time (fall-spring)

Lights On: 6 A.M.

Lights Off: 8 P.M.

Smith S422B	Supply storage
Smith S422B	Hatching broth storage
Smith S422B	10% sucrose storage
Smith S422B	Morgue freezer small
Smith S429	Morgue freezer large
Smith S413	Egg storage (store on shelving above benches) in the main open lab

Insectary Standard Operating Procedures for Insectary Maintenance, Containment, Transport, and Shipping

These procedures are mandatory to put us in compliance with USDA ACL2 (arthropod containment level-2; similar to BSL-2) regulations.

1. All Vosshall Lab members who are involved in work with mosquitoes must read the Vosshall lab Mosquito Rearing Standard Operations Procedure Manual prior to commencing work with mosquitoes
2. The manager of the insectary will then walk new users through the proper procedures
3. All users must wear appropriate clothing and Personal Protective Equipment (PPE) to decrease the incidence of accidental release or cross-contamination of transgenic lines
4. It is the responsibility of all users to make sure that all containment apparatuses are in proper working order and to immediately repair any that are not. If a user cannot satisfactorily complete the repair, Leslie or the lab manager must be notified
5. It is the responsibility of all users working with transgenic mosquitoes to make sure that any accidental release within the procedure room or environmental room is immediately addressed by capturing or killing all released mosquitoes

Transport of Mosquitoes to ensure no accidental releases occur when lab members move between environmentally controlled room and other lab spaces with mosquito adults

1. Before removing any cage with mosquitoes from the Tropical room S425 or Procedure room S422B, lab members must check if there is any damage to the cage that will allow mosquitoes to escape.
2. The cage must be then placed in container before it is removed from the room. Lab members may use a clear plastic bag to contain the cage. It is essential to check that the bag does not have any holes that will allow mosquitoes to escape.

Proper shipping and receiving of transgenic mosquitoes

1. All transgenic mosquitoes must be shipped in a triple barrier: a container, within a container, within a container according to these government regulations:

7 CFR Part 340.8(4)

Insects, mites, and related organisms. Insects, mites, and other small arthropods shall be packed for shipment as specified in this paragraph or in paragraph (b)(3) of this section. Insects (any life stage) shall be placed in an escape-proof primary shipping container (insulated vacuum container, glass, metal, plastic, etc.) and sealed to prevent escape. Such primary container shall be placed securely within a secondary shipping container of crushproof

Styrofoam or other material of equivalent strength; one or more rigid ice packs may also be placed within the secondary shipping container; and sufficient packing material shall be added around the primary container to prevent movement of the primary shipping container. The secondary (Styrofoam or other) container shall be placed securely within an outer shipping container constructed of corrugated fiberboard, corrugated cardboard, wood, or other material of equivalent strength.

2. Where relevant, the CDC or USDA permit number must appear on the outside of the shipping container.

3. Send packages by Fedex, disclosing the contents on a customs declaration if the shipment is overseas.

Lab Member Requests for Strains and Supplies

1. Adequate supplies of clean hatching trays, oviposition cups, sugar feeder bottles and wicks, and netting
2. Assembled plastic cages by prior request
3. Oviposition vials by prior request
4. Hatching broth that meets quality control measures and in amounts as needed for each week
5. 10% sucrose for sugar feeders
6. Mouse blood feeding services by a regular weekly schedule and/or by special request
7. Production-scale availability of Orlando (ORL)
8. Production-scale availability of Liverpool (LVPib12)
9. On request, scale up mutant strains in active use
10. Maintain archival mutant strains (NPYLR1, orco2, orco5, orco16, Gr1, Gr2, Gr3, HP-1, wrw, ppk28, Ir7b.1, Ir76b, Ir25a, Ir8a, TRPA1, Gr19, and others to be produced in the future)

Sucrose for sugar feeders

GOAL: to prepare 10% liquid sucrose solution to sustain cages of adult mosquitoes

1. Weigh out 50 g of sucrose per 500 mL bottle and 100 g sucrose per 1L bottle
2. Add sucrose to clean bottle—be sure that bottle is in good condition with an intact thread seal that is not chipped
3. Label bottles with date of preparation (month/day/year); initials of person making solution; and “10% SUCROSE”
4. Add 500 mL or 1 L of milliQ (18 mOhm) water to the appropriate bottle
5. Shake well to dissolve
6. Seal with bottle with gasketed lid—be sure that both lid and gasket are in good condition
7. Put in pan and insert into autoclave—be sure that lids are partially open to avoid bottles exploding due to autoclave pressure
8. Autoclave on Steris Test S:00:01:00 cycle for 33 minutes
9. Remove from autoclave and immediately tighten lids
10. Store at room temperature in S422B

Hatching broth

GOAL: to prepare deoxygenated broth containing fish food for hatching eggs.

1. Prepare fresh weekly—discard any broth that is older than 7 days.
2. Label bottles with full date (month/day/year), initials of person preparing the hatching broth, and "HATCHING BROTH"
3. We currently prepare hatching broth in different "single-serving" volumes: small (400 mL), medium (850 mL), and large (1.5 L).
4. Fill Ball jar bottles with the following volume of tap-distilled water

Small	400 mL
Medium	850 mL
Large	1500 mL

5. Add the following number of pellets of Tetramin fish food ground into a fine powder in a mortar/pestle.
   In cases where you need ½ pellet, manually break it in half first.

Small	0.5 pellet
Medium	1 pellet
Large	1.5 pellet

6. Seal bottle loosely with lid—be sure that lid and gasket are in good repair so that bottles seal tightly after autoclaving
7. Shake to mix powdered fish food.
8. Check that lid is slightly loose or bottle will explode in autoclave due to pressure build-up.
9. Autoclave on Steris Test S:00:01:00 cycle for 33 minutes
10. Immediately after removal of bottles from the autoclave, seal lids very tightly to prevent oxygenation of the hatching broth.
11. Successfully prepared hatching broth bottles/jars will give off the characteristic sound of a sealed bottle/jar when opened: Pfffft. If the bottle/jar does not give off that sound, air has leaked into the jar and the hatching broth may not be optimal for rearing.
12. Store at room temperature in top shelf in S422B room number
13. Users to rinse hatching broth bottles/jars and place them in the S422B room bin. They are washed in our dishwashers, NOT brought to the washing facility.

MOSQUITO REARING PROTOCOL *Aedes aegypti*

This protocol serves as the standard operating procedure in the Vosshall lab for rearing and maintenance of lab stocks. Starting with a large number of eggs is intended to reduce potential genetic bottlenecks which can have negative consequences on mosquito fitness. Rearing animals for genetics, behavior or other experimental paradigms may differ from the following protocol in some details (number of animals, etc.), but nonetheless requires consistency and great care to avoid contamination of strains.

General notes

- Great care must be taken to avoid cross-contamination of different strains; even a single contaminating egg from a different strain will ruin a stock.
- Rearing of both larvae/pupae and adults occurs in a humidified insectary at 25-28°C, 70-80% RH with a 14h:10h light:dark photoperiod.
- Always wear fresh gloves when handling eggs. Change gloves often, and always between handling different strains.
- All waste from these procedures is considered biohazardous and must be disposed of in the appropriate receptacle in S422B.
    - Solid waste (paper towels, egg papers, Whirl-Pak bags, used diapers, pipettes, used oviposition vials, etc.) must be disposed of in the autoclave trash bin.
    - Liquid waste must be either frozen overnight (for small containers such as pupal cups) or placed into the kill tank containing sodium hypochlorite.
    - All glass or plasticware must be rinsed and be free of all eggs, larvae, pupae, adults, or leftover food prior to placing in the bin for washing. All tape must be removed.
- Stocks should be turned over (hatched, blood-fed, and eggs collected) at least every 3 months.
- Troubleshooting: if you find that pupation is not synchronized (<85% pupation by afternoon of day 8), make sure you are properly thinning larvae and that larval pans are properly fed (not too much, not too little).

Rearing from egg paper to adults

Day	Required Activity
1	Hatch Eggs
2	Remove egg paper
3	Thin Larvae
4 - 6	Feed Larvae
8	Collect Pupae
11	Remove Pupal Cups

Day 1 Hatch Eggs

- Prepare work area by wiping down bench with 70% ethanol and placing a clean diaper on bench.
- Plan to use between 3 and 6 well-stocked egg papers depending on age; recently collected eggs will hatch at higher rates while eggs stored for longer (up to 3 months) will have decreased viability.

- Label a full-size larval rearing pan or two half-size larval rearing pans (from storage closet S413) with your name, hatch date, and strain/genotype of eggs to be hatched. Use your personally-assigned color tape only.
- Rinse pan briefly with distilled water to remove any possible soap residue from cleaning.
- Open a fresh Ball jar of hatching broth (stored in S422B), ensuring that the seal is intact and pour 1L into larval pan.
- Open the Whirl-Pak bag containing egg papers on a fresh paper towel.
- Use forceps previously cleaned by wiping with a KimWipe soaked with 70% ethanol and dried, to carefully remove egg papers and place into larval pan

- Place lid(s) on pan(s), and place on a shelf in tropical room (S422A)
- Clean your working area by discarding diaper, paper towels, and empty Whirl-Pak bags in biohazard waste, wipe down bench and forceps with 70% ethanol. Rinse out hatch broth jars and place in the bin for washing.
- Discard gloves if finished. Change gloves if moving on to hatching additional strains and repeat the entire procedure above.

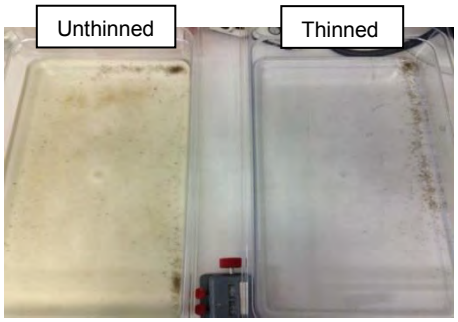
Day 2 Remove egg paper

- Clean forceps by wiping with a KimWipe soaked with 70% ethanol, wipe dry
- Using forceps, remove egg paper from larval pan. Wrap egg paper in a paper towel and discard in biohazard trash
- After every pan, clean forceps by wiping with a KimWipe soaked with 70% ethanol, wipe dry
- Add 2 L of distilled water to each large pan or 1L to both small pans using the large (5 L) water container above the sink
- Using a mortar and pestle, thoroughly crush one tablet of Tetramin, into a fine powder (no lumps) and add to each pan.
- Close lid and return pan to shelf

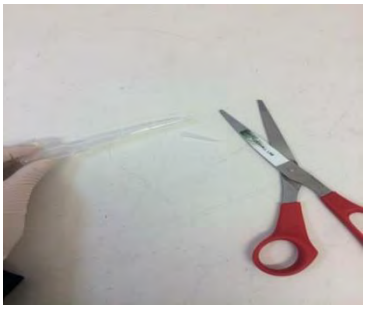
Day 3 Thin larvae

- Prepare work area by wiping down bench area in S422B with 70% ethanol
- For each original hatched pan, two new pans will be generated, each with 450 larvae.
- Label two new full-size larval pans or four new half-size larval pans with date, genotype, and your name.
- Add 3 L tap-distilled water to each pan
- Feed each pan with 2 tablets of Tetramin fish food, ground into a fine powder with a mortar/pestle.

- For the thinning procedure, you will be picking 450 larvae at random from the original pan.



- Using scissors, remove 15-20mm from the tip of a disposable plastic pipette



- Transfer a total of 450 larvae into each new full-size pan or 200 larvae into each new half-size pan. This is most easily done by transferring larvae in batches of 10 and using a click-counter to keep track of each transfer (45 or 20 clicks total).
- Return the two new pans to the insectary.
- Dispose of the contents of the original pan by tipping into the kill tank.
- Rinse empty pan by swirling tap water and tipping contents into kill tank.
- Place old tray and lid in the bin for dirty plasticware adjacent to the kill tank.

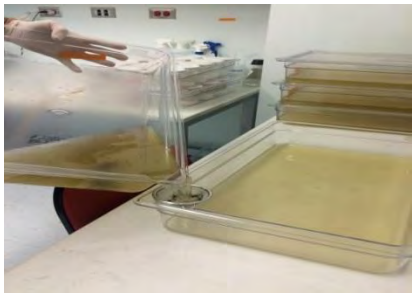
Days 4-6 Feed larvae

- Larvae must always have ample food. However, care must be taken not to overfeed as this will lead to bacterial growth and death. Water should remain relatively clear and odor-free.
- Check pans in the morning and evening to ensure that there is food present.
- On day 4, add 3 Tetramin tablets to each large pan or 1.5 tablets to each small pan by manually breaking them up and sprinkling throughout the pan. Avoid localizing to any one area (i.e., the front or the back).

- On days 5-6, ensure larvae are well fed (but not over-fed). Generally, 3 tablets per day should suffice for 450 larvae (though variations in animal number or strain may lead to differences).

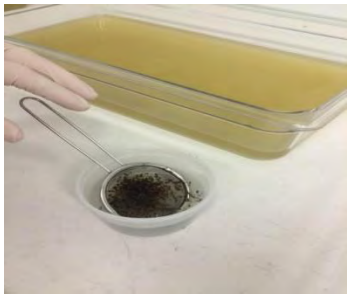
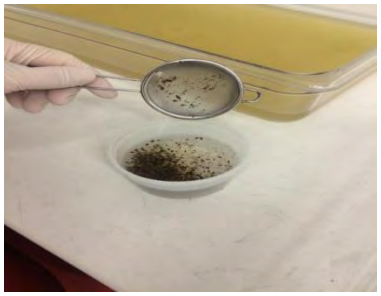
Days 7-8 Collect pupae

- The pupal stage lasts approximately 48 hours before adult mosquitoes eclose, and pupae must be picked and put into a cage before this happens.
- Typically, the first pupae will appear on day 7, with most of the pan pupating by day 8.
- The simplest and easiest way to collect pupae from a pan is by straining as described below. Alternatively, for pans that are not pupating synchronously (i.e., there are many larvae left), pupae can be picked into a pupal cup using a disposable plastic pipette with ~20mm removed from the tip.
- In the afternoon of day 8, prepare to strain pupae into a pupal cup.
    - Prepare work area by wiping down bench with 70% ethanol.
    - Get an empty full-size larval pan and a sterilized strainer from the wall of S422B.
    - Position strainer on the corner of the empty full-size pan



    - Slowly and carefully pour the contents of the pupating pan through the strainer (pupae and larvae will collect in the strainer)
    - If any pupae remain in the pan, add distilled water, swirl, and pour through strainer. Repeat as necessary.
    - Tip contents of collection pan into kill tank; rinse both collection and original pans with tap water into kill tank and place into the washing bin.
    - Transfer pupae to a large pupal cup by inverting the strainer above the cup and pouring distilled water over the strainer. There may be a few larvae mixed in, but if you have synchronized your rearing, there should be few if any larvae. As long as you take the pupal cups out of the cage once most pupae have eclosed, it should not be a problem if larvae are present from the beginning. They will

probably die for lack of food or will not eclose. Pupal cups should be no more than 3/4 full of water to prevent sloshing. Water should be clean; if it contains excess waste or food, decant wastewater into a new pupal cup and replace with clean distilled water.

- Used strainers must be placed into the small freezer morgue overnight.
- Set up a standard BioQuip BugDorm cage (30 x 30 x 30 cm). Check that the cage is well sealed and entrance sock well-secured to cage to prevent mosquito escapes.
- For each strain, there should be two pupal cups (each with ~450 pupae) in one cage, resulting in ~900 adult mosquitoes.
- With clean gloves, prepare 2 sugar feeders per cage. Fill glass sugar feeder to the brim with 10% sucrose. Add cotton wick.

- Label cage with your name/date/genotype of mosquito and place in insectary
- Before moving onto a new strain, re-clean the bench by wiping down with 70% ethanol and change gloves.

Day 11

- Remove pupal cups from cage on day 11 by putting a lid onto the pupal cup while it is still in the cage, removing from the cage, and placing into the small freezer in S422B.
- Keep the adults well fed after this point. Change sugar feeders once per week or whenever the wick looks moldy or the sugar feeders look dry.

Insectary Housekeeping

Guidelines for Lab Members

The mosquito insectary is a labor-intensive facility. We depend on our dedicated staff to keep the daily operations running. However, it is very important that individual users cooperate with the basic rules listed below:

1. Put cages, nets, and pupal cups in the freezer morgue
2. Rinse pans, cups, Ball jars, sucrose feeder bottles and put in the bin in the insectary
3. Rinse sucrose bottles and put in the autoclave room bin
4. Keep the benches clear
5. Label your cages
6. Discard old cages
7. Replace sugar feeders if you they become moldy
8. Participate in the monthly insectary mop-out. If you are not available on your assigned date/month, please switch with another user.
9. Sign up for mouse blood-feeding in advance so that we can plan for your needs
10. Sign up for production rearing in advance so that we can plan for your needs
11. Sign up for cages if you need a large supply number
12. Put cups from egg laying and pupae in the freezer morgue
13. Do not put egg papers in the kill tank
14. Sign up for oviposition vials if you need a large supply number
15. Sign up for hatching broth if you need to use more than 5 L or 3 large hatching broth jars (1500 ml each), or 10 medium jars (500 ml each)

Mosquito Disposal

Proper Disposal of mosquito life stages

Adults

1. Killed by desiccation, freezing, or autoclave
2. Carcasses are wiped out and all dry insect waste placed in the biological waste bin

Larvae and Pupae

1. Pour contents of the pans in the kill tank, rinse the pan and pour contents into the kill tank
   OR (if kill tank full)
2. Add 10% bleach to the larval pan and allow to sit overnight
   OR
   Freeze the whole pan

Eggs

1. All papers and gloves that come in contact with eggs will be disposed of in biological waste bags
2. The biological waste bag will be green tagged and placed in the biohazard waste closet S410.2
3. The biological waste will be picked up and autoclaved by lab safety

Cleaning of Cages and Larval Pans

Adult Cages

1. Remove mosquitoes
2. Cages are cleaned as follows
   a. Bug Dorm-cleaned in the Vosshall lab dishwasher using after rinsing them well on the “China Crystal” setting
   b. BioQuip cages-cleaned in the washing facility
   c. Bucket cages-cleaned in the washing facility

Larval Pans

1. Remove liquid
2. Rinse
3. Send to the washing facility

Kill Tank

Set Up and Use of Kill Tank for Aquatic Stages

1. Empty in the morning by turning on the pump; do not allow the pump to run dry, as soon as all liquid is gone, turn off the pump, otherwise the motor will be ruined
2. Add ½ gallon of bleach
3. Allow to sit overnight before emptying
4. Change filter every Friday or as needed. Debris will cause clogging of the filter and liquid cannot go through. Filters are in a drawer labelled on the bay across from S422B

Morgue Jobs

1. Every morning take cages, pupal cups, and sucrose containers out of the freezer morgue. Rinse them off well before putting them in the dishwasher
2. Bug dorm cages: remove the sleeve from the cage and put it to the washer; take out and rinse the sugar and pupal cups and put into the dishwasher. If there are too many pupal cups and sucrose containers put them in the large steel racks to be put in the

washers in the washing facility. The cage is taken apart and rinsed to remove excess blood and mosquitoes and put into the lab's dishwashing machines. Sugar wicks go into the biohazard bags

3. Silver metal cages, large and small: open and remove sugar and pupal cup as above; bang cage to free up loose mosquitoes and wipe with paper towel; cages are then sent to the washing facility
4. Bucket cages: remove top and put the netting into the wash; the safety ring is put into the dishwashers in the lab; the cage is rinsed and cleaned to remove blood and mosquitoes and is sent to the washing facility
5. Pupal containers with netting and rubber band: the netting is removed and put in the wash first removing the rubber band; containers are put in the biohazard waste bag
6. Large pans, large lids, and medium pans: rinse well and send to the washing facility
7. Small pans, medium and small lids: rinsed and washed in the lab's dishwasher
8. The biohazard waste bag is emptied when full. It is taped shut and removed and sprayed down with alcohol and then gets a green biohazard label and put in the biohazard waste closet S410.2 for lab safety to pick up
9. Working benches are all washed up with 70% ethanol

Release Inside Insectary/Procedure Room

To prevent mosquitoes from entering the general lab area in the event of an accidental release in the procedure room or environmental room:

1. Use shop vacuum to catch released mosquitoes. Seal the vacuum nozzle with plastic wrap and tape. Place the vacuum in the cold room for 24 hours, in the procedure room spray the vacuum with 70% ethanol and wipe with paper towel and discard contents in biological waste container
2. Place a sign with "Do Not Enter—Insect Release" on the procedure room entrance
3. Set up extra BG sentinel traps in the procedure room

Entry and Exit Procedure Room

There is an interlocking system that only allows one of the vestibule doors to open at a time prevent the escape of mosquitoes and into the general lab

Procedure Room Entry

1. The red button to the right must be pressed and held as the 1st vestibule door is pulled open
2. The outer door must be allowed to close completely; the red button to the right of the inner vestibule door must be held as the inner vestibule door is pushed open. The air curtain will automatically turn on.

3. Step into the procedure room and close the door completely. The air curtain will automatically turn off.

Procedure Room Exit

1. The red button to the left must be pressed and held as the door to the vestibule is pulled open. The air curtain will automatically turn on.
2. The inner vestibule door must be allowed to close completely behind you. The air curtain will automatically turn off. The red button to the left of the outer vestibule door must be pressed and held as the door is pushed open
3. Step out of the vestibule and close the door completely behind you

Entry and Exit Tropical Room

To prevent escape into the general lab you must use extra care, because the Tropical Room does not have an air interlocking system

Tropical Room Entry

1. Pull open the Tropical Room exterior door
2. Make sure that the door closes behind you once you enter the screened vestibule
3. Proceed into the Tropical Room through the screen door
4. Make sure that the screen door is closed behind you

Tropical Room Exit

1. Pull open the screen door and enter the vestibule
2. Make sure that the screen door closes behind you
3. Check for any loose mosquitoes in the vestibule and kill them before exiting
4. Proceed through the Tropical Room exterior door

Female blood-feeding

Egg production requires that females be fed to repletion on mammalian blood. Cages can be blood-fed on anaesthetized mice, artificial membrane feeders, or on human volunteers. The standard in 2014 is to use mice for general colony maintenance.

The current schedule is that staff members blood-feed cages on request several days a week. The schedule will be posted to indicate how many cages you need fed on each day that is offered. If your needs are not being met, let Leslie or the lab manager know right away. We can always make arrangements to increase our mouse colony, offer more feeding days, and/or establish membrane-feeding procedures. Please do not resort to feeding cages on yourself only because our facility is not meeting your needs.

Blood-feeding: anesthetized mice

The use of mice in mosquito blood-feeding is regulated by IACUC protocol 14756, approval date November 04, 2014; expiration date November 4, 2017.

Animals:

- Mouse (*Mus musculus*) (Jackson Labs)
- Strain/Breed: Swiss Webster
- Sex: F
- Age: 5 to 52 weeks
- Weight: 20-40 g

Census:

A total of 360 mice will be used over the course of a 3 year time period. 60 mice will be used routinely every 6 months for blood feeding.

Past experiences in the lab have shown that a single mouse is sufficient to feed up to 250 female mosquitoes. The number of mice will be scaled according to the number of female mosquitoes present in each cage. For instance, 2 mice would be the required to feed approximately 500 female mosquitoes. It will be ensured, that an individual mouse is not subject to overfeeding, reducing the risk of the animal perishing from excessive blood loss. *Aedes aegypti* will feed with the mice inside the cage, this method allows for enough surface area for mosquito feeding and ensures little crowding. *Anopheles gambiae* will feed with mice resting above the cage; this feeding method is proven to be effective and sufficient for *Anopheles gambiae* mosquitoes, and reduces the chance of mosquito escapes. Using either type of blood feeding method, mosquitoes will feed until satiety.

Any given mouse must be given at least 1 week rest before re-feeding a cage.

Transport of Mice to the Lab: Mice will be transported to and from the CBC/Smith 422B in containers supplied by the CBC.

Total number of animals used for three-year study: 360animals

Anesthesia:

Dosage and Dilution:

- 1ml ketamine (100mg/ml)
- 0.5ml xylazine (20mg/ml)
- 8.5ml of nuclease free water

Inject each mouse intraperitoneally with 0.2 ml of ketamine/xylazine dilution, place in a separate cage, and allow it to succumb to the anesthesia. If after 10 minutes, the mouse is still responsive, administer an additional 0.1ml of the mixture. The maximum dose of anesthesia that will be administered to any individual mouse will be 100mg/kg of ketamine and 10 mg/kg of xylazine.

Procedure:

Description of Feeding Method: When mice are non-responsive after foot-pad pinching, they are placed on the netted top of the mosquito cage (*Anopheles gambiae* blood feeding method), or placed inside of the cage (*Aedes aegypti* blood feeding method*)*.

The mouse will be left on the cage for a minimum of 5 minutes and a maximum of 20 minutes. The optimum feeding will be when mosquitoes are engorged and excreting excess fluids. Post-feeding mice will be placed back in their cages and monitored to make sure they fully recover from anesthesia. Mice will have a recovery time of 20 minutes after they were used for mosquito blood feeding and before their return to CBC.

Return procedure of mice: put the mice back in the transport container and return to the CBC. Mice that are kept in the lab in the transport containers that have no food or water should be returned to CBC within 3-4 hours. If the mice are kept in the lab in a cage, and food and water is provided, they must be returned to the CBC within 12 hours. Mice are not to be kept in the laboratory overnight. This is a violation of the IACUC protocol and AALAC standards.

Blood-feeding: human volunteers

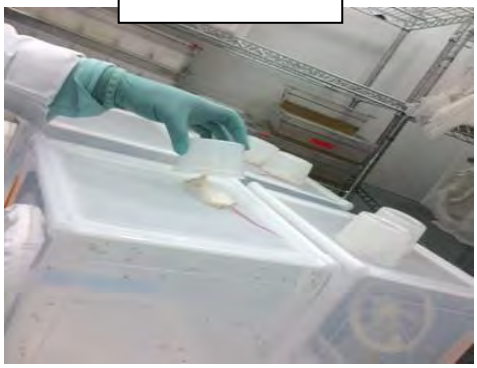
The use of human volunteers in mosquito blood-feeding is regulated by IRB protocol LVO-0652, which is renewed annually in October. No one can blood-feed a mosquito or participate in behavioral experiments with mosquitoes unless they are registered as a subject in this IRB protocol. The current text of the approved protocol and the consent form can be found in a binder above the lab manager's bench. Please consult with Leslie if you have any questions or want to be added to or removed from the protocol. If you are not comfortable discussing any aspect of this IRB protocol with Leslie, please contact Sid Strickland in the Dean's Office or the IRB office in the RU Hospital.

Mating, Blood feeding and Egg-Collection

Day	Required Activity
1 (5-10 days post-eclosion)	Blood-feeding
5	Egg collection
7	Egg conditioning
10	Hatching or storage

- After emergence, females and males will begin mating freely after ~2 days. If virgin females are required, they must be completely separated from males within 24 hours of emergence.
- For colony maintenance, mosquitoes can be blood-fed 5-10 days post-emergence.
- **Day 1: Blood-feeding**
    - The morning of blood-feeding, remove the sugar wick from the cage.
    - Place anesthetized mouse in cage in S422B, and monitor until the majority (>90%) of mosquitoes have fed to repletion (typically 15-20 minutes).

Feeding
Anopheles gambie



Feeding
Aedes aegypti

    - After blood-feeding, remove the mouse, and add a new sugar feeder to the cage.
- **Day 5: Egg collection**
    - To prevent contamination, it is absolutely critical that you only work with 1 strain at a time. Mosquito eggs are very sticky, and in the case of *Aedes aegypti*, also very hardy. A stray egg that sticks somewhere is easily introduced into another strain if you are not extremely scrupulous and clean in your work at this stage.

- Four days after blood-feeding, prepare two large oviposition cups by filling each halfway with distilled water. Each cup is ringed with 6 overlapping Whatman papers (55mm in diameter)

- Label oviposition cups with name date and strain and place two oviposition cups into each cage.

- **Day 7: Egg conditioning**

  - Clean the bench by wiping down with 70% ethanol
  - Put down a clean diaper
  - Remove oviposition cups two days after introducing them to the cage
  - Egg papers can be dirty at this stage. To clean them for storage, follow the following procedure
    - Tip out water from cup into the kill tank.
    - Fill the cup halfway with distilled water
    - Gently tip/swirl water to rinse off the eggs to remove debris and dead mosquitoes.
    - Tip out rinse water into the kill tank; repeat rinse as necessary to remove most debris.
  - Take out one egg paper at a time using forceps that have been cleaned with 70% ethanol and dried with a KimWipe.
  - Place egg papers face up onto a paper towel in a single layer. Do not allow egg papers to overlap.

- Allow the papers to sit on the paper towel for 5 minutes to wick away residual moisture.
- Prepare a half-sized tray lined with a double layer of dry paper towels
- Place individual egg papers into tray with forceps; ensure that egg papers do not overlap.

- Place lid on tray and keep in the insectary for 3 days.
- When collecting eggs for multiple strains, you must change gloves, dispose of old diaper, clean bench with 70% ethanol, sterilize forceps with 70% ethanol and place a new diaper on the bench between every strain.

- **Day 10: Hatching or storage**
  - At this point, egg papers can either be hatched immediately or dried down for storage (up to 3 months).
  - For hatching; see above protocol.
  - Egg storage
    - Clean the bench by wiping down with 70% ethanol
    - Put down a clean diaper and put on fresh gloves
    - For each set of 3 egg papers, cut a standard single-fold paper towel into 3 equal sections along the long axis.
    - Put down one section of paper towel, put 1egg paper on top, then put paper towel on top of that. Repeat until you have a stack of egg papers interleaved between pieces of paper towels.

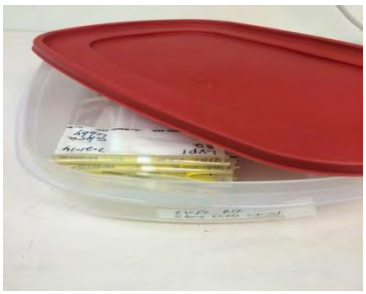
- Each egg paper should end up sandwiched between a layer of paper towel so that they dry properly

- Label a Whirl-Pak bag with your name, genotype/strain, and the date of storage.

- Place each set of egg papers/paper towels in a separate labelled Whirl-Pak bag and seal by folding over three times and securing with the wires.

- Each Whirl-Pak bag should be a “single serving,” meaning that all egg papers in that bag are hatched at the same time. Some people may put 3 egg papers/bag, while others will put up to 6 egg papers/bag. Being consistent about the number of egg papers stored per bag will minimize stray bags with old eggs and make the egg inventory process much easier.

- Place Whirl-Pak bags into plastic food storage containers with tight-fitting lids and leave at room temperature out of direct sunlight. Label each container clearly with your name, date, and the genotype/strain.



- Each genotype or strain should have its own separate container.

- Keep track of lab strain/archival mutant strain rearing schedule in a Google Lab Rearing Calendar. Stocks must be turned over within 3 months of egg storage. Reminders should be programmed into the Google Lab Rearing Calendar.

1. IMPORTANT: Only store egg papers in Whirl-Pak bags, not loose on paper towels in a container.
2. IMPORTANT: Do not mix strains/genotypes in the same container. Contamination is a real and scary issue with any stored eggs.
3. IMPORTANT: Whatever you do, do NOT store eggs in hatching pans! We need these for rearing, and also as we learned in the Great Flood of 2014, hatching pans are NOT water proof. Roman Corfas lost a large number of egg papers because the hatching

pans were flooded and eggs started to hatch. Use only water-tight food storage containers for egg storage.

4. Rotate stocks of production rearing and mutant strains every 3 months. Discard egg papers that are older than 3 months.
5. Keep track of lab strain/archival mutant strain rearing schedule in a Google Lab Rearing Calendar.

Procedure for ovivials

1. Once egg-laying is complete (after 2 days from the original introduction of females into ovivials/after blood-feeding), remove the animals from the ovivial and pull out egg paper
2. Follow the Egg Collection procedure previously explained in this SOP manual

Sexing mosquitoes

Sexing at pupal stage

This can be done by visual size separation. Female pupae are generally larger than male pupae.

Photo of *Aedes aegypti* pupae: male (bottom) and female (top)

Sexing at adult stage

Mosquito antennae are decorated with sensilla (fine sensory hairs). The male mosquito has significantly more long sensilla than female antennae. The “feathery” appearance of the male antennae will allow you to tell males apart from females by eye without a microscope.

Adult mating

1. To assure that female mosquitoes are virgin before mating for crosses, female and male mosquitoes must be separated within 24 hours of eclosion
2. A single male may mate with multiple females
3. The smallest volume of enclosure containment suitable for mating is the Solo 16 oz paper cup soup container
4. Males and females need to be at least 2 days old post-eclosion before mating
5. Leave male and female mosquitoes together for 24-48 hours to be certain that mating has occurred