Short-Term Freezing of Anopheles stephensi (Mosquito) Larvae

I. Materials

a. Mosquito eggs on egg paper
b. Insectary chamber, capable of programable temperature, light, and humidity
c. Distilled water
d. Fish food
e. Small hatch bin (a solid, polycarbonate or greater strength container, that can hold water to a depth of 5 or more cm, with dimensions approximately 10 cm x 20 cm)
f. Two (or more) Large Hatch bins (dimensions approximately 20 cm x 40 cm)
g. Plastic transfer pipettes
h. Strainer
i. Gloves
j. Small (2 oz) disposable cups
k. Insect rearing cage with nylon mesh screen and port
l. Scissors
m. Circulating water pump
n. Glass insect feeder
o. Parafilm
p. Filter paper
q. Defibrinated calf blood
r. Microscope slides
s. Olympus SZX16 Advanced Stereo Microscope (or similar)
t. Penetrating cryoprotective agents (see examples, section III.A
u. Non-penetrating cryoprotective agents (we use 0.5 M Trehalose)
v. Square culture (petri) plates
w. FalconTM Cell Strainers, 70 um (or similar)
x. Thermoelectric Laboratory Cold Plate (AHP-1200CPV or similar)
y. Kim wipes

II. Phase I: Establish and maintain mosquito colony

A. Phenotypic Analysis of developing mosquitoes (Figure 1)

Larvae develop from eggs to first instar larvae (L1) around 2-3 days after transferring egg paper. L1 develop into L2/L3 over the next 4 days (approximately). During that time, they show the ability to hatch, feed and further develop. L4 transition into pupa and emerge as adults approximately 2 days after pupation.

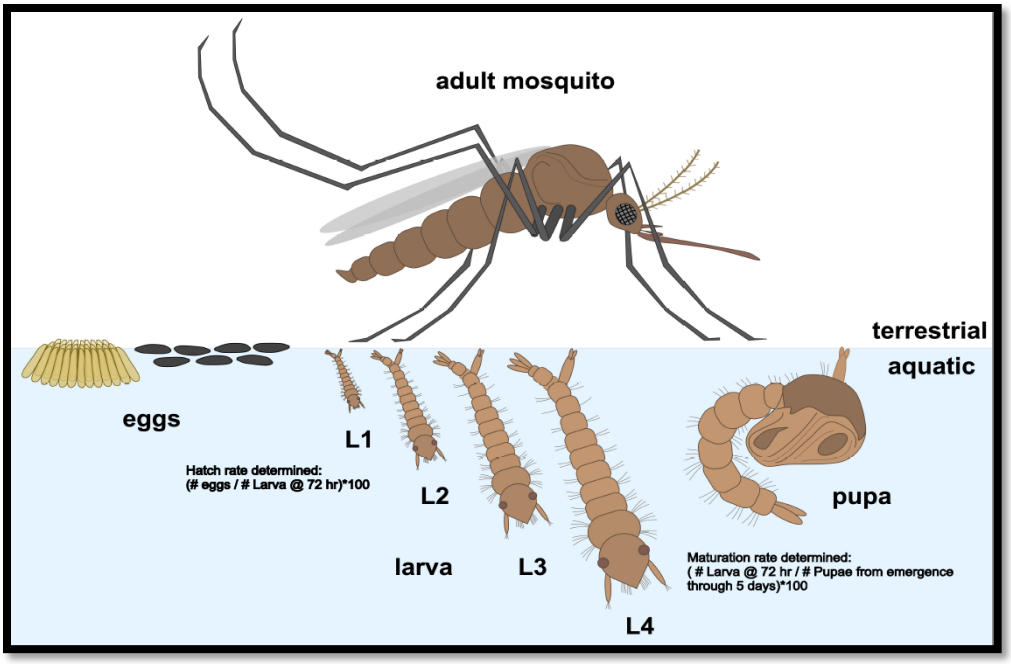

Figure 1: An. stephensi life stages showing hatch and maturation rate data collection points

B. Husbandry (modified from MR4 Methods in Anopheles Research. MR4 M*ethods in Anopheles Research Laboratory Manual*, BEI Resources, 2015)

1. Hatch eggs

- Obtain egg paper with referenced eggs from BEI or other qualified source.
- Take clean small hatch bin, label with date, and dispense ~500mL of distilled water. Dispense slowly to avoid bubbles (eggs will not hatch in a synchronized manner).
- Put on gloves and place the egg paper into the water.
- Add a pinch of fish food and cover the pan
- Place pan in insect chamber and maintain under controlled conditions of temperature (28 °C), relative humidity (70%) and light (12:12 L:D diurnal cycle).

2. Larvae splitting and feeding

- Move larvae into a larger bin using a strainer, roughly 24 hours after eggs are hatched. Follow the same instructions as far as pouring DI water slowly; from here tap water can be used.
- Make sure to thoroughly rinse out the small bin where eggs were hatched.
- For the following days, make sure that bins are not over saturated with larvae (~ 60% saturation). Higher than that, larvae will stop growing. So split as much as necessary
- Add food and cover bin.
- During this period, changing the water may be necessary to avoid mold and other microbial growth. Use strainers and transfer into a fresh bin if necessary

3. **Picking up pupae**
    - Pour an ounce of tap water into a clear, plastic, disposable 2oz (about 59.15 ml) cup.
    - Use a transfer pipette or a small mesh-made-scoop to collect the pupae into the cup.
    - Transfer the cup into an insect rearing cage by placing cup through top port. Add sugar and water cups through the port.
    - Males will emerge first, so it is important to pick up pupae for several days (~5) to ensure females emerging from pupae are also picked.
4. **Blood feeding mosquitoes (glass feeder protocol)**
    - The optimal time to blood feed mosquitoes is 3-5 days post-eclosion
    - Remove sugar and water 12-16 hrs. prior to blood feed (Note: the colony will significantly die off if starved for more than 24 hrs.)
    - Fill water basin with tap water and attach heating element/pump. Turn on and pre-warm water to 37 – 42C.
    - Prepare glass feeders. Use scissors to cut the length of parafilm or hog gut sufficient to cover mouth of glass feeder. (Note: hog gut is often stored frozen. Allow time to thaw before use or thaw in tap water). Stretch tightly across mouth without tearing and use rubber band to hold membrane in place. (Note: If hog gut is used, be careful not to let it dry out. A petri dish with wet paper towels may be used for this purpose. Do not place prepared glass feeders in petri dish with standing water as the water may enter the feeder through the membrane and dilute the blood.
    - Briefly turn off the water pump and use plastic tubing to connect all feeders in a circuit that leads back into the water bath. (Wet each end of each piece of plastic tubing before use to ensure easier fit.) Turn pump back on and ensure A) a steady flow of water around circuit and B) no dripping. Do not, however, place feeders on top of cartons yet, as mosquitoes may begin to drink and fill up on water.
    - Use transfer pipette to measure appropriate amount of blood into well of glass feeder. 1-2 ml per feeder is more than enough.
    - Place feeders on top of cartons and begin feeding. Optional: tear off a long strip of tape to hold glass feeder firmly in place against roof of mosquito carton.
    - Visually inspect mosquitoes to see if they are feeding. Look for females clustered around feeder with bright red full bellies. Ensure that the temperature of the water is approximately 37 C. Some variability in temperature is permissible but note that parafilm may melt/deform if bath is run too hot.
    - Allow mosquitoes to feed for up to one hour. Return all mosquito cartons to insectary and disassemble water bath apparatus.
    - Let glass feeder and membrane soak in 10% bleach solution for at least 15 minutes. Dispose of membrane and rubber band in trash. Wash glass feeder and let dry.

- Collect eggs onto filter paper and continue the process to maintain colony.

C. Obtaining and counting larvae

1. At 16-18 hours post-egg hatching, remove *An. stephensi* larvae hatch bin.
2. Transfer a small amount of water from the hatching habitat to a new bin.
3. Cut the tip of a plastic bulb pipette with scissors, approximately 1cm above the tip, to widen the inlet area of the pipette.
4. Remove 7-20 larvae at a time by pipetting, and count larvae through the clear walls of the pipette.
5. Transfer to a new bin containing hatch bin water.
6. After larvae are counted, transfer them back to the original habitat, along with the water from the new hatch bin.

D. Phenotypic Characterization of Larvae at each Instar by Advanced Stereo Microscope (Larval Microscopy)

1. Cut the tip of a plastic bulb pipette with scissors, approximately 1cm above the tip, to widen the inlet area of the pipette.
2. Remove 5-7 larvae from their hatch bin, and transfer in one drop to a glass microscope slide.
3. Analyze larvae using an Olympus SZX16 Advanced Stereo Microscope, at variable magnification settings and determine (a tabulation might help):
    a. Microscope settings
    b. Photographic and descriptive phenotypic characterization of larvae at each instar:
    c. Number of larvae at first instar (L1)
    d. Characteristics of L1 larvae and on through pupation

III. Phase 2: Freezing and Recovery of L1 Larvae

Note: for each of these initial conditions, 150-400 larvae are used per condition. Maturation rate (L1-pupae) is determined for evaluation during initial experiments.

A. Cryoprotectant Selection

Prepare cryoprotectants (or Cryoprotective Agents, CPAs) in nano pure water at room temperature and determine toxicity of cryoprotectants to the L1 larvae. Potential cryoprotectants are listed below.

Potential Cryoprotectants

1. 1.5 M ethylene glycol (EG),
2. 1.5 M methanol (ML),
3. 1.5 M dimethyl sulfoxide (DMSO), or
4. 1.5 M methyl acetamide or methyl formamide (MA or MF)

B. Determination of the Toxicity of Cryoprotectants

1. Collect 50 or more L1 larvae from the insectary bin using a transfer pipette with a blunt end and transfer onto a filter dish.
2. Immerse the larva in 1.5 M EG, 1.5 M ML, 1.5 M DMSO, or 1.5 M MA/MF, or water alone (control) while in the filter dish for 1, 2, 3, 4, 5, 6, or 8 h at room temperature (22-25°C).
3. After the appropriate exposure time, drain respective CPA from each dish and rinse larva by immersion in tap water while they remain in the filter dish.
4. Continue Step three with two additional washes, or empirically based on sheen of the L1 larvae.
5. Culture the L1 larvae in water with fish food (as described in Section 1.B) and assess for survival based morphological changes from L1-L4 larvae.
6. Determine the percent survival (Percent survival = # of pupae collected/initial egg count) for each time point for a given CPA solution.
7. Record a minimum of three replicates of each to assess toxicity.

C. Freezing larvae

In this example, a mixture of 7M methyl formamide (MF) in 0.5 M of trehalose was chosen as the optimum CPA. Further, our studies with newly emerged versus late L1 stage larvae supported using 14–24-hour old L1 larvae for freezing. A diagram of the protocol for this process is provided (**Figure 2**).

1. Prepare the CPA solution of 7 M MF in 0.5 M trehalose in a square culture dish and place dish on cooling table set at 28°C.
2. Place 14-20 hr emerged L1 larvae in clean water. Count larvae.
3. Transfer larvae to a nylon mesh basket submerged in clean water that is in a square culture dish set on the cooling table. Use ~ 200 L1 larvae per condition group for optimum data sets.
4. Begin slow cooling on the cooling plate from 28°C to 0°C over 30 minutes.
5. Retract the basket from the water, blot dry the L1 Larvae with a Kim wipe, and transfer the basket to the culture dish containing the CPA solution.
6. Hold for 5 minutes at 0°C
7. Begin rapid cooling of L1 Larvae on cooling table, and cool to -15°C.
8. Leave L1 larvae on cooling table (10 min – 2 hr), or transfer to -20°C freezer (longer time periods) until warming.

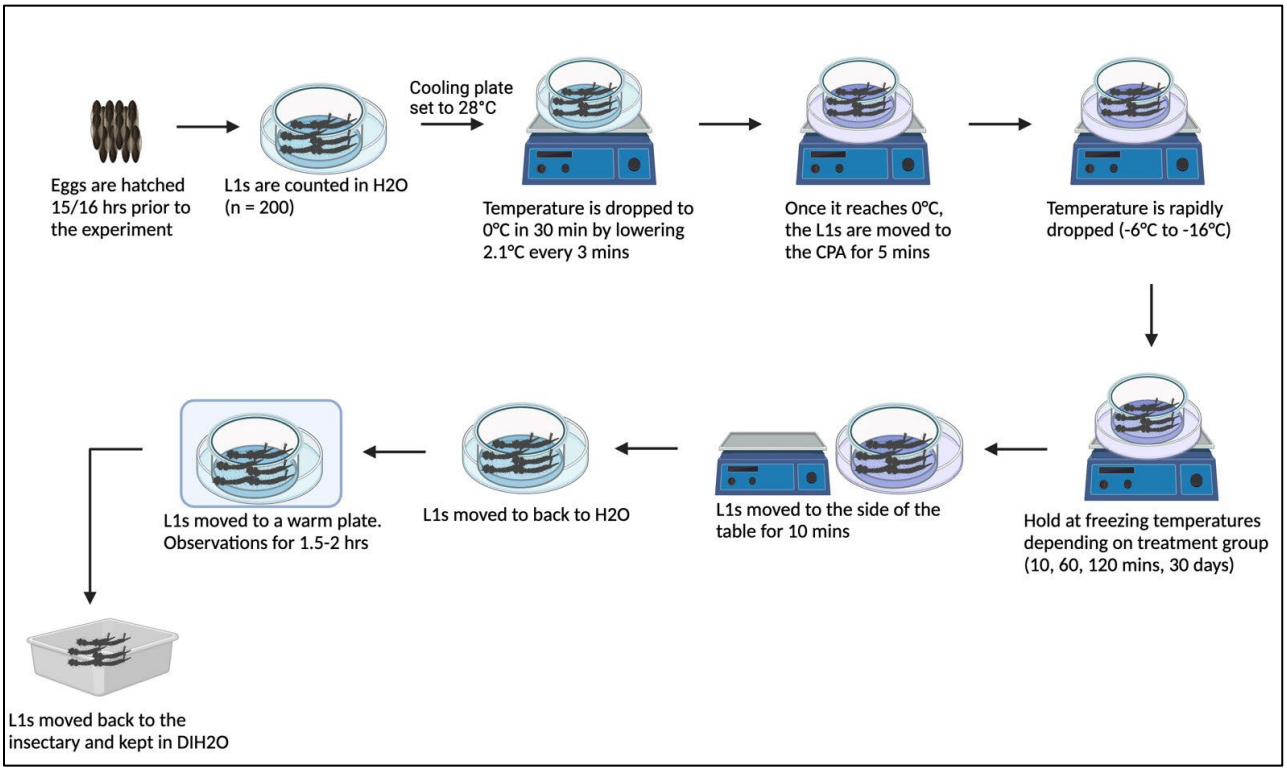

Figure 2. Diagram of L1 larvae freezing process

D. Warming larvae

The current warming method is described below. We note that additional warming trials may need to be conducted for further optimization; viability using this method and maintaining freezing conditions for 10 min is 60%.

1. Remove larvae from the cooling table and hold on the side of the table, at 4°C for 10 min.
2. Remove the mesh basket and submerge into a square culture dish containing water held at 4°C and incubate for an additional 5 min.
3. After this time, move the L1 larvae to a square dish with water held at 28°C for 1-2 hrs. Perform initial viability assessment by watching for movement (including mid-gut movement) using a dissecting microscope.
4. Return L1 larvae to husbandry conditions for recovery and development through life stages (**Figure 1**).
5. Determine recovery and analysis of viability as described below.

E. Viability studies

1. Determine the number of pupae that emerge from each freezing incubation time point; calculate the percent recovery as done for percent survival, based on number of L1 larvae from each time point.
2. Determine the percentage of recovered pupae that survive and develop into adults.
3. Determine the ratio of males to females (sexual differentiation) in adult mosquitoes.
4. Perform phenotypic similarity analyses (via microscopy; section I.C. of this protocol)
5. Determine the number of eggs subsequently produced from adults maturing after recovery from cryopreservation.
6. Determine the number of larvae (F2) hatching from eggs produced by adults that matured after recovery from freezing to confirm the colony has returned to healthy, normal husbandry conditions.