Larval Bioassay method (drafted by Bill Brogdon and updated by Nsa Dada)

Time mortality data gathered from the following larval bioassays is a measure of **the time it takes for a compound to penetrate the mosquito, traverse intervening tissues, get to the target site and act on that target site**. Anything that prevents or delays the compound from doing it's job is contributing to resistance.

Materials: 150 ml glass beaker
Pipetters and tips
Absolute alcohol
Chemical(s) to be tested
Stirring rod or similar equipment
Timer
Late 3rd or early 4 th Instar Mosquito larvae

Preparation:

1. Make sure beakers are clean.

2. Mix stock solutions. If stock solutions are already made, make sure you allow enough time for them to come to room temperature before use. Gently swirl the stock solution to mix it before use.

To make stock solutions, dilute the appropriate amount of technical grade insecticide in absolute alcohol (Table 1). Suggested amounts can be found in Table 1. Once this stock solution is made, it can be stored in the refrigerator in lightproof bottles until needed.

3. Label your beakers.

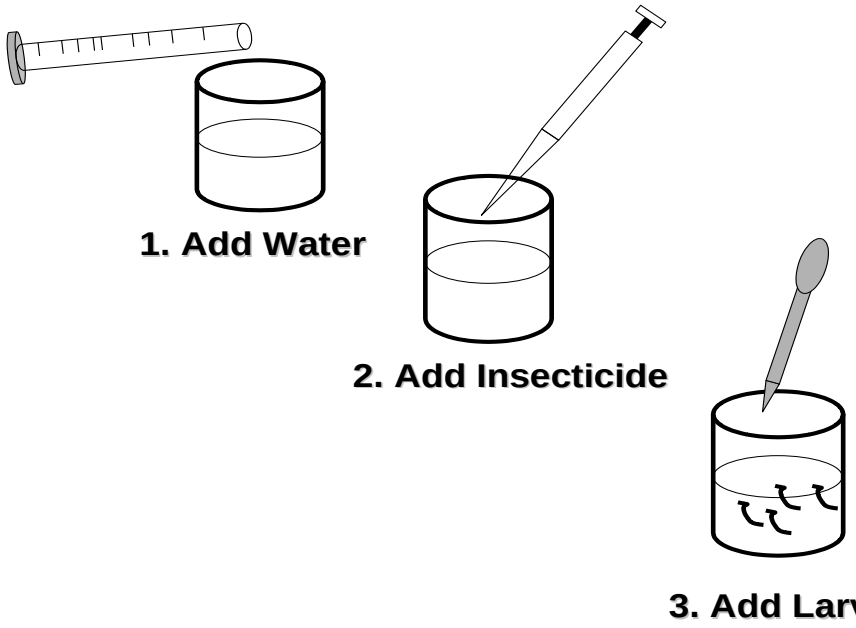
Dosing the beakers:

1. Add 100 ml of distilled water to each of the beakers.

2. Add 1 ml of the appropriate premixed stock solution (mixed in section 2 above) for the chemical you are working with and mix with the distilled water already in the beaker.

3. Be sure to make control beakers to run along side your insecticide beakers. Follow the same steps as above but **do not** add any chemical other than 1 ml ethyl alcohol to the beaker.

4. It is a good idea to let the beakers Abreath@ at least 10-15 minutes before use, to allow the alcohol to evaporate from the beaker.



More than one batch of mosquitoes can be run in a beaker in a day. After each repetition, discard the solution and begin again. Use care to reuse the same beakers with the same insecticide.

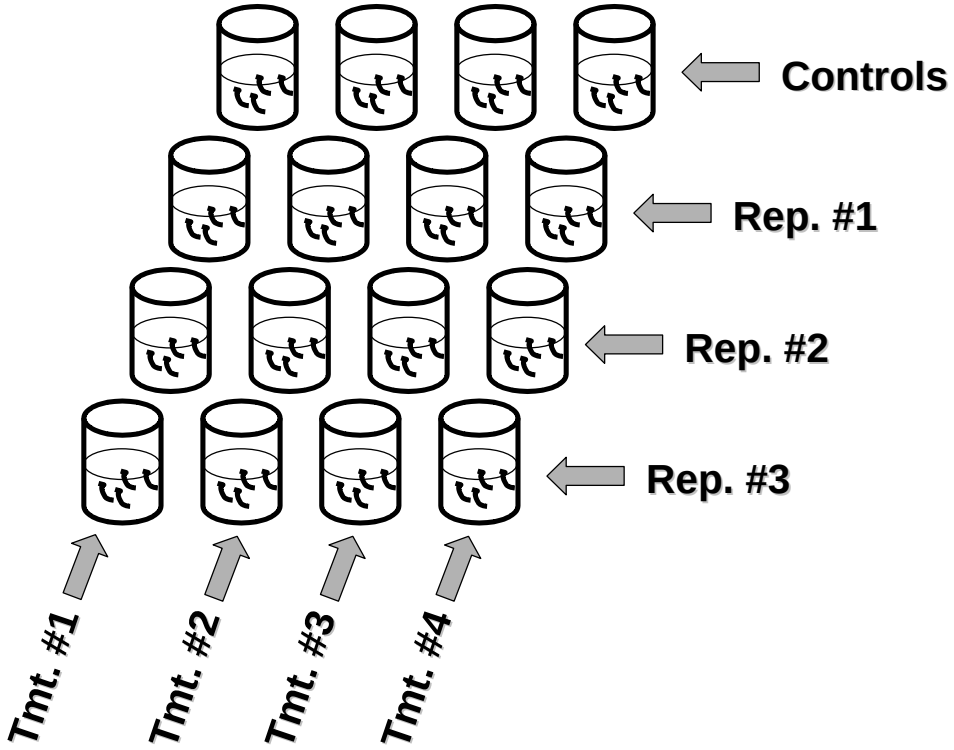
Running the Assay:

1. Aspirate 15 to 25 mosquitoes into each beaker. Use a number you can easily count with accuracy as the mosquitoes swim about. With practice you can use many more mosquitoes than at first. It is best if you collect all of the mosquitoes together in a pipette and introduce them into the bottle at once; this will reduce the amount of additional water that is added to the beaker and minimize possible contaminates/organic material from the larval water. In the case of especially organic rearing water, larvae may first be transferred into clean water prior to sampling.

2. Examine the beaker to be sure all mosquitoes survived the transfer process. Especially avoid exuviae, which can be easily confused with dead larvae. Remove all exuvae and pupae as necessary.

3. Start a timer and record how many mosquitoes are dead or alive (which ever is easier to count) every 15 min. until all are dead or 1.5 - 2 h have elapsed. Tapping the beaker or gently touching the larvae with a stirring rod or other small instrument works well to identify alive mosquitoes. You may want to continue for a total of 2 h when working with Malathion or Fenthion as these chemicals tend to take longer to kill mosquitoes than pyrethroids, for example.

4. Recount the total number of mosquitoes in each beaker and calculate the percent mortality for each 15 minute interval. To get an accurate count, empty the beaker through a screen to collect the larvae and count on a paper towel. Plot percent mortality (y axis) against time (x axis) using a probability scale for the percent mortality. If probability paper or a suitable statistical computer program is unavailable, a standard graphical plot is functional.



Clean up:

When you are finished with your beakers or they have become too old to use, triple rinse them with acetone and wash them with warm soapy water. Place them in an oven to thoroughly dry before using them again. If you are uncertain whether they are completely clean, introduce some susceptible mosquitoes into the beaker after you dried them. They should not die right away. If they do, clean the beakers again.

Table 1. Stock solutions and dosages we currently use:

Stock* (Techgrade AI + Alcohol)		**Amount of Stock Added to tests**	**Final concentration/beaker****
		Insecticides	
Permethrin	100 µg/ml	1 ml	100 µg/beaker
Resmethrin	100 µg/ml	1 ml	100 µg/beaker
Malathion	100 µg/ml	1 ml	100 µg/beaker
Synergists			
DEF	??µg/ml	??µg	??µg/beaker
PBO	??µg/ml	??µg	??µg/beaker
Control		1 ml	0 µg/beaker

*Example: To make stock permethrin (1 mg/ml), add 10 mg technical grade permethrin to 10 ml alcohol. To make a stock with liquid techgrade insecticide, add 10 µl of techgrade insecticide to 10 ml of alcohol. This yields 1 mg/ml from which serial dilutions can be made.
** Final concentrations are based on a density of 1 for the technical grade insecticide. If insecticide is purchased as a solid instead of a liquid, then the proper amount can be weighed to come to a final concentration in µg/beaker.

Calibrating a beaker:

To decide on what concentration of insecticide to use when running a larval bioassay, you will have to calibrate the beaker. To do so you will need to select susceptible mosquitoes to use as a base line. You will also need to select a range of concentrations to use.

1. Prepare beakers as above making several sets with a range of different concentrations.

2. Run assays on susceptible mosquitoes.

3. When you graph the results you should see that with increasing concentration the line gets straighter, steeper and moves toward the Y axis. If you are in the correct range, the line will reach a point where increasing the concentration does not change the line. This is the Asaturation@ point for the mosquitoes. Increasing the concentration does not cause the insecticide to penetrate the mosquito and get to the target site any quicker. This is the concentration you want to work with. If you find this concentration works too quickly, you can use a lesser concentration that gives you a straight line and kills the mosquitoes in a reasonable amount of time.

4. You may have to repeat using different concentrations (larger or smaller) or smaller increments between concentrations to find the right saturation point. For example you may start out with increments of 100 ug, starting with 100 ug/beaker and going to1000 ug/beaker. If you did not see a clear saturation point you may need to run more beakers with < 100 ug/beaker or > 1000 ug/beaker. On the other hand, if you do see a clear saturation point, you can refine what that value is by running more beakers at smaller increments near where you see the break. You can go back and rerun beakers using an increment of 100 fold concentrations. We utilized pesticide solutions of 100 μg/ml, 10 μg/ml, 1 μg/ml, and 0.1μg/ml.

Interpreting beaker data:

Like all resistance tests, larval bioassay data need to be compared to data from Asusceptible@ or base line sources. A resistance threshold for each insecticide can be determined by drawing a straight line down from the point at which all of the susceptible colony mosquitoes died. If any of your mosquitoes survived beyond this threshold, you may interpret this to mean that these survivors represent that proportion of the population that has something allowing them to delay the insecticide from reaching the target site and acting or in other words, they have some degree of resistance.

Using Synergists:

One of the advantages of the larval bioassay is that synergists can be added to the beakers (along with the insecticide) if resistance is found. This will help you determine what mechanism(s) is acting. PBO (piperonyl butoxide) eliminates oxidase activity and DEF (S.S.S-tributlyphosphorotrithioate) eliminates esterase activity. If the same population with resistance is run in beakers with one of the synergist added, that population should go back to looking like a susceptible population if the mechanism was based on an elevated level of the particular enzyme that synergist elimintates.

Chemical Name	**Company / Catalog # [1]**	**Price / Amount [2]**
Propoxur	ChemService / F2045	$42.60 / 1 g
Permethrin	ChemService / PS758	$58.90 / 100 mg
PBO (Piperonyl Butoxide)	ChemService / PS100	$39.00 / 100 mg
Resmethrin	ChemService / PS1000	$84.90 / 100 mg
Naled (Dibrom)	ChemService / PS605	$45.60 / 1 g
Malathion	ChemService / PS86	$47.10 / 1 g
DEF (S.S.S-Tributlyphosphorotrithioate)	ChemService / PS562	$42.60 / 1 g
Fenthion	ChemService / PS655	$50.50 / 1 g

ChemService
P.O. Box 3108
660 Tower Ln.
West Chester, PA 19381
1-800-452-9994
fax 610-692-8729

[1] Mention of a Company name is not an endorsement of that company nor does it mean this is the sole or cheapest source of the Chemical.

[2]Prices are from 1996 catalogs and may not be accurate. Check with the vendor for current prices.

Equipment / Instruments and Supplies

Beakers - 150 ml ($165-182.30 for 48 beakers)
Pipetors (10-100 ul and 100-1000 ul - $215 each)
Pipetor tips (box of 96 $48-$55, bag of 1000 $20.61-$28.59)
timers ($11.65 - $27.00)
glass containers to store chemicals in (30ml size $58.96 for 48)

Set up including 2 chemicals about $850

Equipment/Instruments and Supplies *

Baxter
601 S.W. 33rd Ave.
Ocala, FL 32670
1-800-234-5227

PGC Scientifics
P.O. Box 7277
Gaithersburg, MD 20898-7277
1-800-424-3300
fax 1-800-662-1112

Fischer Scientific
P.O. Box 4829
Norcross, GA 30091
1-800-766-7000
fax 1-800-926-1166

VWR
P.O. Box 669967
Marietta, GA 30066
1-800-932-5000

Carolina Science and Math
1-800-334-5551 ext 5310
fax 1-800-222-7112

Cole Parmer Instrument Co.
7425 N. Oak Park Ave.
Niles, IL 60714
1-800-323-4340
fax 708-647-966