Supplemental Material for the video entitled

“Testing for Resistance in Larval Mosquitoes: An Overview”

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How to Use the Video and Supplemental Information:

This video provides an overview of resistance testing for mosquito larvae. The results of a survey of mosquito control organizations carried out by the National Association of County and City Health Officials (NACCHO) in 2017 (https://www.naccho.org/ uploads/downloadable-resources/Mosquito-control-in-the-U.S.-Report.pdf) indicated that training in pesticide resistance testing was one of the greatest needs of mosquito control organizations in the United States of America. Resistance testing of adult mosquitoes, such as the CDC bottle bioassay, is not discussed in this video. Larviciding, the application of control agents against the larval stage of the mosquito life cycle, is one of the approaches used for multi-faceted integrated mosquito management emphasized by the American Mosquito Control Association. Larviciding is the primary means of mosquito control in some parts of the United States and can be a significant component of mosquito control programs in other regions of the United States where the application of chemical pesticides against adult mosquitoes is used primarily for mosquito abatement.

The approach illustrated in the video is based on a training workshop focusing on larval mosquito bioassays developed by Tianyan Su, Ph.D., of the West Valley Mosquito and Vector Control District, Ontario, CA for the Mosquito and Vector Control Association of California (MVCAC). Many of the tables and figures featured in this video were developed by Dr. Su for the MVCAC's training program for mosquito and vector control districts in southern California. This video introduces the rationale for running bioassays using larval mosquitoes, and summarizes the set-up and interpretation of the results of bioassays using larval mosquitoes. It is intended to provide an overview of the theory and approach to assessing resistance to larval mosquito control agents. Future videos will focus on each of the four larval mosquito control agents used routinely for mosquito abatement: *Bacillus thuringiensis* subsp. *israelensis*, *Lysinibacillus sphaericus*, spinosad and methoprene.

This supplemental material provides the tables and many of the figures featured in the video, a transcript of the voiceover in the video, examples illustrating the making of the stock solutions and calculations used in bioassays, an example datasheet, an example of probit paper, information about additional considerations when testing insect growth regulators such as s-methoprene, three reprints of papers on resistance testing in mosquito larvae, and the World Health Organization's (2005) "Guidelines for Laboratory and Field Testing of Mosquito Larvicides". The three reprints illustrate the methods used for testing hydrophobic insect growth regulators (Dame et al. 1998. J. Am. Mosq. Control Assoc.), dose-response curves for susceptible and resistant mosquito populations as well as crosses using these populations (Wirth et al. 2012. J. Med. Entomol.), and comparisons of resistant field populations to susceptible laboratory populations (Su et al. 2019. J. Med. Entomol.).

Topics Covered in the Video

Topic	Time code
Outline of topics covered	0:00:00
Five capabilities of a mosquito control organization	0:01:17
Uses of the larval mosquito bioassay	0:02:37
The dose-response curve	0:04:01
Choosing test concentrations	0:04:48
Linearizing the dose-response	0:05:22
How the dose-response changes as resistance evolves	0:06:36
Three types of larval mosquito control agents	0:08:00
How frequently should you run bioassays?	0:10:13
Supplies needed	0:11:30
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Stock solutions and bioassay cup concentrations of the mosquito control agent	0:14:10
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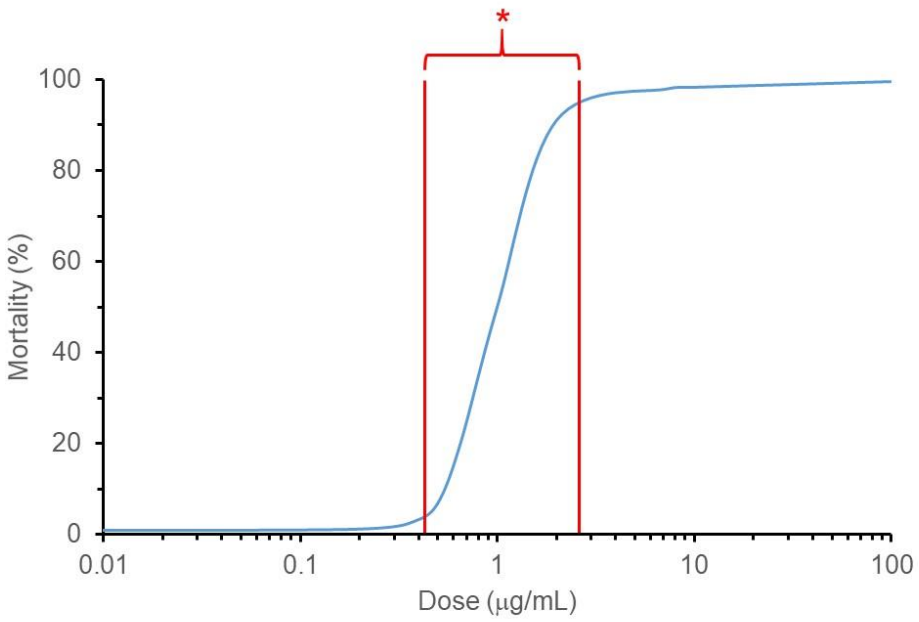
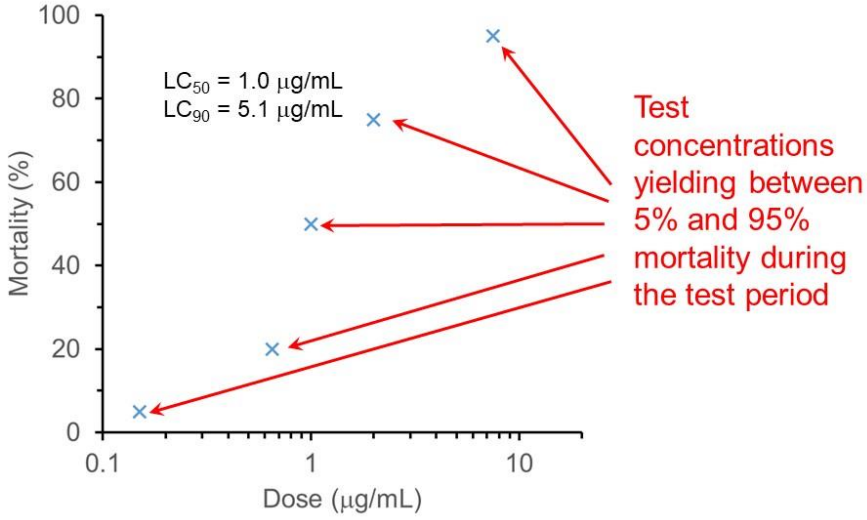
Feeding mosquito larvae	0:21:21
Reading the bioassay	0:22:53
Interpreting bioassay results	0:24:34

Selected Tables and Figures in the Video

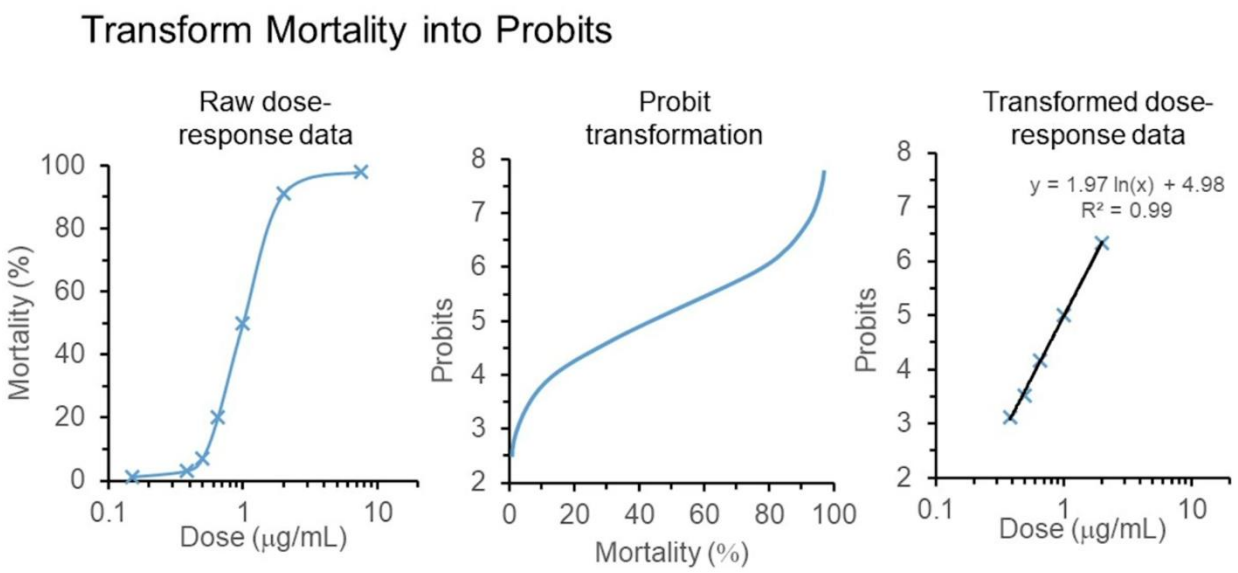
AGENCIES CARRYING OUT VECTOR CONTROL MUST BE ABLE TO:

1. surveil local mosquito populations
2. use surveillance data of mosquitoes and the pathogens that they transmit to inform vector control activities
3. have an action plan to control mosquitoes at all life stages
4. use integrated mosquito management that incorporates multiple approaches for vector control
5. conduct pesticide resistance testing

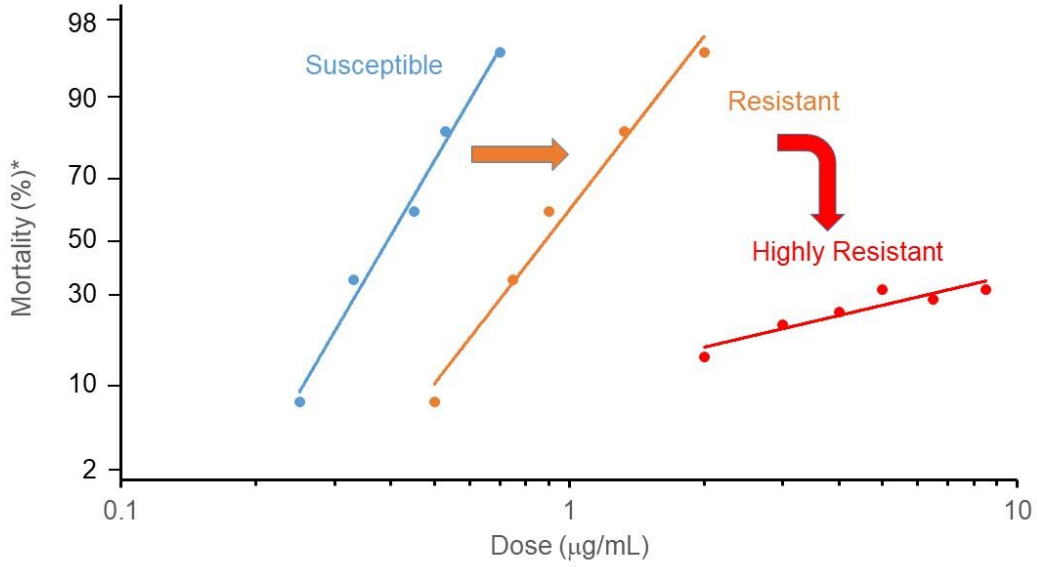




Identify 4 to 5 concentrations of the control agent within the range of concentrations across which mortality changes markedly (illustrated by the range noted by * in the upper diagram). Typically choose two concentrations above and two concentrations below the LC_{50} for the control agent against the mosquito population of interest.



Goal: Use probits to transform the S-shaped distribution of the raw dose-response data into a straight line.



* back-transformed from probits

The changes in dose-response curves for a mosquito population as the level of resistance increases. As the level of resistance increases in the susceptible population, the dose-response of the resistant population shifts to the right and the slope of the regression line might decrease. As the mosquito population evolves very high levels of resistance, the dose-response flattens out and one is unable to obtain meaningful levels of larval mosquito mortality at even very high doses of the control agent.

Active Ingredients	Commonly Used Formulations for Laboratory Bioassays* (% Active Ingredient)
Bacterial larvicides *Bacillus thuringiensis* subsp. *israelensis* [Bti] *Lysinibacillus* (*Bacillus*) *sphaericus* [Ls]	Primary or Technical Powders (~98%) Aqueous Suspension (AS, SC: 11.61%) Water Dispersible Granules (WDG: 37.4% [Bti] or 51.2% [Ls])
S-methoprene	Technical liquid (~95%) Liquid (5%, 20%)
Spinosad	Emulsifiable Concentrate (EC: 20.6%) Granules (30-day: 2.5%)

*Some of these products are formulated as granules, pellets, tablets or briquets which are not as amenable as are technical powders, liquids and formulations that suspend readily in water for conducting laboratory bioassays in small water volumes.

Three types of larval mosquito control agents that are routinely tested using bioassay. Bacterial larvicides must be ingested by mosquito larvae. S-methoprene is an example of a larval mosquito control agent that requires only contact with the target mosquito. Spinosad is an example of a larval mosquito control agent that can be ingested or works through contact to the target mosquito.

Suggested concentration ranges for initial bioassays using urban and suburban populations of *Culex* spp.

Active ingredients	Products	Concentration ranges
B.t.i.	**Primary Powder (7,000 ITU/mg)** **Aqueous Suspension (1,200 ITU/mg)** **Water Dispersible Granules (3,000 ITU/mg)**	**0.015 – 0.075 mg/liter** **0.15 – 0.75 mg/liter** **0.05 – 0.25 mg/liter**
L. sphaericus	**Primary Powder (1,000 ITU/mg)** **Water Dispersible Granules (650 ITU/mg)**	**0.0015 – 0.0250 mg/liter** **0.0025 – 0.0250 mg/liter**
Spinosad	**Emulsifiable Concentrate (20.6%)** **Granules, 30-day (2.5%)**	**0.0025 – 0.0250 mg/liter**
s-methoprene	**Technical grade liquid (≥ 98%)** **Liquid (5% or 20%)**	**0.25 – 25 μg/liter**

Initial Solution (stock)			
%	mg/liter (= ppm)	Aliquot (mL)	Final concentration (mg/liter) in 100 mL
1.0	10,000	1.0	100
Serial dilutions ↓ 2 mL of 1% stock solution into 18 mL distilled water		0.5	50
		0.1	10
0.1	1,000	1.0	10.0
↓ 2 mL of 0.1% stock solution into 18 mL distilled water		0.5	5.0
		0.1	1.0
0.01	100	1.0	1.0
↓ 2 mL of 0.01% stock solution into 18 mL distilled water		0.5	0.5
		0.1	0.1
0.001	10	1.0	0.1
		0.5	0.05
		0.25	0.025
		0.1	0.01

An example of the serial dilutions of a 1% stock solution (200 mg of product in 20 mL of distilled water) to obtain a final concentration of 0.1 mg/liter of product in each 100-mL bioassay cup. 1.0 mL (or 1000 μL) of a 0.001% stock solution would be added to each bioassay cup to obtain the desired concentration in the bioassay cup.

Bioassay set-up and result reading

	B.t.i., *Lsph* and spinosad	IGRs - JHA*, JHM
Materials	Technical grade, liquid or small granules	
Concentrations	4-5 (whole product for *B.t.i.* or Lsph, AI+ for spinosad)	4-5 (AI)
Amt. of water per cup (ml)	100	100
Larval food	adequate to support 24-48 hrs.	adequate to support pupation
Mosq. larvae	late 3rd - early 4th	late 4th
Replicates	3 x 25 larvae/cup	3 x 25 larvae/cup
Temp. (C)	25-27	25-27
Duration	24 hrs. (*B.t.i.*, spinosad) or 48 hrs. (*Lsph*)	All larvae die or emerge as adults
Results	mortality of larvae	mortality of larvae, pupae & adults

* JHA, JHM = juvenile hormone analog; juvenile hormone mimic
AI = active ingredient

Typical experimental conditions for bioassay testing of larval mosquito control agents. Bioassays are run for either 24 hours (*B.t.i.* or spinosad) or 48 hours (*L. sphaericus*). When testing insect growth regulators, the bioassay is run for 5-6 days or until all larvae either die or complete immature development (emerge as adults).

In addition to the number of surviving individuals, the following information should be recorded:

Mosquito species
Larval instar
Location of population tested
Control agent tested
Range of concentrations tested
Initial number of larvae in each cup
Duration of bioassay

Mortality in the Untreated Control

Repeat test if mortality in the control treatment is > 5% when testing larvicides with quick action such as microbial agents or occasionally conventional or EPA reduced risk pesticides.

Repeat test if mortality in the control treatment is > 10% in larvicides with slow action such as IGRs.

Treat "UTC - mortality" as an actual data point;

Correction by Abbott's Formula, then convert back to number of dead, and subject to probit analysis.

$$\text{Corrected mortality} = \frac{\text{Mortality in T (\%)} - \text{Mortality in UTC (\%)}}{\text{100\% - Mortality in UTC (\%)}} \times 100$$

Mortality for larvicide treatments in 24-hr or 48-hr bioassays should be corrected if mortality in the untreated control exceeds 5%. Abbott's formula could be used to correct mortality in the treatments of a mosquito control agent if mortality in the control treatment ≤ 20%. If pupation is > 10% in bioassays of microbial larvicides, then the mosquito larvae used in the bioassay might have been too old (i.e., had ceased feeding prior to pupation); the bioassay should be repeated. Levels of pupation > 10% are expected to occur in the untreated control and in treatments at low concentrations of insect growth regulators.

Data analysis and interpretation

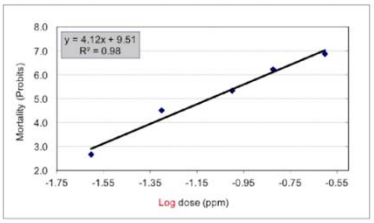
Probit analysis – mortality as probit units vs. log concentration
(LeOra Software, Berkeley, CA)
POLO PC
POLO Plus

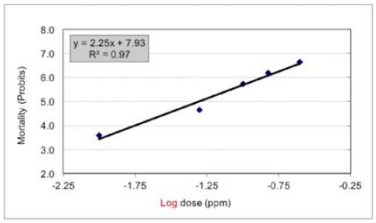
	Microbials	IGRs (JHA, JHM)
Data type	# larvae exposed # larvae died	# larvae exposed # larvae, pupae & adults died
Analysis	Probit analysis (software or probit paper)	
Results	Mortality (95% CI for LC_{50} and LC_{90})	Inhibition of emergence (95% CI for IE)
Data quality	χ^2 (data heterogeneity); Coefficient of determination (R^2)	
Slope (LC_{90}/LC_{50})	heterogeneous vs. homogeneous populations; slope ± standard error	
Tolerance	Resistance Ratio = $LC_{resistant}$ / $LC_{susceptible}$ < 5	
Resistance level	Resistance Ratio = $LC_{resistant}$ / $LC_{suscep.}$ ≥ 5 (Low: 5-20; Mid: 21-100; High: >100)	

The parameters measured routinely in bioassays using microbial larvicides or insect growth regulators. Statistical programs capable of analyzing dose-response data using probit analysis provide analyses of the slope of the dose-response regression line, goodness of fit of the bioassay data to the linear probit model, LC values, as well as facilitate the calculation of other relevant metrics. The LC90/LC50 ratio can provide a quick estimate for heterogeneity before running probit analysis to calculate slope. A greater LC90/LC50 ratio as in methoprene as compared with *B.t.i.*, means a smaller slope of the dose-response line (i.e, a flatter dose-response line) and higher heterogeneity in terms of response to the pesticide tested. Smaller LC90/LC50 ratios are common in (1) inbred populations, (2) highly selected populations, and (3) an acute toxicant.

Susceptibility of *Cx. quinquefasciatus* to *B.t.i.*

Water dispersible granules (3000 ITU/mg) (RR = 1.42 at LC_{50}, 0.86 at LC_{90})





Field:
LC_{50} 0.075 (95% CI: 0.062-0.091) ppm
LC_{90} 0.163 (95% CI: 0.130-0.233) ppm

Lab:
LC_{50} 0.053 (95% CI: 0.031-0.078) ppm
LC_{90} 0.189 (95% CI: 0.122-0.446) ppm

The log dose for mortality at a probit value of 5.0 in each figure has to be back-transformed to obtain the LC_{50}. 50% mortality occurs at a probit value of 5.0. The LC_{50} for the field population: $10^{-1.125}$ = 0.075 mg/liter (or ppm). The LC_{50} for the lab population: $10^{-1.275}$ = 0.053 mg/liter.

Factors that influence the outcome of bioassays in mosquito control agents:

- Test materials
- Mosquito species
- Larval instars and bio-fitness
- Water quality
- Duration of exposure
- Room/water temperature
- Larval food addition
- Criteria for result reading

Transcript of Voiceover in the Video

Time (from start)	Text
0:00:00	In this video, we will cover the following topics: (1) the purpose and benefit of carrying out bioassays. What information do you acquire about the efficacy and direction of control efforts in your mosquito control program? (2) What supplies do you need to run bioassays that examine the level of resistance in a population of mosquitoes to various control agents used against the larval stage of the mosquito life cycle? (3) How do you set up a bioassay that has an experimental design that is appropriate for making conclusions about the susceptibility of mosquitoes in your district to larval mosquito control agents? (4) How do you read the results of the bioassay to determine mortality in the mosquito larvae? (5) How do you interpret the bioassay results, including robustness of the results, correcting for mortality in the control treatment, interpreting dose-response curves and resistance ratios, and assessing the level of resistance that has evolved in a particular mosquito population.
0:01:17	Controlling mosquitoes requires that local health departments and vector control organizations have five core capabilities. In order to make informed decisions about vector control that reduce the incidence of vector-borne diseases and improve quality of life, agencies carrying out vector control must be able to: (1) surveil local mosquito populations, (2) use surveillance data of mosquitoes and the pathogens that they transmit to inform vector control activities, (3) have an action plan to control mosquitoes at all life stages, (4) use integrated mosquito management that incorporates multiple approaches for vector control and (5) conduct pesticide resistance testing. Practicing informed integrated mosquito management will increase the cost-effectiveness of mosquito control activities, assist in choosing the most effective environmentally-friendly method or methods for vector control given the local conditions, and maintain the effectiveness of the comparatively few larvicide and adulticide mosquito control agents in the vector control toolbox.
0:02:37	The methods used to assess resistance include conventional cup and bottle bioassays, microplate assays and molecular assays. The standard laboratory bioassay when coupled with biochemical or molecular studies can help to determine the mechanism of resistance. In addition to resistance management and susceptibility monitoring, the methods that are described in this video can be

used for:
- Research investigating the efficacy of new insecticides and new insecticide formulations.
- Product quality control to investigate the consistency of mosquitocidal activity among different batches of the same product.
- Assessing the stability of mosquitocidal activity of product that has been stored for extended periods after purchase. And last
- Study of the biotic and abiotic factors affecting larvicidal activity and efficacy of particular mosquito control agents.

0:03:36 If we are interested in the mortality of mosquito larvae caused by a particular control agent and we do not know the activity of that control agent against the mosquito population of interest, then to find out the activity range of the control agent the mosquito larvae are exposed initially to a wide range of test concentrations, together with an untreated control.

0:04:01 A standard dose-response to a larval mosquito control agent should look like this. Mortality is plotted on the y-axis and the logarithm of dose is plotted on the x-axis. Note that there is no mortality of larvae at doses on the left side of the graph. On the right side of the graph, mortality reaches a point at which mortality is 100% as dose increases. Including doses in both of these regions of the dose-response curve in your laboratory bioassay is not informative. You want to identify the region in the center of the dose-response where mortality increases as a function of dose. It is within this range of doses that you want to target for your bioassay.

0:04:48 You want to use a range of 4 to 5 concentrations, yielding between 5% and 95% mortality during the bioassay to determine LC50 and LC90 values. LC stands for lethal concentration. The 50 and 90 refer to the concentrations that cause 50% and 90% mortality, respectively. Typically, we want to identify two concentrations above and below the dose that causes 50% mortality for our bioassay test.

0:05:22 We linearize the dose-response by plotting the number of individuals dying for a known number of larvae exposed at each concentration of the control agent being tested by plotting mortality as probits.

The percentage mortality at each of the doses tested in the bioassay is based on a binomial outcome: larvae are either dead or alive. The raw dosage-mortality data shown in the left panel approximate a cumulative normal curve that is sigmoidal or S-shaped. By converting the Y-axis to normal equivalent densities using the probit transformation, shown in the middle

panel, we linearize the cumulative normal distribution of mortalities as is shown in the right panel. It is much easier to use a line than a curvilinear relationship to carry out inverse prediction where we are essentially using mortality, the Y variable, to predict dose, the X variable, measured on a logarithmic scale. We are interested in the position of the dose-response relative to dose, the slope of line, the fit of the data points to the linear model among other parameters that we will discuss later.

0:06:36 The dose-response of a population of mosquitoes that is susceptible to the control agent across a range of doses might look like this. Mortality increases with an increasing dose of the control agent. As the population becomes increasingly resistant to the control agent, the dose-response curve will shift to the right. The population depicted in the line on the right is comparatively less susceptible to the control agent than is the population whose dose-response curve is depicted by the line on the left. When a mosquito population becomes highly resistant to a particular control agent, the dose-response moves further to the right and may flatten out. This indicates that one is unable to obtain meaningful levels of larval mosquito mortality at very high doses of the control agent.

0:07:28 What agents are used to control mosquitoes in the larval stage?

Mosquito larvae occur in standing water. The control agents are typically applied to the water surface and active ingredients used to control mosquito larvae fall into three general categories: control agents that must be ingested or eaten by mosquito larvae, control agents that work by contact with mosquito larvae or control agents that work both through contact and ingestion by mosquito larvae.

0:08:00 First, there are products derived from bacteria that produce protein precursors that must be ingested by the mosquito larvae and then broken down in the larval digestive tract to form toxins. These products are known as bacterial or microbial larvicides. Commercial formulations of these biologically-derived agents are made from different strains of *Bacillus thuringiensis israelensis* or *Lysinibacillus sphaericus*, formerly known as *Bacillus sphaericus*. These materials are highly specific in that they are toxic only to mosquitoes and their close relatives at operational application rates.

Commercial formulations come in a variety of forms: liquids and different types of granules and briquets. The commercial formulations differ in their toxicities. The international toxicity units or ITUs indicate the toxicity of the formulation to mosquito

larvae. The higher the international toxicity units (ITUs), the more toxic is the formulation per unit mass. It is important to know the ITU of the formulation that is undergoing bioassay.

0:09:12 A second category of larval mosquito control agents has a mode of action that requires only contact with the mosquito larvae. Juvenile hormone mimics are an example of a control agent that is absorbed through the cuticle.

Control agents such as monomolecular films that spread across the water surface, change the surface tension and drown the larvae could also be included in this category.

0:09:38 A third type of control agent works by contact and ingestion. Spinosad is derived from a bacterium and consists of neurotoxins for several groups of insects. The percentage of spinosad in formulated products differs. For example, spinosad can range from 2.5 to 20.6% of commercial formulations. In order to calculate the concentration of the active ingredient in a bioassay, the investigator needs to know the percentage of the active ingredient in each formulation being assayed.

0:10:13 How frequently do I need to run bioassays?

Mosquito control agents used routinely in mosquito control operations should be evaluated on a regular basis. Bioassays are run to establish the baseline level of resistance in local mosquito populations to a new product to be incorporated into a mosquito control program.

This is more relevant to adulticides than to larvicides because the former are often used to control other insects besides mosquitoes. It could be the case that local mosquito populations have been selected for resistance against a widely used control agent, even if that control agent has never been used locally to control mosquitoes.

Bioassays need to be run more frequently than once or twice each year if you observe that a particular control agent has failed to reduce mosquito populations in the field or if you are aware that mosquitoes in your area are known to carry genes that confer resistance to a particular control agent. For some control agents, resistance to multiple control agents, or cross resistance, is possible.

0:11:30 To test the levels of resistance of mosquito larvae, you need the following items: mosquito larvae in the appropriate instar for testing. Most bioassays that assess the susceptibility of mosquitoes to comparatively rapid mortality, within 1 or 2 days

to a control agent, use early fourth instar larvae in 1-day trails and late third instars in 2-day trials. For control agents that require ingestion by the larvae, you do not want to use late fourth instars that have ceased feeding prior to pupation. Sometimes younger instars, such as second instars, are used in bioassays. An important point to keep in mind is that you want to maintain consistency in the instar and husbandry of larvae across all of the treatments in the bioassay.

Other supplies that you need to run a bioassay include:
- Plastic, wax-lined or styrofoam cups containing 100 or 200 milliliters of water. When evaluating methoprene or other compounds that are hydrophobic, one needs to take precautions to avoid binding of the control agent to the bioassay cups. Silanized glass vessels are recommended. Styrene cups also are an alternative bioassay cup.

- tap water that has been dechlorinated by aging for 24 hours or distilled water. Tap water can be aerated during aging to enhance dechlorination.
- Either formulated or technical grade forms of the control agent
- 20-ml glass vials to mix different concentrations of the active ingredient being tested.
- Pipettes and
- screens for transferring larvae

0:13:17 You want to create a range of concentrations of the active ingredient to test against the mosquito larvae, resulting approximately 5 to 95% mortality within the exposure period. Remember that you also want to include an untreated control treatment in each bioassay.

As you can see, the range of doses that you will want to target in your bioassay differs among the control agents. The range of doses to be tested reflects the inherent toxicity of each formulated control agent. Four to five doses should be tested in each bioassay.
Often concentrations for Bti and *L sphaericus* products are based on entire formulation with ITU/mg specification, while products based on spinosad and methoprene are based on active ingredient levels.

0:14:10 Most of the time, a stock solution or suspension is made at 1% concentration for a volume of 20 ml, then serially diluted at 10 times taking 2 ml of the stock solution adding it to 18 ml distilled water) to appropriate levels depending on treatment concentration ranges needed for a given insecticide.

As you can see in this table, if you started by mixing 200

milligrams of your control agent in 20 milliliters of water to obtain a 1% solution, the concentration of that initial suspension or solution would be equal to 10000 milligrams per liter or equivalently 10000 ppm.

You would carry out 10-fold dilutions of each stock solution by adding 2 mls of well shaken stock solution to 18 ml of distilled water. You continue the dilution process until you obtain a stock solution with the appropriate concentration of the control agent for you to obtain the range of concentrations desired in the 100 ml bioassay cups.

0:15:16 So for example, if you have a 0.001% stock solution, or 10 milligrams per liter of the control agent, you would add 1000, 500 or 100 microliters of the stock solution to cups holding 100 ml of water to obtain final concentrations of 0.1, 0.05 and 0.01 milligrams of the control agent per liter.

If your bioassay cups hold more or less than 100 ml, then you need to adjust the amount of stock solution added to each cup accordingly.

0:15:56 Let's run through an example. Suppose we want to test four concentrations of a water dispersible granule formulation of Bti against mosquito larvae. This formulation is 3,000 ITUs/mg. Concentrations used in bioassays of this control agent are typcially based on the entire formulated product. A 1% stock solution, 200 mg of product in 20 ml of diluent, usually distilled water, which is equivalent to 10 milligrams per milliliter or 10,000 ppm, is made and then serially diluted as needed. 100 to 1000 microliters of diluted stock solution are added to 100 ml of water in replicate bioassay cups. Quite often the treatment concentrations do not go on a 10 times scale, particularly for pesticides with acute toxicity: a smaller range of concentrations of the control agent is typically required.

Now let’s suppose the highest concentration that we want to test is equal to 0.1 milligrams per liter of formulated ingredient. To obtain 0.1 milligrams per liter of the formulated product in each 100 ml bioassay cup, add 1000 microliters of a 0.001% stock solution to each cup. To do this, serially dilute the 1% stock solution three times. If we were interested in the amount of Bti in each cup, then we would need to correct for the concentration of Bti in the formulated product. Based on the product label, we know that each liter of this formulated control agent contains 37.4% Bti.

Now, we also want to have a range of test concentrations that might include the following three concentrations: 0.01 mg/liter, 0.025 mg/liter, and 0.05 mg/liter. We would make solutions of

these concentrations by adding 100, 250 or 500 microliters of the 0.001% stock solution to each bioassay cup. In this example, 50% mortality of mosquito larvae is expected to occur at about 0.038 mg/liter.

0:18:35 Be careful not to contaminate the water in the cups of untreated control treatment with the control agent. Typically, you want to start processing the bioassay cups with the control, then the lowest concentration of the control agent, working your way up to the treatment containing the highest concentration of the control agent in your bioassay. Doing so will help to reduce inadvertent contamination among treatments in the bioassay.

0:19:01 If you plan to compare the level of resistance in a particular field population of mosquitoes to a susceptible population of the same species, then you will need either to maintain a susceptible mosquito colony in your laboratory or to acquire susceptible individuals from reputable source.

0:19:21 To set up the bioassay using the conditions described in this table, add 25 late 3rd instar or early fourth instars to each bioassay cup. Mosquito larvae are transferred from the rearing pan to the bioassay cup using a small screen. The amount of water transferred with the larvae should be minimized. You want to have a minimum of three replicate cups for each concentration in the bioassay, including the untreated control. You can use additional replicate cups if you have an adequate supply of larvae which have been raised under the same conditions. The more replicate cups that you use, the more robust are the findings of your bioassay test. After you have added the larvae to the bioassay cups, then add the different concentrations of the control agent to the cups. In order to avoid contamination, disposable cups are preferred to reusing bioassay containers. Remember to follow all required procedures for disposing of materials used in bioassays.

It is recommended that you repeat the bioassay a minimum of two more times on subsequent days using fresh stock solutions for each test.

0:20:39 The bioassays are run under a known temperature regime, typically 25 to 28ºC. Sometimes slightly warmer conditions are maintained in mosquito insectaries. A continuously recording temperature sensor can be purchased fairly cheaply. Alternatively, a recording maximum-minimum thermometer can be used to keep track of the temperature extremes during the experiment, as ambient water temperatures are critical during exposure for ingestion and cuticle absorption of pesticides by mosquito larvae.

The photoperiod recommended by the WHO for bioassays is 12 hours of light and 12 hours of dark.

0:21:24 Larvae will need to be fed for long-term bioassays such as required to test insect growth regulators. Investigators differ whether or not to feed mosquito larvae during short-term bioassays of 24 hours. For bacterial larvicides that must be ingested by larvae, the food levels and organic matter in the environment can influence the outcome of bioassays. Whether or not you provide food for the larvae, you want the food supply to be consistent across replicates in each bioassay and among bioassays, especially if you are interested in long-term trends of resistance in your mosquito populations. Food level and type need to be consistent and should be quantified.

For short-term bioassays of 24 hours in length, it is preferable not to add food – but if you do add food, then a couple of drops of 10% rabbit pellet solution can serve as phagostimulant and avoid starvation of larvae, but not cause water quality issues. For 48-hour bioassays, larvae could be well fed for 24 hours before placing larvae into the bioassay cups and then fed on the morning of the second day of the test. For tests of juvenile hormone mimics, a small amount of solid food such as 100 mg of rabbit pellets per bioassay cup is suggested to provide nutrients till pupation.

0:22:53 After a prescribed period of time, mortality of the mosquito larvae is assessed. Distinguishing between dead and alive larvae is usually straight-forward. Alive larvae will maintain their position in the water column or respond to movement. Dead larvae usually lie, unmoving on the bottom of the bioassay cup. Moribund larvae are problematic. Is the larva alive or dead? You need to be consistent when dealing with moribund larvae – ones that can't maintain normal position, do not show the characteristic diving reaction when the water is disturbed and often exhibit a shrunken body and dark coloration. If the larva does not move when touched with a dissection probe or some comparable item at the siphon or cervical region, then consider the larva to be dead. It can be risky to count the cadavers, as dead larvae can be consumed by survivors during test period.

You want to record the number of surviving mosquito larvae on a datasheet. Include all relevant information about the conditions of the bioassay on the datasheet. Some of the relevant information that should be included is the following: the mosquito species, the instar of the larvae used in the bioassay, the location where the mosquitoes were collected,

the control agent being tested, the range of concentrations that were tested, the number of mosquito larvae placed initially into each replicate bioassay cup, the duration of the bioassay or the time since the start of the bioassay that data are recorded.

0:24:34 If the control mortality is between 5% and 20%, the mortalities of treated groups should be corrected according to Abbott's formula. Tests with control mortality greater than 20% or pupation greater than 10% should be discarded.

0:24:56 Concentration–response data are subject to probit analysis using one of the many computer programs that offer probit analysis. For example, you could use POLO-PC or POLO Plus or almost any statistical software to calculate LC50 and LC90 levels, their 95% or 99% confidence intervals, the coefficient of determination or R^2 to quantify the fit of your bioassay data to the linear probit model, the slope of the dose-response line, and population heterogeneity indicated by the ratio of LC90/LC50. These parameters vary upon the quality of the data in the bioassay, mosquito species, population genetic diversity, and mode of action of pesticides tested. Finally if you are comparing the dose-response of a field population to that of a susceptible laboratory colony, you calculate resistance ratios which is LC_{field}/LC_{lab}.

If the resistance ratio is less than 5, the field population remains susceptible to the control agent as compared to the laboratory population. Resistance ratio can be categorized into three levels: low levels of resistance if the resistance ratio is between 5 and 20. Mid or intermediate levels of resistance occur when the resistance ratio is between 21 and 100. High levels of resistance are indicated by resistance ratios greater than 100. These categories are somewhat arbitrary. In practice, one wants to detect the evolution of resistance in mosquito populations early on in the process and implement measures to counteract the further intensification of resistance as soon as possible.

0:26:50 Let's look at some bioassay data collected for *Culex quinquefasciatus* exposed to four different larval mosquito control agents.

Dose-response lines for mosquito larvae exposed to Bti with a toxicity of 3000 ITUs/mg are shown here.

The LC50 value for the field-collected population is 0.075 mg per liter. The LC50 value of the susceptible laboratory colony is similar but slightly lower, 0.053 mg per liter. If you compare the 95% confidence intervals for these two values, you can see that the 95% confidence intervals overlap suggesting that the

susceptibilities of the two populations to this control agent do not differ significantly. Notice also that the resistance ratio of the two LC50s is only slightly greater than 1.

The coefficient of determination, or R^2, describes how well the bioassay data fit the linear dose-response model. 0% indicates that the model explains none of the variability of the response data around its mean. A value of 1.0 indicates that the model explains all, 100%, of the variability in the response data. In other words, the points fall on the regression line. You can see that the fit of the linear model to the data from both bioassays is very good since the R^2 values are near to 1.

0:28:29 This second group of graphs shows the response of these same mosquito populations to *Lysinibascillus sphaericus*. Notice that the LC50 values are much smaller than in the previous graphs. These mosquito populations are much more susceptible to *L. sphaericus* than to *Bti* …even though the potency of the formulation for *L. sphaericus* is much lower, only 650 ITUs/mg, as compared to 3000 ITUs/mg in the Bti formulation. The 95% confidence intervals for the LC50s overlap indicating that the LC50s of the two populations are not statistically significantly different.

0:29:13 The third group of bioassay results is for the same population and its susceptibility to spinosad. Notice that the LC50s are considerably lower than for the two bacterial larvicides.

This last group of bioassay results is for the insect growth regulator, methoprene. Notice that the regression lines are flatter than those shown previously and the IE50s are in the parts per billion or micrograms per liter range. Also notice that 95% confidence intervals for the field and laboratory populations do not overlap. The resistance ratio is 7.6 indicating that the field population shows some resistance to this mosquito control agent as compared to the susceptible laboratory colony.

0:30:04 There is a number of factors that influence the outcome of bioassays of mosquito control agents. These factors include:
- the material being tested. For the same control agent, different formulations have different inherent toxicities. The mode of action differs appreciably among bacterial larvicides versus spinosad versus insect growth regulators.
- The mosquito species is important. Mosquito genera and species can differ appreciably in their inherent susceptibilities to particular larval mosquito control agents. Given factors like the selection pressure from control agents, the extent of genetic exchange among populations, and other factors, different populations of the same mosquito species can differ

significantly in their susceptibilities to a particular control agent.
- Age of the larvae and effects of husbandry practices on the quality of the larval mosquitoes is also important.
- Water quality. The amount of organic matter in the water in a field site can influence the efficacy of some control agents.
- Duration of exposure
- Temperatures that the bioassays are being run
- Whether food is added to the replicate bioassay cups...the type of food...how much food is added...can all affect the outcome of the bioassay. In nature, high levels of enrichment and food can reduce the effectiveness of bacterial larvicides that are ingested by mosquito larvae by reducing the amount of bacterially-derived proteins ingested per unit time.
Last, the criteria used for assessing the effect of the control agent on the larval mosquitoes are important. Typically, mortality is the criterion used. However, for IGRs, inhibition of eclosion to the adult stage is one of the criteria used to evaluate the effectiveness of mosquito control.

0:32:02 Based on the bioassay data results, we can now determine the correct dosage of mosquito control agent for effective control.

Bioassay, a simple straight-forward process to quantitatively measure the interaction of a lethal agent versus a target organism, is an important tool for multiple purposes ranging from resistance detection and management, active ingredient screening, commercial product evaluation, studies on product stability under harsh conditions, and the efficacy of particular formulations of mosquito control agents in the diversity of larval developmental sites in your district.

Summative Assessment: Worked Examples

After watching the video and obtaining software to carry out probit analyses, answer the following questions.

1) You find that your ability to control *Culex quinquefasciatus* populations in your district with a water-dispersible granule formulation of *L. sphaericus* seems to have declined after 6 applications of the control agent. You want to compare the dose-response of a field population to that of a susceptible laboratory colony. The LC_{50} of the laboratory colony is around 0.008 mg/liter for the 650 ITU/mg formulation. You suspect that the LC_{50} of the field population for this formulation of the control agent might be around 0.12 mg/liter. You want to expose the laboratory population to the following concentrations of the control agent in 48-hour bioassays: 0.001, 0.005, 0.01, 0.02 and 0.025 mg/liter. You want to expose the field population to the following concentrations of the control agent: 0.01, 0.05, 0.1, 0.2, and 0.75 mg/liter. How would you achieve each of these concentrations of the control agent in 100-mL bioassay cups?

2) After repeating bioassays with a new formulation of a microbial larvicide on three dates against *Aedes aegypti*, you obtain the bioassay data in the table below. Compute the LC_{50} and LC_{90}, the 95% confidence limits for both LCs, the slope of the dose-response curves and the heterogeneity (χ^2/df) of the dose-response.

Concentration (mg/L)		0	0.01	0.025	0.05	0.1	0.25
Date	Replicate	# of dead larvae at 24 hr					
1	1	0	7	11	19	20	20
1	2	0	5	14	20	20	20
1	3	0	2	8	18	20	20
2	4	0	4	12	17	20	20
2	5	0	9	10	19	20	20
2	6	0	6	17	20	20	20
3	7	0	3	4	18	19	20
3	8	0	1	3	19	19	20
3	9	0	0	4	17	19	20

Answers to the Summative Assessment: Worked Examples

1) Nine concentrations (0.001, 0.005, 0.01, 0.02, 0.025, 0.05, 0.1, 0.2, and 0.75 mg/liter) of the control agent are needed to run both bioassays. You mix 200 mg of the formulated *L. sphaericus* product in 20 mL of distilled water. You would dilute this 1% stock solution by shaking well, then taking 2 mL and adding this sample to 18 mL of distilled water. Carry out three more serial dilutions by repeating this process three more times using the new stock solution at each step of the process. After completing the 10-fold serial dilutions you will have the following stock solutions: 18 mL each of 1%, 0.1%, 0.01% and 0.001% stock solution; 20 mL of a 0.0001% stock solution. To obtain the desired concentrations of the mosquito control product in bioassay cups containing 100 mL of water you make the following additions:

Using the 0.0001% stock solution, add 100 μL (0.001 mg/liter), 500 μL (0.005 mg/liter), 1000 μL (0.01 mg/liter) to replicate bioassay cups to obtain the final concentrations shown in parentheses. Two sets of replicate cups containing the 0.01 mg/liter concentration are needed (one set for each bioassay).

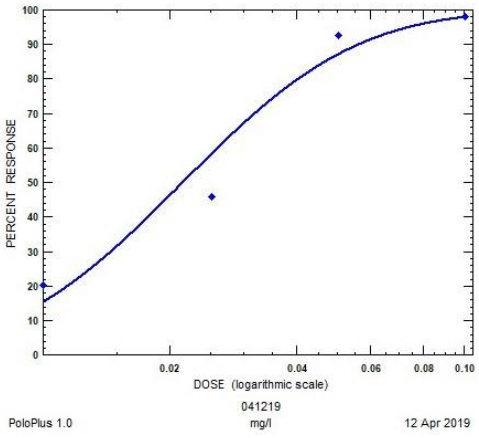
Using the 0.001% stock solution, add 200 μL (0.02 mg/liter), 250 μL (0.025 mg/liter) or 500 μL (0.05 mg/liter) to replicate bioassay cups to obtain the final concentrations shown in parentheses.

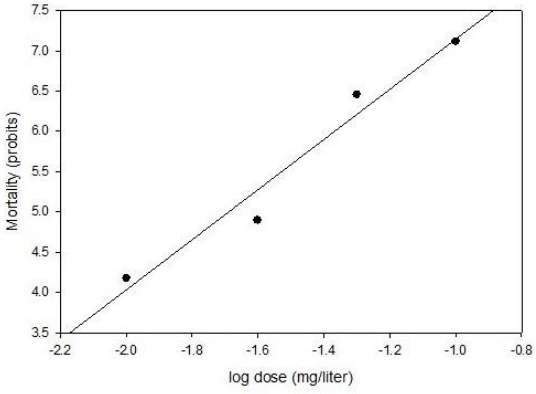
Using the 0.01% stock solution, add 100 μL (0.10 mg/liter), 200 μL (0.20 mg/liter) or 750 μL (0.75 mg/liter) to replicate bioassay cups to obtain the final concentrations shown in parentheses.

Remember to keep the stock solution well-shaken and not to contaminate treatments of lower concentration by re-using pipettes that were used to dispense a treatment of higher concentration.

2) The following results were obtained for the bioassay example.

LC_{50} (95% Confidence limits) = 0.020 (0.014-0.029)
LC_{90} (95% Confidence limits) = 0.053 (0.037-0.076)
Slope (± SE) = 3.07 (± 0.19); Heterogeneity = 9.8 (χ^2/df = 19.6/2)





BIOASSAY OF CONTROL AGENTS AGAINST MOSQUITO LARVAE IN THE LAB

Experiment no.: _____ Investigator: _____ Location: _____ Treatment date: _____

Material: _____ Formulation: _____ Temp: _____

Species: _____ Larval instar: _____ Larvae/cup or vessel: _____

Water: Tap _____ Distilled _____ Volume of water: _____ ml Food: _____ Date stock solution made: _____

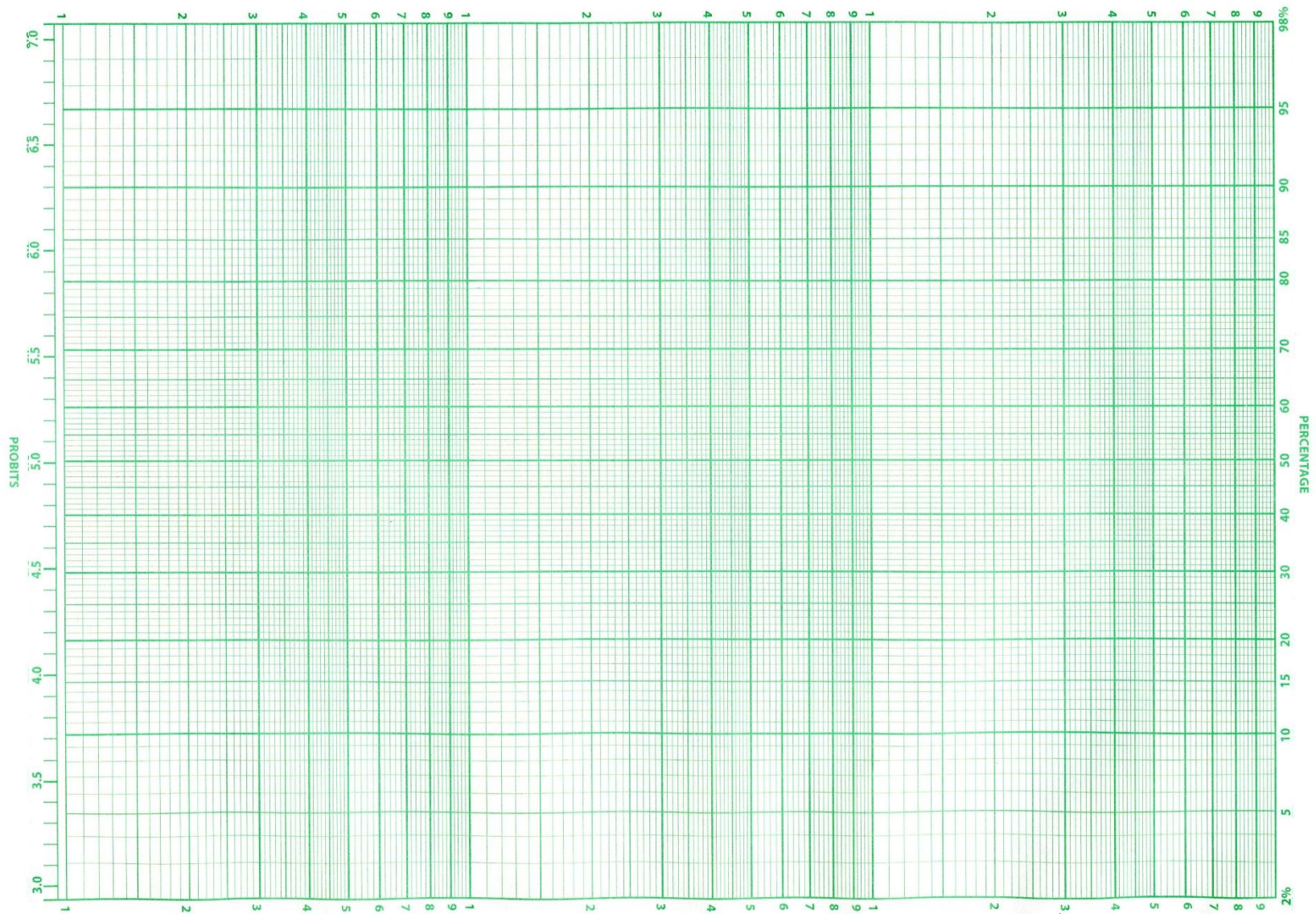
		No. of survivors at various concentrations (mg/liter) post-exposure (hr)														
		24 hr								48 hr						
		Concentration								Concentration						
Date	Replicate	0.00 (UTC)								0.00 (UTC)						
	1															
	2															
	3															
	4															
	5															
	6															
	7															
	8															
	9															
	10															
	11															
	12															
	Total															
	Average															
	% mortality															

LC50 (CL 95%) _____ LC50 (CL 95%) _____

LC90 (CL 95%) _____ LC90 (CL 95%) _____

LC99: _____ LC99: _____

Slope: _____ Heterogeneity: _____ Slope: _____ Heterogeneity: _____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Methoprene Testing: additional considerations

Testing for resistance in mosquito larvae to insect growth regulators (IGRs) requires an approach that differs from the standard 24-hour bioassays carried out using bacterial larvicides, such as *Bacillus thuringiensis* subsp. *israelensis*, or chemical insecticides. For example, methoprene is markedly hydrophobic. Consequently, there is a potential for product loss through binding on container surfaces (i.e., bioassay cups, vessels for mixing stock solutions and pipettes used to transfer solutions to the bioassay cups). Volatilization into the atmosphere is also a concern when running bioassays with methoprene.

David Dame, PhD, provides the following recommendations for carrying out bioassays using insect growth regulators such as methoprene.

Methoprene testing differs from the approach used for standard bioassays for three reasons:

1) Methodology. The test may run for 5-6 days, not a 24-hour period. The live mosquito larvae often eat dead and dying larvae, potentially biasing the results. The amount of food per larva is variable because each day the test containers have differing numbers of survivors and amounts of detritus, causing variation in container content. These factors affect survival and necessitate a relaxation of significant difference determination, corrected in part by conducting more replicates. Although some product testers do all "replicates" on the same day and with mosquito larvae from the same batch (i.e., duplicates), the results are only suitable for statistical analysis if the replicates are done on separate days and/or with different batches of mosquito larvae.

2) Methoprene stability. Methoprene dissipates into the atmosphere, reducing the amount in solution, contaminating nearby substrates and causing variable lowering of methoprene concentration, especially if containers are not covered. When added to water, methoprene is hydrophobic and readily binds to surfaces, causing further reduction in concentration of the solution.

3) Methoprene solubility. Methoprene has a low level of solubility in water, causing problems with preparation of solutions, especially if high levels of resistance exist. This can be countered by using acetone in the preparation of stock solutions. But acetone causes larval mosquito mortality above 0.01 ppm, so it is necessary to conduct both water solution and acetone solution controls to determine true impact.

Relatively accurate analysis can be achieved by increasing the number of true replicates conducted and by using precautionary steps to control the initial concentration of methoprene. An approach to stabilizing the methoprene concentration is illustrated in the accompanying reprint of Dame et al. (1998. *J Am Mosq Control Assoc* 14: 200-203). Stock solutions were prepared twice. The first preparation of each treatment in the bioassay was held (refrigerated) in its container for 24 hours, after which it was replaced by the 2nd preparation. The first fill was assumed to bind all the available links on the interior of the stock solution bottle, leaving no open links to impact the 2nd filling. This procedure was repeated for each replicate bioassay. Pipettes used for serial dilutions and to treat the test containers were each filled

with the serial dilution appropriate for the treatment, and then emptied after a few minutes (rather than 24 hours) and refilled with more of the same solution to provide pipettes whose interior surfaces were devoid of open linkages. It is important to separate the pipettes by methoprene treatment concentration and to label the pipettes by each treatment in the bioassay.

The glass test containers were purchased silanized (no open links). The appropriate volume of serial dilutions of methoprene was pipetted into the 100 ml of specially prepared test water (see text) in the test containers and allowed to stand for 30 minutes for the acetone to volatilize before the larvae and food slurry were added. Test containers (i.e., silanized-glass bioassay cups) were not reused.

Because of the variability mentioned in the paragraph on Methodology, the acceptable maximum level of mortality in the controls was raised to 50% for the concentration series tested (Abbott's formula allows a maximum of only 20%) and 44% for any individual test container. Probit analysis was conducted with results that met these control mortality requirements. [For comparison, in standard 24-hour product test seldom does a control container approach or reach 20% mortality. Whereas, in 5-6 day tests control mortality exceeding 20% is relatively common.]

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MOSQUITO (*AEDES TAENIORHYNCHUS*) RESISTANCE TO METHOPRENE IN AN ISOLATED HABITAT

DAVID A. DAME,[1] GEORGE J. WICHTERMAN[2] AND JONATHAN A. HORNBY[2]

ABSTRACT. Salt-marsh mosquitoes (*Aedes taeniorhynchus*), collected on 2 barrier islands in Lee County, Florida, that had been treated from 1989 to 1994 with 150-day methoprene briquets, were bioassayed with technical *s*-methoprene in the laboratory. Susceptibility of the indigenous Captiva strain (median lethal concentration [LC_{50}] estimate, 6.71 ppb) collected from Captiva Island was 14.9-fold lower than the naive Flamingo strain (LC_{50} estimate, 0.45 ppb) from Everglades National Park. The Lover's Key strain (LC_{50} estimate, 6.66 ppb) was 14.8-fold less susceptible than the naive strain. Determinations of the susceptibility of nearby foci of the mainland mosquitoes exposed in the past several years to methoprene have not been completed, but probit analysis of laboratory exposures revealed that the only mainland strain tested (Burnt Store) was no less susceptible (1.06-fold) than the naive Flamingo strain. These findings support the theory that the observed resistance might be restricted to the barrier islands. The known resistance foci (generated with briquet formulations) are located west of the mainland where there is minimal likelihood of inflow of genome from the mainland. On the other hand, the mainland mosquitoes, which were exposed to liquid formulations of methoprene from 1987 to 1994, are believed to have substantial gene flow between exposed and nonexposed populations and thus a reduced likelihood of selection for resistance.

KEY WORDS Briquet formulation, insect growth regulator, IGR, insecticide, restricted gene flow, selection, susceptibility, salt-marsh mosquito

INTRODUCTION

Operational use of methoprene in Florida extends back to the first applications of Altosid SR10® (Zoecon Corp., Palo Alto, CA) in Lee County in 1974. Several mosquito control programs in Florida have used methoprene for control of immature mosquitoes for more than 20 years. This study originated from an investigation into factors related to observed reduction in the efficacy of operational use of methoprene to control *Aedes taeniorhynchus* (Weid.) on the east coast of Florida (G. A. Curtis, D. A. Dame and G. F. O'Meara, unpublished). Difficulties in controlling *Ae. taeniorhynchus* on the barrier islands in Lee County prompted a request to include the Captiva strain in the east coast studies in which susceptibility analyses were being conducted.

The findings of the preliminary observations warranted additional studies to confirm and, if possible, determine the nature and distribution of the observed reduction in susceptibility of *Ae. taeniorhynchus* on Captiva Island. This report covers laboratory studies conducted on strains subsequently collected in Lee County to initiate an assessment of the geographical extent and possible habitat relationship of resistance to methoprene within the county. Although these studies are not yet completed, the results to date are considered sufficiently important to release the findings.

MATERIALS AND METHODS

Test insects: Adult female *Ae. taeniorhynchus* were collected early in the summer of 1995 from salt-marsh habitats on 2 barrier islands that had received applications of methoprene 150-day briquets annually from 1989 to 1994. The first-generation progeny of these barrier island collections are referred to as the Captiva and Lover's Key strains in this report.

Known methoprene-susceptible females were collected in 1995 and again in 1996 at Flamingo, FL, in Everglades National Park, which had no history of methoprene usage. First-generation eggs from these females are referred to as the Flamingo strain.

In 1996, collections were conducted on the Lee County mainland from an area that had received multiple applications of liquid formulations of methoprene (1987–94). First-generation eggs from this strain are known as the Burnt Store strain.

The parental Captiva, Lover's Key, and Burnt Store mosquitoes were trapped in the natural habitat at locations separated by 15–20 km (Fig. 1) and transported to the Lee County Mosquito Control laboratory in Ft. Myers, where they were transferred to holding cages and subsequently received blood meals from young chickens or through membranes. The resulting eggs were collected on cheesecloth pads moistened with water and placed on top of the cage outside the screen, through which the females oviposited. These eggs were stored and then hatched as needed to provide larvae for testing. Flamingo collections were taken to either the Lee County Mosquito Control laboratory or the Florida Medical Entomology Laboratory, where they were handled in the manner described above.

To provide larvae for testing, each day portions of egg pads were immersed for 2–5 h in deoxygenated reverse-osmosis (RO) water to which larval food had been added. The resulting larvae were

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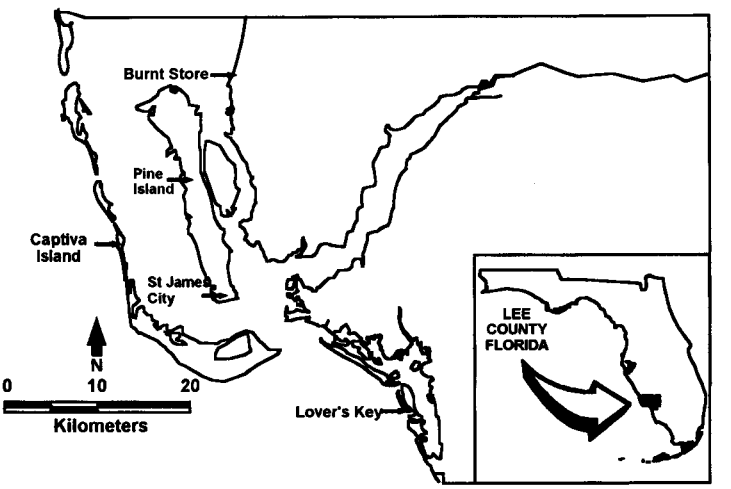

Fig. 1. Location of test strain collections (Captiva Island, Lover's Key, Burnt Store).

manually transferred to rearing trays (10 × 12.5-in. photographic developing pans) at a density of 125 per tray. Equipment was color coded to assure separation of the concurrently reared strains. The larvae were maintained at an ambient temperature of 28.6 ± 0.2°C. Finely ground liver powder (Bacto Liver Powder, Difco Laboratories, Detroit, MI) was sprinkled onto the surface of the rearing water at the rate of 50 mg/tray for the 1st and 2nd days and 150 mg/tray daily thereafter. Larvae were transferred to exposure dishes when they reached late 3rd or early 4th stage.

Treatment containers and solutions: The larvae were exposed to methoprene in culture dishes (3.5-in. diam) holding 100 ml of water. To prevent distortion of results by the known affinity of methoprene to adsorb to glass surfaces, each test container was pretreated with Sylon™ CT silanizing reagent (Sepulco Inc., Belleforte, PA) and rinsed to remove water-soluble and acetone-soluble Sylon CT residues.

The stock solution containers were preconditioned by first filling each container with a solution of the same concentration that it would ultimately hold and after ca. 24 h the conditioning solution was replaced with a freshly prepared solution. Stock solutions were prepared with technical *s*-methoprene (Lot 950531749, 95.63% purity, Zoecon Corp., Dallas, TX) and reagent grade acetone. Test solutions were prepared from these stock solutions using marked reusable pipettes, which were not rinsed between uses in order to maintain their preconditioned status.

These procedures were followed to minimize the number of attachment sites available on the glassware for adsorption of methoprene molecules in the preparation and during the actual experiment. Such attachment reduces the amount of methoprene available to the test insects. Stock solutions were refrigerated when not in use during the 1-month-long testing periods. Test solutions were prepared daily, as needed, and discarded the same day.

Larval exposures: Larvae were transferred from the rearing trays into holding cups before being placed in the test containers. To reduce the physiologic shock resulting from the direct transfer of larvae from rearing water to clear water, a standardized quantity of water from the rearing trays was mixed with the RO water in both the holding medium and the final test medium. In the holding cups the ratio of RO water to rearing water was approximately 1:1 and 3–4 drops of food slurry (10 mg liver powder/ml) were added to the mix. The test medium routinely consisted of 95 ml RO water and 5 ml rearing water, to which 4 drops of food slurry were added just prior to introduction of the larvae.

Twenty-five late 3rd- or early 4th-stage Flamingo and Captiva and/or Lover's Key larvae were exposed at each concentration in each replicate in the 1995 test series. A wide range of concentrations was used to assure that the observations would include the results of several exposures above and several below the 50% mortality level. With Burnt Store larvae reared in 1997 from the 1996 mainland site collections, triplicate sets of 15–28 larvae each were exposed on test days. Because of limited availability of Burnt Store larvae, exposures in that test series were limited to 5 concentrations that were expected to produce midlevel mortality of the naive Flamingo strain.

To minimize the toxic effect of the small amount of acetone in the test medium, larvae were placed in the treatment dish no sooner than 30 min after application of up to 1 ml of prepared solution and immediately after addition of a slurry containing 0.9 mg of liver powder per larva. The level of acetone was reduced to a maximum of 0.01 ppm in the exposure medium because of its observed detrimental affect on the larvae (an alternative solvent,

Table 1. Results of POLO PC probit analysis of the effect of *s*-methoprene on emergence of adults of barrier island (Captiva, Lover's Key) and naive (Flamingo) strains of *Aedes taeniorhynchus* exposed as late 3rd- and early 4th-stage larvae (95% confidence limits and standard error in parentheses for median lethal concentration [LC_{50}] and slope, respectively).

	Flamingo	Captiva	Lover's Key
No. subjects	1,117	343	550
No. controls	181	72	95
Slope	0.981 (0.103)	0.868 (0.134)	1.387 (0.157)
LC_{50} (ppb)	0.45 (0.25–0.73)	6.71 (2.52–16.0)	6.66 (3.20–13.4)
LC_{50} ratio[1]		14.9	14.8

[1] Ratio of barrier island strain to naive strain.

ethanol, was not utilized because it was also found to be deleterious). Daily series of the Captiva and Lover's Key tests included an untreated control containing 0.01 ppm acetone with each strain in addition to the untreated control without acetone.

Following introduction of larvae in tests with Captiva or Lover's Key strains, the culture dishes were secured with 100-mm petri dish covers to reduce evaporation; however, this step was bypassed in the 1997 tests with Burnt Store larvae. Pupae that developed within 6 h of introduction were removed and discarded due to possible inadequate exposure time (the record of the number of introduced larvae was adjusted accordingly when this occurred). Dead larvae were removed daily and pupae were transferred to covered holding cups containing fresh water. The larval exposure dishes and pupal holding cups were maintained at an ambient temperature of 28.5 ± 0.2°C. Adult emergence was observed 2 or 3 days after pupae were collected; complete separation from the pupal exuvium was considered to be adequate evidence for successful emergence.

Larval survival in this type of test is highly variable for 2 primary reasons. First, a minute amount of harmful acetone is introduced into each treatment dish as diluent; but without the solvent the technical material could not be serially diluted satisfactorily because the maximum solubility in water is ca. 1 ppm. Second, the duration of exposure in insect growth regulator tests varies from 1 to 4 days, the range of time required before the last larva pupates; this period is dependent on the specific age of the introduced larvae and the resulting nutritional and biological conditions within the treatment dish. These conditions in turn are dependent on the number of survivors that remain in the dish each day and the resulting utilization of the available nutritional materials and buildup of excreta and both beneficial and pathogenic microorganisms.

Because of these factors, each test series included at least one untreated control unit with 0.01 ppm acetone, in which survival from larva to adult ranged from 0 to 100%. Test series in which the survival to the adult stage among the untreated controls fell below 50% and individual tests in which larval mortality exceeded 44% were excluded from the probit analyses. The exclusion of those replicates in which the control mortality exceeds 50% overall or 44% in the larval stage (vs. 20% maximum for 24-h Abbott's formula adjustments [Abbott 1925]) represents an adjustment for test methodology and duration. The selected data were submitted to probit analysis (POLO PC) derived from Finney (1971) and based on the number of adult survivors compared to the initial number of larvae.

RESULTS

Barrier island strains: The results provided in Table 1 reveal the naive Flamingo strain to be 14.9-fold and 14.8-fold more susceptible than the Captiva and Lover's Key strains, respectively, based on the median lethal concentration (LC_{50}) estimates. The analysis for 10% lethal concentration (LC_{10}) and 90% lethal concentration (LC_{90}) levels provided similar estimates of relative susceptibility at the 95% level of confidence. These findings confirmed the results of the 1994 study (G. A. Curtis, D. A. Dame and G. F. O'Meara, unpublished) in which the difference between Captiva and Flamingo strains was estimated to be ca. 10-fold.

The mainland strain: The LC_{50} of the mainland Burnt Store strain was not significantly different from that of the naive Flamingo strain at the 95% level of confidence, producing a comparison ratio of 1.06. However, the LC_{50} estimate fell above the actual exposure level, so additional estimates were used for interpreting the analyses. The LC_{10} and 20% lethal concentration (LC_{20}) estimates both fell within the actual exposure range and the strains were not significantly different at the 95% level of confidence. The 30% lethal concentration (LC_{30}) and 40% lethal concentration (LC_{40}) estimates fell outside the exposure range, but here also the strains were not significantly different at the 95% level of confidence.

DISCUSSION

No previous studies have revealed significantly increased tolerance to methoprene among natural mosquito populations, although resistance has been observed in laboratory mosquitoes selected for many generations by methoprene exposure (e.g.,

Culex spp., Brown and Brown 1974, Georghiou 1974).

The data generated in this study confirm the existence of resistance in both the Captiva and the Lover's Key strains. Presumably, in both cases the resistance is due to extended exposure and selection in a genetically isolated site. Both strains had been exposed to applications of 150-day briquets for 5–6 seasons (1989–94), which had given satisfactory operational results until 1993–94. These strains presumably had been fully susceptible to methoprene prior to 1989, when they probably were genetically indistinct from the population at St. James City on nearby Pine Island, Florida (Fig. 1), where early experimental studies had been conducted with methoprene (Dame et al. 1976).

However, it is impossible to draw conclusions from these barrier island data concerning the susceptibility of mainland mosquitoes. Both of the barrier island resistant strains are located west of the mainland (Fig. 1), a factor which may tend to reduce migration from the mainland into the affected barrier island populations and thereby enhance selection. Earlier studies (Provost 1952, 1957) demonstrated long range *Ae. taeniorhynchus* flights oriented from the Lee County barrier islands eastward onto the mainland. As a result of the geographical orientation, and probable isolation from influx from the mainland, selection pressure may materially exceed that which would occur on the mainland where more genetic mixing would be expected to occur. Thus, there would appear to be a good possibility that selection towards resistance would be much less intense on the mainland than on the barrier islands.

The limited study conducted on the mainland Burnt Store strain revealed no indication of resistance or tolerance. Unlike the barrier island strains, for which the LC_{10} estimates in 1995 were significantly different following exposure to briquet formulations for several seasons than the naive strain at the 95% level of confidence, the Burnt Store mainland strain was not significantly different from the naive strain at any of the 1997 estimated LC levels and the slopes were similar. This strain had been exposed in nature to multiple applications of liquid formulations of methoprene from 1987 to 1994. Although the availability of a susceptible genome on the mainland may account for the lack of resistance in the Burnt Store strain, we are unable to rule out the possibility that the type of formulation and application technique might also be important factors.

Nevertheless, the resistance observed on the 2 separate barrier islands has not yet been detected on the mainland. Insofar as is possible, further studies will be conducted to elucidate the dynamics of methoprene resistance in *Ae. taeniorhynchus* in Lee County.

ACKNOWLEDGMENTS

Laboratory space, equipment, supplies, and staff were generously made available by the Lee County Mosquito Control. The study benefited materially from the assistance of George O'Meara and technician Leonard Evans of the Florida Medical Entomology Laboratory for providing Flamingo eggs and testing materials. Funding and technical support from Sandoz Agro made possible the propagation and testing of the resistant strains. Preparation of silanized glassware by Sandoz Agro staff in Dallas, TX, was instrumental in speeding up the process of experimentation. Without the support of the Lee County Mosquito Control staff, especially technician Brian Cotterill and Director Willam Opp, the studies would not have been possible. We are grateful for the helpful assistance of those who reviewed various stages of the manuscript: G. Alan Curtis (Indian River Mosquito Control, Vero Beach, FL), George F. O'Meara (Florida Medical Entomology Laboratory, Vero Beach, FL), Doug VanGundy (Zoecon Corporation, Dallas, TX), and Tom G. Wilson (Colorado State University, Ft. Collins, CO).

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Inheritance, Stability, and Dominance of Cry Resistance in *Culex quinquefasciatus* (Diptera: Culicidae) Selected With the Three Cry Toxins of *Bacillus thuringiensis* subsp. *israelensis*

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ABSTRACT Mendelian crosses were used to study the mode of inheritance of Cry toxin resistance in a *Culex quinquefasciatus* Say (Diptera: Culicidae) colony (CqAB11A) that evolved insecticide resistance under laboratory selection with a deletion mutant of *Bacillus thuringiensis* subsp. *israelensis* de Barjac lacking the Cyt1Aa toxin component but containing its three major Cry toxins, Cry4Aa, Cry4Ba, and Cry11Aa. High levels of resistance were observed to Cry toxins. F1 offspring of reciprocal crosses to a sensitive colony showed intermediate levels of resistance with no maternal effect, indicating autosomal inheritance. Dose–response data for backcross offspring deviated significantly from the monofactorial model when tested with Cry4Aa + Cry4Ba + Cry11Aa, Cry4Aa + Cry4Ba, or Cry11Aa. However, tests with Cry11Ba from *B. thuringiensis* subsp. *jegathesan* (Seleena, Lee, Lecadet) fit the monofactorial model. Dominance of F1 offspring was calculated at different concentrations of Cry-toxin suspensions and, as reported for other Cry-resistant *Culex*, generally decreased as concentration increased. A subset of colony CqAB11A was reared without selection pressure for 18 generations with little change in susceptibility, indicating a highly homozygous population. Consistent with reports for other Cry-resistant *Culex*, the data show these mosquitoes evolved resistance to *B. thuringiensis* Cry toxins at multiple loci in response to selection pressure and that cross-resistance to Cry11Ba was conferred by one of those loci.

KEY WORDS genetics, resistance, Cry toxin, dominance, mosquito

The bacterium *Bacillus thuringiensis* (Bt) is distinguished by the production of crystalline proteins at sporulation, many of which are toxic to insects (Lacey 2007). Depending on the strain of *B. thuringiensis* and the specific endotoxins it produces, different insecticidal effects are observed. For example, Cry4Aa, Cry4Ba, and Cry11Aa from *B. thuringiensis* subsp. *israelensis* de Barjac are toxic to nematoceran dipterans, including many mosquito and blackfly vectors of important human diseases, whereas Cry1 and Cry3 endotoxins are active against lepidopterans and coleopterans, respectively (Federici et al. 2010). When B. *thuringiensis* cells lyse at sporulation, a crystallized endotoxin is released into the environment where it can be ingested by susceptible insect larvae in their natural feeding process. After ingestion, the crystal endotoxin dissolves inside the insect midgut environment and releases the insecticidal proteins, which are activated by natural proteases. The activated toxins bind to receptors on the brush-border membrane of the insect midgut epithelium, destroying the epithelium and killing the larvae by destroying this tissue through the formation of cationic pores that result in cell death (Bravo et al. 2007).

Although widespread use against lepidopteran pests of the closely related bacterium *Bacillus thuringiensis* subsp. *kurstaki* (de Barjac and Lemille) has resulted in the evolution of insecticide resistance in several field populations of *Plutella xylostella* (L.) (Tabashnik et al. 1990, Shelton et al. 1993) and a few greenhouse populations of *Trichoplusia ni* (Hübner) (Janmaat and Meyers 2003), larvicides based on *B. t.* subsp. *israelensis* have been successfully used for >20 yr with no evidence of field control failure in mosquito populations (Becker and Ludwig 1993, Becker 1997). This failure to evolve insecticide resistance is in sharp contrast to the widespread resistance that evolved in many mosquito populations in response to conventional insecticide treatments. The lack of resistance to *B. t.* subsp. *israelensis* is attributed to the diversity and mechanisms of action of the toxins produced by this species, which includes the three major Cry toxins noted above, and Cyt1A, a cytolytic toxin, all of which are packaged in a single, large parasporal body (Ibarra and Federici 1986). A unique characteristic of *B. t.* subsp. *israelensis* is its relatively high toxicity, on the order of 10 ng/ml against fourth instars of various mosquito species; activity that is higher than would be

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expected from the activities of the individual toxic components. Numerous studies have shown that the various toxins interact with one another synergistically (Crickmore et al. 1995, Poncet et al. 1995) and that synergy is responsible for masking the evolution of resistance in *B. t.* subsp. *israelensis*–selected mosquitoes (Wirth et al. 1997). The Cyt1Aa component plays a critical role in both synergy and in resistance suppression (Wirth et al. 1997). For example, studies have demonstrated that mosquitoes selected with *B. t.* subsp. *israelensis* Cry toxins in the absence of the Cyt1Aa toxin can evolve high levels of resistance (Georghiou and Wirth 1997, Wirth et al. 2004), but those high levels of resistance can be overcome by combining Cyt1Aa with the Cry toxins (Wirth et al. 1997). More importantly, resistance evolves more slowly and to much lower levels in mosquitoes exposed to a single Cry toxin presented in combination with Cyt1A (Wirth et al. 2005).

Although we currently have a basic understanding of how the *B. t.* subsp. *israelensis* toxin characteristics influence the evolution of Cry resistance in mosquitoes, little is known of the genetics underlying this resistance. Data on the genetic basis of insecticide resistance is important to our general understanding of an insect population's response to selection pressure, and this information also can lead to the development of tactics for avoiding or managing insecticide resistance. For example, the data generated from laboratory studies on the genetics of *B. thuringiensis* subsp. *kurstaki*–resistant crop pests were used in the development of the Environmental Protection Agency–mandated refuge strategies implemented for genetically engineered Bt crops (Glaser and Matten 2003). Furthermore, this information is invaluable for designing recombinant bacterial insecticides that are refractory to driving resistance evolution in treated populations. Although field resistance to *B. t.* subsp. *israelensis* is not yet a problem, it would be shortsighted to assume that it cannot occur. In fact, significant levels of resistance have evolved in several field populations of *Culex pipiens* L. complex treated with *Bacillus sphaericus* Neide, an unrelated microbial control agent (Sinègre et al. 1994, Rao et al. 1995, Silva-Filha et al. 1995, Yuan et al. 2000, Mulla et al. 2001) and a suspected case of *B. t.* subsp. *israelensis* resistance was recently reported from New York (Paul et al. 2005). Here, we examined the Mendelian patterns of inheritance of Cry-toxin resistance and cross-resistance in a colony of *Culex quinquefasciatus* Say with high levels of resistance that evolved under laboratory selection pressure by using a deletion mutant of *B. t.* subsp. *israelensis.* This mutant strain synthesizes the three major Cry toxins that naturally occur in *B. t.* subsp. *israelensis,* but lacks Cyt1Aa. Traditional Mendelian backcross experiments were used to determine whether phenotypic expression of resistance was monogenic or polygenic. Dominance values were calculated at various treatment concentrations and the stability of resistance and cross-resistance in the absence of selection pressure was examined. The patterns of inheritance are discussed in relation to patterns observed for other *C. quinquefasciatus* laboratory colonies with Cry resistance.

Materials and Methods

Mosquito Colonies. Two *C. quinquefasciatus* colonies were used in these experiments. Colony CqSyn is a synthetic susceptible colony that originated in 1995; it was formed by pooling multiple field collections and used for baseline bioassays to estimate resistance levels and as the susceptible colony for the genetic crosses (Wirth et al. 2004). The second colony, CqAB11A, originated in 1990 from a different synthetic population and has been maintained under selection pressure since that time with a *B. t.* subsp. *israelensis* deletion mutant expressing the natural Cry toxins (Cry4Aa + Cry4Ba + Cry11Aa) but not the Cyt1Aa toxin component (Georghiou and Wirth 1997).

Recombinant Bacterial Strains. A crystalliferous *B. t.* subsp. *israelensis* that synthesizes Cry4Aa + Cry4Ba (Delécluse et al. 1993b), Cry11Aa (Wu et al. 1994), or Cry11Ba from *B. thuringiensis* subsp. *jegathesan* (Seleena, Lee, Lecadet) (Delécluse et al. 1995) were used in bioassays. The deletion mutant of *B. t.* subsp. *israelensis* that synthesizes Cry4Aa + Cry4Ba + Cry11Aa but lacks Cyt1Aa was used in both selection and bioassays (Delécluse et al. 1993a). All recombinant materials were in the form of lyophilized crystal/spore powders. The bacterial strains were grown on liquid media as described previously (Park et al. 1998). Sporulated cells were washed in distilled water and sedimented, and the resulting pellet was lyophilized and stored at 4°C.

Selection and Bioassay Procedures. Stock suspensions of crystal/spore powder were prepared by weight in deionized water and homogenized by incorporating glass beads into the suspension and agitating on a vortex mixer to obtain a fine particle suspension. Stocks were prepared monthly, and 10-fold serial dilutions were prepared weekly. All stocks and dilutions were stored at −20°C when not in use. Early fourth instars were used for bioassay tests and selections. Bioassays exposed groups of 20 larvae to different concentrations of crystal/spore suspension in 100 ml of deionized water in 237-ml (8-ounce) plastic cups. At least seven different concentrations, plus a water control, were replicated a minimum of five times on five different days. Mortality was determined 24 h after treatment, and data were analyzed using a Probit program for the PC (Raymond et al. 1993). Resistance ratios were determined by comparing concurrently obtained dose–response values for CqSyn and CqAB11A and calculated by dividing the LC_{50} (or LC_{95}) of the selected colony by the corresponding value for CqSyn. Dose–response values with overlapping fiducial limits were not considered significantly different.

Selection consisted of feeding the recombinant bacterial suspension to groups of 1,000 early fourth instars in 1 liter of deionized water in enamel metal pans for 24 h. The treatment concentration was adjusted to

Table 1. Dose–response values and resistance ratios for the colony CqAB11A (R), CqSyn (S), and F1 offspring of reciprocal crosses to CqSyn by using suspensions of recombinant powders expressing toxins Cry11Aa, Cry11Ba, Cry4Aa + Cry4Ba, or Cry4Aa + Cry4Ba + Cry11Aa

Toxin(s)	Colony	LC_{50} (FL), μg/ml	LC_{95} (FL), μg/ml	Resistance ratio	
				LC_{50}	LC_{95}
Cry4Aa, 4Ba, 11Aa	CqSyn	0.0249 (0.0175–0.0345)	0.345 (0.178–0.699)	1.0	1.0
	CqAB11A	1.21 (0.796–1.83)	64.4 (26.7–163)	49.1	187
	R X S	0.210 (0.171–0.255)	5.01 (3.54–7.67)	8.5	14.5
	S X R	0.122 (0.0805–0.186)	4.39 (2.09–9.46)	4.9	12.7
Cry4Aa, 4Ba	CqSyn	0.122 (0.02313–0.646)	1.35 (0.0705–25.5)	1.0	1.0
	CqAB11A	126.3 (37.5–444)	41974 (1176–1.8×10^6)	634	15,011
	R × S	2.62 (1.64–4.18)	173 (65.9–470)	21.5	128.1
	S × R	2.98 (1.75–5.07)	171.7 (56.0–539)	24.4	127.2
Cry11Aa	CqSyn	1.64 (0.664–3.99)	130 (25.1–695)	1.0	1.0
	CqAB11A	13,004 (1,646–12×10^6)	428,483 (∞)	11,708	506,310
	R × S	108.5 (36.9–340.5)	38,052 (1,150–1.7×10^6)	66.6	292
	S × R	83.4 (18.2–390)	28,820 (170–5.5×10^6)	52.2	222
Cry11Ba	CqSyn	0.0486 (0.0369–0.0638)	0.294 (0.181–0.496)	1.0	1.0
	CqaB11A	0.957 (0.583–1.56)	92.1 (35.4–248)	24.6	427
	R × S	0.222 (0.130–0.379)	6.07 (2.11–17.8)	4.6	20.6
	S × R	0.186 (0.148–0.228)	5.54 (3.88–8.59)	3.8	18.8

ensure 50–80% mortality. Survivors were removed to clean water, fed, and used to continue the colony. Throughout their selection history, 500–1,000 adults were used to establish any single generation, to avoid population bottlenecks. After 28 generations of selection pressure, generations were allowed to overlap.

Genetic Crosses. Reciprocal mass crosses were prepared between susceptible (S) CqSyn and the resistant (R) CqAB11A colonies. Virgin male and female adults were obtained by isolating pupae in scintillation vials. A minimum of 300 males and 300 females were used for each mass cross. The following crosses and backcrosses were prepared, with the female parent listed first: 1) CqSyn × CqAB11A; 2) CqAB11A × CqSyn; 3) (CqAB11A × CqSyn)F1 × CqSyn.

We accepted the assumption that CqAB11A was homogeneous for resistance because the resistance levels, in combination with the stability of resistance in the absence of the insecticide, were consistent with a high proportion of homogeneity for *B. thuringiensis* resistance. Backcross offspring were tested with the various recombinant powders by using 12–14 different concentrations of crystal/spore suspension to provide robust dose–response data and tested for goodness-of-fit to a monofactorial modal as described previously (Wirth et al. 2010).

Dominance Calculations. The single concentration method of Hartl (1992), as described by Liu and Tabashnik (1997), was used to estimate the degree of dominance of the resistance or cross-resistance trait, $h = (w_{12} - w_{22})/(w_{11} - w_{22})$, where h is the degree of dominance, w_{11} is the fitness of the homozygous resistant parent, w_{12} is the fitness of the heterozygous offspring, and w_{22} is the fitness of the homozygous susceptible parent. The fitness of the homozygous resistant parent at any treatment concentration was assumed to be 1. The fitness of the susceptible parent and the heterozygous F1 were estimated from the survival rate of the larvae at a specific treatment concentration divided by the survival rate of the resistant parent at the same concentration. Using this formula, an h value of 0 indicates fully recessive inheritance, an h value of 1 indicates a fully dominant trait, and an h value of 0.5 represents a codominant trait. When $0 < h < 0.5$, the trait is partly recessive, whereas when $0.5 < h < 1$, the trait is partly dominant.

Stability of Resistance. Three pans of larvae from CqAB11A were allowed to develop without exposure to insecticide and used to establish generation one of the unselected line. The unselected line was reared for 18 generations without exposure to insecticide with generations maintained separately. Susceptibility to insecticides was evaluated at generations 3, 7, 10, and 18.

The proportion of survivors of exposure to a 10-fold series of concentrations up to 200 μg/ml for each insecticide were used to calculate the change in frequency of resistant genotypes in the populations in the absence of insecticide exposure as described by Tabashnik et al. (1994). The rate of change in the absence of insecticide exposure (R) can be calculated from the following formula: R = (log [final proportion surviving treatment] – log [initial proportion surviving])/n, where n is the number of generations not exposed to insecticide. A negative R value indicates a decline in the proportion surviving exposure to insecticide. Values close to 0 or 0 indicate stable resistance.

Results

When assayed with Cry4Aa + Cry4Ba + Cry11Aa, colony CqAB11A had LC_{50} and LC_{95} values of 1.21 and 64.4 μg/ml and resistance ratios of 49.1 and 187, respectively (Table 1). F1 offspring of reciprocal crosses with CqSyn were intermediate in resistance to their respective susceptible and resistant parents, with LC_{50} values of 0.210 and 0.122 μg/ml. These values were not significantly different from each other based on overlap of their fiducial limits. Higher resistance was observed using recombinant powders expressing Cry4Aa + Cry4Ba. Lethal concentration values at 50 and 95% were 126.3 and 41,974 μg/ml, resulting in

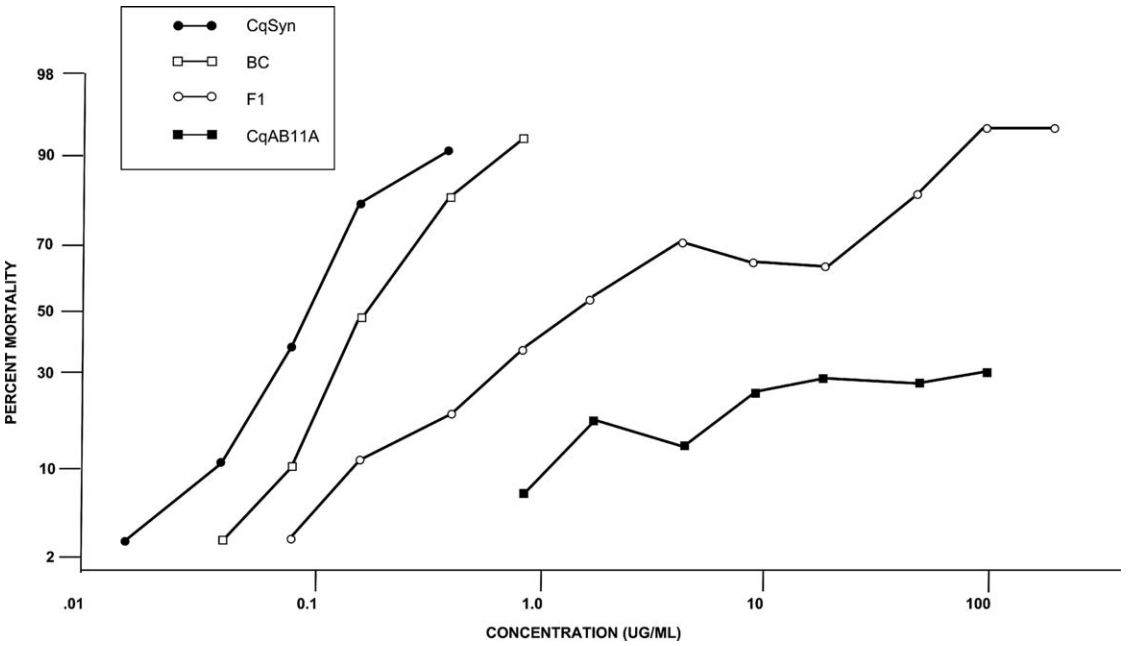

Fig. 1. Results of dose–response testing with *B. t.* subsp.. *israelensis* toxins Cry4Aa + Cry4Ba against two parental colonies (CqSyn, CqAB11A), the F1 (CqAB11A × CqSyn), and offspring of the backcross (BC) [(CqAB11A × CqSyn) F1 × CqSyn].

resistance ratios of 634 and 15,011, respectively. Offspring of reciprocal crosses to CqSyn tested with Cry4Aa + Cry4Ba were intermediate to their respective resistant and susceptible parents and were not significantly different from one another, with LC_{50} values of 2.62 and 2.98 μg/ml. The highest resistance levels were observed in tests with Cry11Aa. Lethal concentration values were 13,004 and 428,000 μg/ml at the LC_{50} and LC_{95} and resistance ratios were estimated at 11,708 and 506,310. F1 offspring showed intermediate levels of susceptibility, with LC_{50} values of 108.5 and 83.4 μg/ml, and these values were not significantly different from each other. Cry11Ba assays on CqAB11A showed LC_{50} and LC_{95} values of 0.957 and 92.1 μg/ml, respectively, and resistance ratios of 24.6 and 427. F1 offspring were again intermediate to their respective parents, with LC_{50} values of 0.222 and 0.186 μg/ml and resistance ratios of 4.6 and 3.8 at the LC_{50} and 20.6 and 18.8 at the LC_{95}. The lethal concentration values of the F1 offspring were not significantly different from each other.

The F1 offspring of the cross CqAB11A × CqSyn were backcrossed to CqSyn and their offspring were bioassayed with the four recombinant powders and tested for goodness-of-fit to a monofactorial model. Tests using Cry4Aa + Cry4Ba + Cry11Aa ($\chi^2 = 31$, df = 13, $P < 0.05$), Cry4Aa + Cry4Ba ($\chi^2 = 29.8$, df = 13, $P < 0.05$) (Fig. 1), and Cry11Aa ($\chi^2 = 18.3$, df = 10, $P < 0.05$) did not fit the monofactorial model. All three dose–response lines deviated significantly from the expected mortality for the monofactorial model at moderately low and moderately high treatment concentrations. In contrast, the dose–response line for Cry11Ba (Fig. 2) showed no significant deviations from the mortality expected under the monofactorial model ($\chi^2 = 22.4$, df = 13, $P > 0.05$).

F1 offspring were generally dominant at low treatment concentrations; however, dominance declined at higher treatment concentrations (Table 2). For example, tests with Cry4Aa + Cry4Ba + Cry11Aa and Cry4Aa + Cry4Ba showed complete dominance (1.0) at 0.002 μg/ml, but dominance declined to semirecessive by treatment concentration of 20.0 μg/ml. Cry11Aa was also dominant at 0.20 μg/ml but declined to codominant at 200 μg/ml. Cry11Ba was fully dominant at 0.02 μg/ml and declined to completely recessive ($h = 0$) by 200 μg/ml.

A subset of CqAB11A was reared without selection for 18 generations and susceptibility to all the recombinant materials was periodically tested (Table 3). R values were calculated at the various treatment concentrations on generation 18 to detect declines in resistance. Most values were very close to 0, indicating generally stable resistance. Negative R values, indicating declines in resistance, were detected toward Cry4Aa + Cry4Ba + Cry11Aa and Cry11Aa; however, these negative values were 0.06 or smaller. Cry4Aa + Cry4Ba and Cry11Ba each showed a single low negative value, and all other R values were positive.

Discussion

C. quinquefasciatus larvae under long-term selection pressure with the *B. t.* subsp. *israelensis* toxins Cry4Aa + Cry4Ba + Cry11Aa evolved moderate levels of resistance to those toxins when presented in combination, but they expressed much higher levels of resistance and cross-resistance to the individual toxins

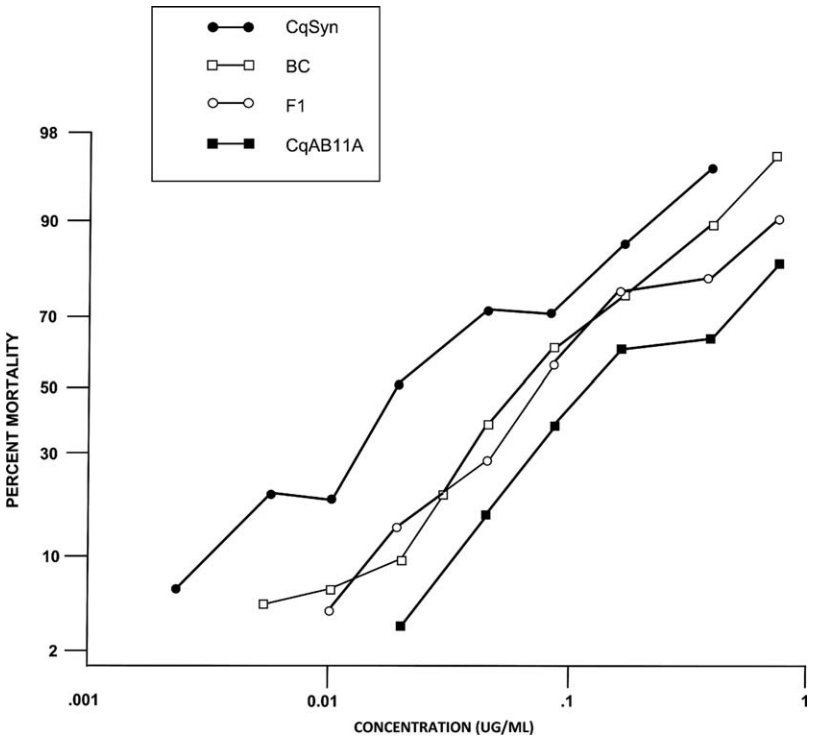

Fig. 2. Results of dose–response testing with *B. t.* subsp. *israelensis* toxins Cry11Ba against two parental colonies (CqSyn, CqAB11A), the F1 (CqAB11A × CqSyn), and offspring of the backcross (BC) [(CqAB11A × CqSyn) F1 × CqSyn].

such as Cry11Aa and Cry11BA, or the mixture Cry4Aa + Cry4Ba. This pattern of resistance evolution in response to selection with mixtures of Cry toxins was reported previously (Georghiou and Wirth 1997, Wirth et al. 1998), but these patterns have persisted under continuing selection pressure and the levels of resistance and cross-resistance have significantly increased. For example, resistance toward Cry4Aa + Cry4Ba + Cry11Aa was 91-fold at the LC_{95} after 28 generations of selection but rose to 187 in this study. Resistance rose more dramatically when the colony was tested with one, or a mixture of two, Cry toxins; from 13.5-fold (Wirth and Georghiou 1997) to 506,310 for Cry11Aa, and from 16.2-fold (Wirth and Georghiou 1997) to 15,011 at the LC_{95} for Cry4Aa + Cry4Ba. A moderate increase in cross-resistance to-

Table 2. Estimation of dominance based on the concentration of *B. thuringiensis* Cry toxin(s) for F1 offspring of reciprocal crosses between CqAB11A and CqSynP

Toxin(s)	Concn, μg/ml	Doninance value for cross	
		CqAB11A × CqSyn	CqSyn × CqAB11A
Cry4Aa + Cry4Ba + Cry11Aa	0.002	1	1
	0.02	0.92	0.48
	0.20	0.56	0.37
	2.0	0.30	0.16
	20.0	0	0.23
Cry4Aa + Cry4Ba	0.02	1.0	1.0
	0.20	0.86	0.95
	2.0	0.54	0.69
	20.0	0.47	0.33
	200.0	0.19	0.24
Cry11Aa	0.20	1.0	1.0
	2.0	0.88	0.91
	20.0	0.54	0.64
	200.0	0.52	0.52
Cry11Ba	0.02	0.94	1.05
	0.20	0.49	0.65
	2.0	0.31	0.26
	20.0	0.23	0.0
	200.0	0.0	0.0

Table 3. Stability of resistance and cross-resistance in the absence of insecticide selection pressure calculated from percent survival of larvae at different treatment concentrations

Toxin(s)	Concn, μg/ml	Generations without selection				Stability (R)
		1	4	13	18	
Cry4A + Cry4B + Cry11A	0.2	79	78	64	26	−0.027
	2.0	44	38	34	13	−0.028
	20.0	13	4	6	0	−0.06
	200	0	1	0	0	0
Cry4Aa + Cry4Ba	2.0	79	90	85	83	0.0012
	20	68	69	53	46	−0.0056
	200	31	50	41	44	0.0056
Cry11Aa	2	100	96	98	96	−0.0011
	20	92	90	85	86	−0.0017
	200	82	79	57	66	−0.0050
Cry11Ba	0.02	98.7	98	100	100	0.0006
	0.20	57	80	97	98.5	0.0133
	2.0	48	55	73	47	−0.0006
	20.0	11	22	47	29	0.0233
	200.0	1	38	20	26	0.0789

ward Cry11Ba was observed, from 347-fold (Wirth et al. 1998) to 427 at the LC_{95}.

When reciprocal crosses were undertaken between CqAB11A and the susceptible colony CqSyn, F1 offspring were not significantly different from each other in their phenotypic expression of resistance and cross-resistance to the different recombinant powders, indicating that resistance traits were not sex-linked and were not influenced by the maternal parent. These data are consistent with our earlier studies on other *Culex* colonies with *B. t.* subsp. *israelensis*-resistance (Wirth et al. 2010) and suggest that autosomal inheritance of Cry resistance may be common in this species. Autosomal inheritance also has been reported for other insect species with resistance to *B. thuringiensis* Cry toxins, including *Plodia interpunctella* (Hübner) (McGaughey 1985), *P. xylostella* (Tabashnik et al. 1992), *Heliothis virescens* (F.) (Gould et al. 1995), *Leptinotarsa decemlineata* (Say) (Rahardja and Whalon 1995), *Pectinophora gossypiella* (Saunders) (Tabashnik et al. 2002), *Ostrinia nubilalis* (Hübner) (Alves et al. 2006), and *Helicoverpa armigera* (Hübner) (Mahon et al. 2007).

The classical backcross method was used to determine whether resistance and cross-resistance were under monofactorial or polyfactorial control in CqAB11A. The F1 dose–response line for Cry4Aa + Cry4Ba + Cry11Aa failed to fit the monofactorial model. The deviations in observed mortality were near the LC_{50} range and could be indicative of nonadditive polygenic inheritance or experimental error (Tabashnik 1991). Although tests with Cry4Aa + Cry4Ba and Cry11Aa also failed to fit the monogenic model, the deviations showed higher than expected mortality at moderately low doses and lower than expected mortality at moderately high doses, including deviations near the LC_{50}, leading to a conclusion of nonadditive, polygenic inheritance (Tabashnik 1991). Assays with Cry11Ba were consistent with monofactorial inheritance of cross-resistance, suggesting that cross-resistance evolved at a locus shared with other Cry toxins or was closely linked with such a locus. Prior studies on resistant colonies of *C. quinquefasciatus*, selected with Cry11Aa or Cry4Aa + Cry4Ba, provide some interesting similarities and differences (Table 4) (Wirth et al. 2010). The Cry11Aa-selected colony evolved the same patterns of inheritance of resistance and cross-resistance observed in CqAB11A; polyfactorial inheritance of Cry11Aa and Cry4Aa + Cry4Ba-resistance and monofactorial inheritance of Cry11Ba cross-resistance. The Cry4Aa + Cry4BA-selected colony also expressed polyfactorial inheritance for Cry4Aa + Cry4Ba resistance, but differed from the other two colonies by evolving monofactorial inheritance of Cry11Aa resistance and polyfactorial inheritance of Cry11Ba cross-resistance. Together, these results indicate that multiple loci are involved in Cry toxin resistance in *C. quinquefasciatus* and that the number of loci involved in resistance to a particular toxin is influenced by the specific components of the Cry toxin mixture used in selection.

Table 4. Comparison of backcross experimental results from three *B. thuringiensis*–resistant colonies, CqAB11A, CqAB, and Cq11A, and tested with various Cry toxins or toxin mixtures

	Mosquito colony		
	CqAB11A	CqAB[a]	Cq11A[a]
Recombinant Bacterial Strain			
Cry11Aa	Polyfactorial	Monofactorial	Polyfactorial
Cry4Aa + Cry4Ba	Polyfactorial	Polyfactorial	Polyfactorial
Cry4Aa + Cry4Ba + Cry11Aa	Polyfactorial	NT[b]	NT
Cry11Ba	Monofactorial	Polyfactorial	Monofactorial

[a] Data from Wirth et al. (2010). Inheritance patterns, dominance, stability, and allelism for insecticide resistance and cross-resistance in two colonies of *Cx. quinquefasciatus* selected with *B. t.* subsp. *israelensis* Cry toxins.

[b] NT, not tested.

Complete or near complete dominance of resistance was observed at lower treatment concentrations in F1 larvae; however, dominance declined to codominant, or in one case, fully recessive inheritance, at the higher treatment concentration. Similar patterns of declining dominance also were observed in two other colonies of *C. quinquefasciatus* selected for resistance with Cry11Aa or to Cry4Aa + Cry4Ba (Wirth et al. 2010). Many other insect species resistant to *B. thuringiensis* Cry toxins have shown patterns of declining dominance, such as *P. xylostella* (Sayyed et al. 2000, Liu et al. 2001), *O. nubilalis* (Alves et al. 2006), *L. decemlineata* (Rahardja and Whalon 1995), and *P. gossypiella* (Tabashnik et al. 2002); however, dominance patterns can vary broadly among resistant insect species. For example, fully recessive inheritance (Tabashnik et al. 1992, Augustin et al. 2004, Mahon et al. 2007), incompletely recessive resistance (Hama et al. 1992, Kain et al. 2004), and incomplete dominance (Sims and Stone 1991, Huang et al. 1999) have been reported. Theoretically, the partly dominant, to dominant inheritance of Cry resistance in these mosquitoes could enhance survival of heterozygous larvae that are exposed to sublethal field treatment concentrations of *B. t.* subsp. *israelensis.* How relevant any such survival might be in nature is questionable, because the native *B. t.* subsp. *israelensis* used in the field expresses the Cyt1Aa protein and its presence overcomes resistance to the other Cry toxins (Wirth et al. 1997).

Resistance was highly stable in the absence of selection pressure and any negative R values were very close to 0, with a single exception. Cry4A + Cry4B + Cry11A showed generally negative R values ranging from −0.027 to −0.06. Otherwise, R values were very close to 0 and were generally positive. Cry11Aa-selected and Cry4Aa+Cry4Ba-selected *Culex* colonies were previously reported to have generally stable resistance and cross-resistance in the absence of selection pressure (Wirth et al. 2010). Given the largely polygenic nature of its Cry resistance, it would not be unexpected for resistance to be unstable toward the most complex, three-toxin mixture. This slight instability may indicate that the colony CqAB11A was not fully homozygous at all resistance loci. In contrast to the high

stability of Cry resistance in *C. quinquefasciatus*, some *B. thuringiensis*–resistant *P. xylostella* colonies showed rapidly declining resistance and R values averaged −0.28 (Tabashnik et al. 1994). Other *B. thuringiensis*–resistant species exhibited slower declines in resistance in the absence of selection. For example, *P. interpunctella* lost resistance slowly (R = −0.02) as did *H. virescens* (R = −0.04) (McGaughey and Beeman 1988, Sims and Stone 1991). Instability of resistance in the absence of selection is generally attributed to reductions in fitness (Tabashnik et al. 1994). For CqAB11A, it is likely that prolonged selection has lead to fixation or near fixation of resistance alleles at most resistant loci. Fixation of resistance alleles is unlikely to occur in the field because mosquito populations are highly mobile, dispersed, and most populations are not genetically isolated. In view of the prolonged selection of this particular *Culex* colony, fitness studies might prove useful in understanding the basis of resistance stability.

The results of this study, and our prior genetic studies with *Culex* (Wirth et al. 2010), show interesting and complex patterns of inheritance of Cry resistance and cross-resistance in *C. quinquefasciatus.* To date, Cry resistance has been consistently autosomal with no maternal effect in the three colonies studied. However, differences in the composition of the toxins used for selection seemed to influence the number of loci involved in resistance and cross-resistance inheritance. It is well known that the different component toxins of *B. t.* subsp. *israelensis* interact to enhance activity (Crickmore et al. 1995, Poncet et al. 1995). Consequently, it is not surprising that those interactions, or the lack thereof because of the absence of a particular toxin, might exert influence during selection. This effect was apparent for Cry11Ba, a toxin to which the three Cry-resistant colonies had never been exposed. *Culex* selected Cry11Aa or a mixture including Cry11Aa showed monofactorial patterns of inheritance of Cry11Ba cross-resistance. The colony selected without Cry11Aa, Cry4Aa + Cry4Ba, evolved polyfactorial inheritance of Cry11Ba cross-resistance.

The cross-resistance data clearly demonstrated that Cry11Ba cross-resistance evolves in response to selection with *B. t.* subsp. *israelensis* Cry toxins. Furthermore, it is known that resistance to Cry11Aa confers cross-resistance to Cry4Aa + Cry4Ba and vice versa (Wirth et al. 1997, 2010). If resistance in these three colonies involves alterations in receptor binding, as is common in many Cry-resistant insects, then Cry11Ba, Cry11Aa, Cry4Aa, and Cry4Ba are likely to share binding sites. Cross-recognition of binding sites has been reported for *P. xylostella*, which has a single receptor that binds Cry1Aa, Cry1Ab, Cry1Ac, and Cry1 F toxins (Granero et al. 1996, Ballester et al. 1999). Furthermore, brush-border membrane vesicle binding studies in *Aedes aegypti* (L.), another mosquito species, suggested that Cry11Aa, Cry4Aa, and Cry4Ba might share a common class of binding sites (de Barros Moreira Beltrão and Silva-Filha 2007). To prove this hypothesis, receptor binding studies and identification of the Cry receptors in *C. quinquefasciatus* is needed.

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Research Article

High Resistance to *Bacillus sphaericus* and Susceptibility to Other Common Pesticides in *Culex pipiens* (Diptera: Culicidae) from Salt Lake City, UT

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Abstract

Biorational mosquito larvicides based on microbial organisms and insect growth regulators (IGRs) have played a vital role in integrated mosquito control, particularly since the invasion of West Nile virus to the United States in 1999. Products that are formulated with technical powder of the bacterium, *Bacillus sphaericus* Neide (recently *Lysinibacillus sphaericus* Meyer and Neide), are among the ones that have been extensively applied to combat *Culex* and other mosquito species. Due to the simplicity of the binary toxins, resistance to this pesticide in laboratory and field populations of *Culex pipiens* L. complex has occurred globally since 1994. A *Cx. pipiens* population with a high level of resistance to *B. sphaericus* (VectoLex WDG) was identified in Salt Lake City, UT, in September 2016. The resistance ratios in this population were 20,780.0- and 23,926.9-fold at LC_{50} and LC_{90}, respectively, when compared with a susceptible population of a laboratory reference colony of the same species. This *B. sphaericus*-resistant population remained mostly susceptible to other commonly used pesticides to control arthropods of public health and urban significance, including ones based on microbial organisms (*Bacillus thuringiensis* subsp. *israelensis*, spinosad, spinetoram, abamectin), IGRs (pyriproxyfen, methoprene, diflubenzuron, novaluron), organophosphate (temephos), neonicotinoid (imidacloprid), phenylpyrazole (fipronil), oxadiazine (indoxacarb), and pyrethroid (permethrin). Results are discussed according to the modes of action of the pesticides tested, and suggestions are made to manage *B. sphaericus*-resistant mosquito populations.

Key words: *Bacillus sphaericus*, *Culex pipiens*, Diptera, Culicidae, resistance

Upon economic development and demographic growth, burdens of vectors and vector-borne diseases have been on the rise globally. Mosquito control by means of environmentally sustainable methods is facing severe challenges for numerous reasons, such as range expansion of vector species, newly emerging and increased distribution of mosquito-borne pathogens, and insecticide resistance development. This last obstacle is due in part to the lack of new active ingredients and formulations. Among the few available mosquito larvicides with microbial origins, *Bacillus sphaericus* Neide, recently *Lysinibacillus sphaericus* Meyer and Neide (Ahmed et al. 2007) plays a unique role in combating peridomestic *Culex* spp. Among many strains that have been isolated and identified from natural soil environments, strains 2362, 1597, 2297, C3-41, and IAB-59 possess high larvicidal activity mainly against *Culex* spp. and some other species in the genera of *Anopheles*, *Culiseta*, *Psorophora*, and *Aedes* (Su 2016a). Since the 1960s, strain 2362 has been the interest of research and development worldwide, with a strong emphasis in the United States. Active strains produce parasporal inclusions during sporulation, containing crystal binary toxins A and B (Bin A 42 kDa and Bin B 51 kDa) that play the predominant role in larvicidal toxicity. Some strains also produce noncrystal mosquitocidal toxins (Mtx) such as Mtx 1, 2, and 3 during the vegetative stage. The Mtx contribute to the larvicidal activity less than the binary toxins but are helpful in minimization of resistance development to the binary toxins. Products based on *B. sphaericus* are highly effective against target larval stages in habitats with high organic matter. These formulations offer desired initial and residual efficacy and possess high safety to nontarget species and the environment. The recycling of *B. sphaericus* in mosquito larval carcasses under laboratory and field conditions (Correa and Yousten 1995) further highlights the uniqueness of this naturally occurring microbial organism. Product development and application have been intensified because of increased mosquito control need since invasion and spread of West Nile virus (WNV) in North America.

Resistance to *B. sphaericus* was reported for the first time in 1994, where a high level of resistance occurred in field populations of *Culex pipiens* L. in southern France in response to repeated treatments (Sinègre et al. 1994). Numerous reports on various levels of resistance in laboratory and field populations ensued from different countries (Su 2016b). The first case of resistance to *B. sphaericus* in *Cx. pipiens* in North America was reported recently, where a population from an urban environment in Northern California exhibited high level of resistance to *B. sphaericus* and low susceptibility to other commonly used pesticides against agricultural and urban pests (Su et al. 2018b). In this article, we report another case of high-level resistance to *B. sphaericus* in an urban population of *Cx. pipiens* that was collected from Salt Lake City, UT, in 2016, after a control failure by VectoLex WSP (*B. sphaericus*) was realized in response to application according to the product label. The susceptibility of this population to other commonly used pesticides belonging to different Insecticide Resistance Action Committee (IRAC) groups was also evaluated, and results are discussed according to their modes of action.

Materials and Methods

Mosquitoes

The second instars of *Culex* spp. in the amount of approximately 3,000–4,000 were collected from two to three typical curbside catch basins (1.5′ W × 3′ L × 3′ D) at each of the following four locations in Salt Lake City and neighboring cities, UT on 21 September 2016, by personnel of Salt Lake City Mosquito Abatement District (2020 N. Redwood Rd., Salt Lake City, UT 84116) and South Salt Lake Valley Mosquito Abatement District (7308 Airport Rd., West Jordan, UT 84084): 1400 W. Talisman Dr., Salt Lake City, UT; 9827 Gainlock Cir. and 11010 S. Redwood Dr., South Jordan, UT; and 13200 S. 800 E, Draper, UT (Fig. 1). Collected specimens were shipped to the West Valley Mosquito and Vector Control District (1295 E. Locust St., Ontario, CA 91761) on the same day of collection by overnight shipping. Field-collected larvae were reared to early third instars when species identification was confirmed to be *Cx. pipiens*. Bioassays on *B. sphaericus* were conducted concurrently on all four field collections, along with a laboratory reference colony of the same species from the San Mateo County Mosquito and Vector Control District (1351 Rollins Rd., Burlingame, CA 94010), which was originated from a field collection in Santa Clara, CA, in 2010. There was no pesticide exposure since colonization. Remaining larvae from the field collection that showed a high level of resistance to *B. sphaericus* in the bioassay were colonized for future bioassays on other commonly used pesticides at F_6 when adequate number of larvae was spared for assays when maintaining the colony at the same time. During these six generations, colony was maintained by approximately 10,000–15,000 larvae at each generation to maintain the genetic representation and resistance stability in a confined population (Amorim et al. 2010).

Pesticides

The product that was used to evaluate susceptibility of field populations to *B. sphaericus* was VectoLex WDG (650 ITU/mg; Table 1). The other pesticides used in the susceptibility profile evaluation were also off the shelf products for the relevance to field operations, including five with microbial origins (*Bacillus thuringiensis* subsp. *israelensis*, combination of *B. t. israelensis* and *B. sphaericus*, spinosad, spinetoram, and abamectin); four insect growth regulators (IGRs; methoprene, pyriproxyfen, diflubenzuron, and novaluron); and one from each of organophosphate (temephos), neonicotinoid (imidacloprid), phenylpyrazoles (fipronil), oxadiazine (indoxacarb), and pyrethroid (permethrin). Detailed information for the products used in bioassays is listed in Table 1. These pesticides are either commonly used mosquito larvicides or ones to control urban and household pests with consideration of unintentional exposure and interactive resistance development in mosquitoes.

Bioassays

Cup Bioassay on *B. sphaericus*

VectoLex WDG was suspended in tap water at 200 mg/20 ml to generate a 1% stock suspension; 10× serial dilutions were made for appropriate treatments. Bioassays were conducted as described in previous publication (Su et al. 2018b). Four to five concentrations resulting in approximately 5–95% mortality plus untreated controls were used in bioassays, with three replicates at each concentration. Twenty-five larvae were placed in 100-ml tap water in a 120-ml disposable Styrofoam cup in each replicate. Three drops of 10% rabbit chow pellet suspension were added to each cup as larval food. Bioassays were conducted at 25 ± 1°C. Early third instars were used, and larval mortality was recorded at 48 h post-treatment. Moribund larvae that showed minimum locomotion in response to disturbance (tapping on the bioassay cups) were also considered dead. Concentration–response data were analyzed using POLO-PC to calculate lethal concentrations (LC_{50} and LC_{90}), their 95% confidence intervals (CIs), and slopes of the concentration-response lines. Data heterogeneity (*H* value) was generated by χ^2/df. The bioassay results were accepted when *H* values were ≤2.5 where good probit model fitness was indicated (LeOra Software 1987). Bioassays were also conducted concurrently on a reference laboratory colony of *Cx. pipiens*.

Cup Bioassays on Other Pesticides

To prepare the bioassay treatments, the emulsifiable concentrate formulations such as Radiant SC, Altosid liquid larvicide, NyGuard IGR, ImidaPro 4SC, and Taurus SC were suspended in tap water by gentle mixing. Small granules as in Advance 375A Ant Bait, Advion RIFA Bait, and VectoBac WDG, or wettable powders as in Dimilin 25W were suspended in tap water by vigorous shaking. Large granular materials of VectoMax CG or pellets of Skeeter Abate were pulverized in a coffee grinder (Hamilton Beach Custom Grind, Southern Pines, NC) at the maximum speed, then suspended in tap water by vortexing for 3 min (Vortex Mixer VX100, Labnet International, Inc., Edison, NJ). For briquets such as Mosquiron 0.12CRD, fine pieces were shaved off using a razor blade and suspended in tap water by vortexing for 5 min (Su et al. 2018b). In bioassays on *B. t. israelensis*, the combination of *B. t. israelensis* and *B. sphaericus*, spinetoram, abamectin, temephos, imidacloprid, fipronil, and indoxacarb, late third instars were used, and larval mortality was recorded at 24 h post-treatment because of fact action of these pesticides. In bioassays using diflubenzuron and novaluron, early third instars were preferred, and results were recorded at 72 h post-treatment in relation to the slow action of chitin synthesis inhibitor. Moribund larvae were recorded as dead. In bioassays of methoprene and pyriproxyfen, late fourth instars were used, and mortality was read when all treated individuals emerged as adults or died prior to emergence, as majority mortality occurred during the transition from late larvae,

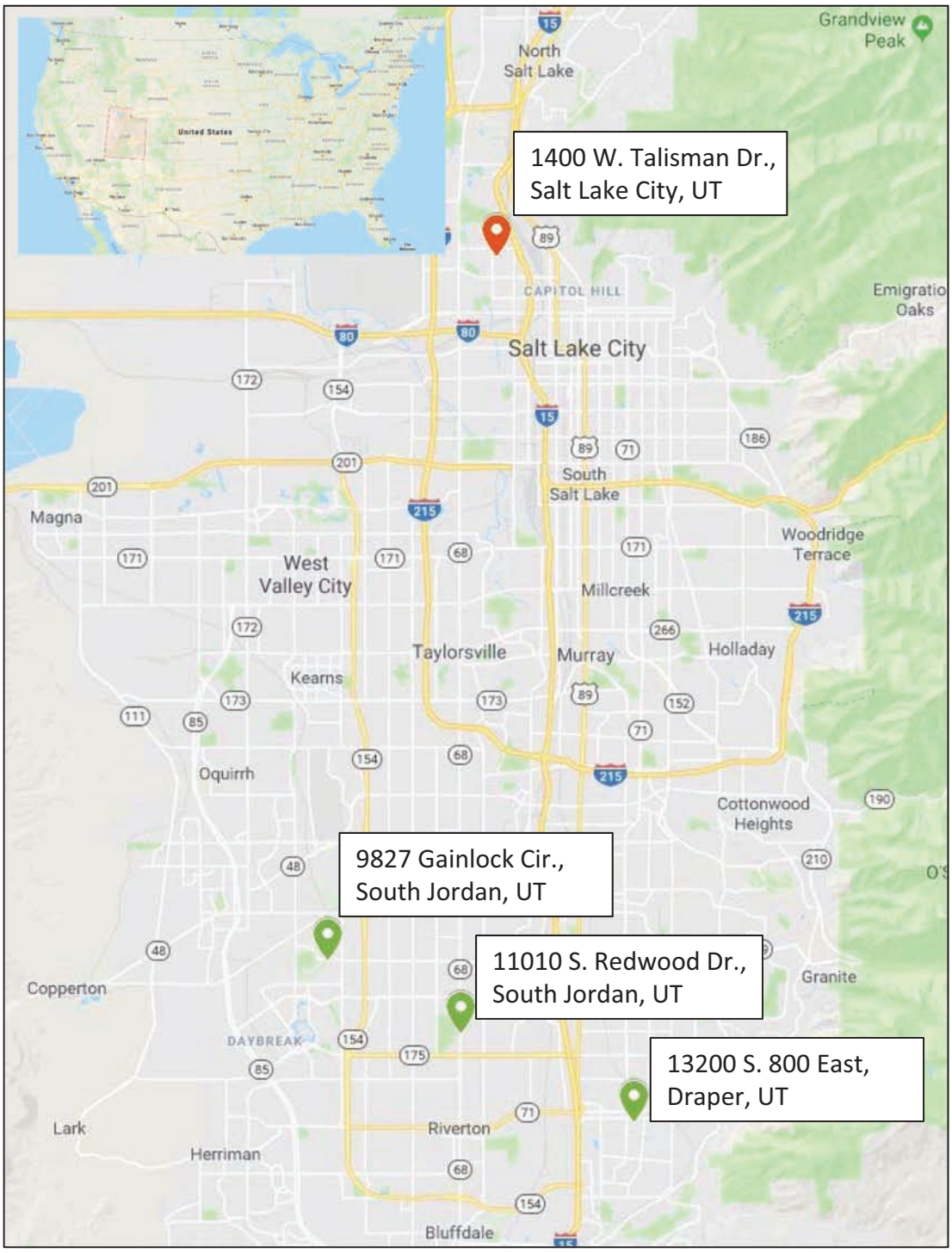

Fig. 1. Sampling sites showing *Bacillus sphaericus*-resistant population in Salt Lake City, UT (red mark), and *B. sphaericus*-susceptible populations in South Jordan and Draper, UT (Green mark).

through pupae to adults. Three drops of 10% rabbit chow pellet suspension were added to each cup as larval food in all bioassays, except those using methoprene and pyriproxyfen where a small piece (approximately 100 mg) of rabbit pellets was added to each bioassay cup to support them until pupation. Concentration–response data were analyzed as previously described. A reference laboratory colony of the same species was assayed concurrently for each pesticide for the calculation of resistance ratio (RR; Su et al. 2018b).

Bottle Bioassay on Adulticide

Bottle bioassays were conducted as described previously (Brogdon and McAllister 1998). As a benchmark for pyrethroid, permethrin was chosen to be assayed against adults. Briefly, technical permethrin (99.9% purity, Chem Service, West Chester, PA) was dissolved in HPLC grade acetone (EMD Millipore, Temecula, CA), and serial dilutions were made to coat the interior of each 250-ml glass bottle (Uline, Pleasant Prairie, WI) at 30 µg/1 ml acetone per bottle. An

Table 1. Information of the pesticides tested against a natural population of *Culex pipiens* L. from Salt Lake City, UT, along with a laboratory reference colony

Category	Products	Active ingredients	Concentration (%)	Lot no.	Manufacturer
Pesticides of microbial origins	VectoLex WDG	*Lysinibacillus sphaericus*	51.2	188-119-PG	Valent BioSciences Corp., Libertyville, IL
	VectoBac WDG	*Bacillus thuringiensis israelensis*	37.4	201-391-PG	Valent BioSciences Corp., Libertyville, IL
	VectoMax CG	*B.t. israelensis* + *L. sphaericus*	4.5 + 2.7	187-575-N8	Valent BioSciences Corp., Libertyville, IL
	Natular G30	Spinosad	2.5		Clarke, St. Charles, IL
	Radiant SC	Spinetoram	11.7	XG14164911	Dow AgroSciences LLC, Indianapolis, IN
	Advance 375A	Abamectin B_1	0.011	81040334	BASF Corp., St. Louis, MO
IGR	Altosid liquid larvicide	Methoprene	5.0	60111357	Wellmark International, Schaumburg, IL
	NyGuard IGR	Pyriproxyfen	10.0	BAB6111	MGK, Minneapolis, MN
	Dimilin 25W	Diflubenzuron	25.0	BA9D30P001	Chemtura Corp., Middlebury, CT
	Mosquiron 0.12CRD	Novaluron	0.12	1012818	Makhteshim Agan North America Inc., Raleigh, NC
Organophosphate	Skeeter Abate	Temephos	5.0	1009280003	Clarke Mosquito Control Products, Inc., Roselle, IL
Neonicotinoid	ImidaPro 4SC	Imidacloprid	40.7	12254PO42	Agrisel USA, Inc., Suwanee, GA
Phenylpyrazole	Taurus SC	Fipronil	9.1	23204	CSI Control Solutions, Inc., Pasadena, TX
Oxadiazine	Advion RIFA bait	Indoxacarb	0.045	SBO45TX	DuPont, Wilmington, DE
Pyrethroid	Permethrin (Technical)	Permethrin (mixture of isomers)	99.9	2601000	Chem Service, West Chester, PA

automatic roller (Fisher Scientific, Fisher Scientific, Hampton, NH) placed in a chemical fume hood (Hemco, Independence, MO) was used to ensure the evenness of the coating. Bottles for untreated controls were coated by acetone only. Bottles were completely dried in the chemical fume hood. Twenty-five 3- to 5-d-old female mosquitoes were aspirated into each bottle. Mortality was read at 5, 10, 15, 30, 45, 60, 90, and 120 min when majority adults died at this coating dose, three replicates were made. Mortality referred to individuals that did not show any movements of body or appendages. Moribund mosquitoes that showed occasional limb (legs and wings) movement were also counted as dead. Bioassays were conducted at 25 ± 1°C and relative humidity 50–60%. Time–mortality data were analyzed for calculations of lethal times LT_{50} and LT_{90}, their 95% CI, and other parameters (Throne et al. 1995). Bottle bioassays were also concurrently conducted on a reference laboratory colony of *Cx. pipiens* for calculation of RRs.

RR Calculation

The RRs were calculated by LC $(LT)_{Field}$/LC $(LT)_{Lab}$. The significance at $P < 0.05$ in susceptibility levels and RRs was determined by separated 95% CIs of LC or LT levels between field population and laboratory colony.

Results

High Resistance to *B. sphaericus*

The field collection from 1400 W. Talisman Dr. in Salt Lake City, UT (red mark in Fig. 1) showed significantly lower susceptibility with LC_{50} 103.9 ppm (95% CI = 76.39–126.64 ppm) and LC_{90} 622.1 ppm (95% CI = 455.09–868.55 ppm) than the other three populations from 9827 Gainlock Cir. and 11010 S. Redwood Dr. in South Jordan, UT, 3200 S. 800E in Draper, UT (green marks in Fig. 1) and the laboratory colony ($P < 0.05$) to VectoLex WDG. The differences in LC_{50} and LC_{90} among these three field populations and laboratory colonies were not significant as indicated by the overlapping 95% CI ($P > 0.05$). Compared with the laboratory colony, the population at 1400 W. Talisman Dr. showed 20,780.0- and 23,926-fold resistance to *B. sphaericus*, whereas no resistance was detected in the other three populations that were collected from South Jordan and Draper, UT (Table 2).

Susceptibility to Other Pesticides

Among the other 13 pesticides tested against larvae and one against adults, the *B. sphaericus*-resistant population only showed significantly lower susceptibility to the combination of *B. t. israelensis* and *B. sphaericus* at LC_{50} level and to permethrin at LT_{90} level (Table 3), and the resistance levels when compared with laboratory reference colony were only 2.36-fold to the former and 4.14-fold to the latter. There were no differences in susceptibility to other 11 pesticides tested between this *B. sphaericus*-resistant population and the laboratory colony of the same species (Table 4).

Discussion

Globalization, emerging, re-emerging, or resurgence of vectors and vector-borne diseases demand cost-effective vector control in an environmentally sustainable manner. Products based on *B. sphaericus* such as VectoLex CG, VectoLex WDG, VectoLex WSP, Spheratax SPH 50G, and Spheratax SPH WSP are among the few tools used in mosquito control operations to combat *Culex* and other mosquito species. The advantages of *B. sphaericus* as one of the most

Table 2. Susceptibility (LC with 95% CI) and RRs to *Bacillus sphaericus* Neide (VectoLex WDG) in F_0 of a natural population of *Culex pipiens* L. from Salt Lake City, UT, in comparison with a laboratory reference colony

Locations	LC_{50} (ppm; 95% CI)	LC_{90} (ppm; 95% CI)	Slope	*H* value[a]	RR at LC_{50}	RR at LC_{90}
1400 W. Talisman Dr., Salt Lake City, UT	103.9[a] (76.39–126.64)	622.1[a] (455.09–868.55)	1.65 ± 0.14	0.80	20,780.0[a]	23,926.9[a]
9827 Gainlock Cir., South Jordan, UT	0.008[b] (0.004–0.013)	0.020[b] (0.013–0.106)	3.21 ± 0.36	1.90	1.60[b]	0.77[b]
11010 S. Redwood Dr., South Jordan, UT	0.008[b] (0.005–0.012)	0.025[b] (0.016–0.073)	2.67 ± 0.29	1.20	1.60[b]	0.96[b]
13,200 S. 800E, Draper, UT	0.004[b] (0.001–0.005)	0.010[b] (0.007–0.024)	2.94 ± 1.02	0.19	0.80[b]	0.38[b]
Laboratory colony	0.005[b] (0.003–0.006)	0.026[b] (0.016–0.087)	2.40 ± 0.38	0.56	1.00[b]	1.00[b]

[a]$H = \chi^2/df$.

[b]Significances in LCs and RRs were indicated by separated 95% CI ranges ($P < 0.05$).

important mosquito larvicides include high activity, tolerance to organic matter, spore recycling, as well as safety to nontarget species and the environment. However, mosquito larvae can develop resistance to *B. sphaericus* in response to sublethal exposures under laboratory and field conditions. Resistance levels vary tremendously in relation to *B. sphaericus* strains, mosquito species, and strains. Presumably this variation is due to historic exposure to other naturally occurring strains, treatment regimens, and gene exchange between treated and untreated populations (Su 2016b). Since 1994, *B. sphaericus* resistance has been reported in *Cx. pipiens* complex in France, India, Brazil, China, Thailand, Tunisia (Su 2016b), and most recently the United States (Su et al. 2018b). The highest level of resistance was revealed in a population of *Culex quinquefasciatus* Say in Nonthaburi Province, Thailand, after five treatments within 4 mo. The RRs at LC_{50}, depending on reference susceptible colonies, were 21,100- to 28,100-fold against VecoLex WDG or greater than 125,000- to 200,000-fold against technical-grade material with 2,000 ITU/mg (Su and Mulla 2004). In North America where the *B. sphaericus* products have been mostly developed and applied against WNV vectors during the past decade, high-level *B. sphaericus* resistance was reported for the first time in a *Cx. pipiens* population collected from Chico, CA, where the RRs were 530.7-fold at LC_{50} and 9,048.5-fold at LC_{90} when compared with laboratory reference colony of the same species. This population from Chico, CA, also showed various levels of resistance or tolerance to abamectin, pyriproxyfen, permethrin, and indoxacarb (Su et al. 2018b). In current studies, the population at 1400 W. Talisman Dr. in Salt Lake City, UT, showed an RR of 20,780.0-fold at LC_{50} and 23,926-fold at LC_{90} to *B. sphaericus*. These levels of resistance were much higher and had much closer RRs at LC_{50} and LC_{90} when compared with what was determined earlier in northern California. These close RRs at LC_{50} and LC_{90} indicate the *B. sphaericus*-resistant population from Salt Lake City was more homogenous compared with the one from northern California (Su et al. 2018b). It is believed that continuous applications of *B. sphaericus* product in the same area for approximately 12 yr was attributable to the high levels of resistance reported here in this article. It is generally thought that recessive genetic factor(s) may govern the *B. sphaericus* resistance in mosquitoes. The mechanism of resistance involves failure of binary toxins to bind to their receptor in the brush-border membrane of the midgut epithelial cells because of compromised integrity of the receptor and/or the glycosylphosphatidylinositol anchor. Other mechanism such as behavioral avoidance in feeding may also play some roles (Nielsen-LeRoux et al. 1995, Darboux et al. 2002, Su 2016b). Limited data have been published (Wirth et al. 2001, Su et al. 2001) regarding *B. sphaericus* susceptibility in natural populations of *Cx. pipiens* in North America prior to Su et al. (2018b) and the current studies. Differences in *B. sphaericus* susceptibility among the populations across California are limited (Wirth et al. 2001), and reduced susceptibility has been observed in larval populations found in dairy wastewater lagoons after continuous applications of *B. sphaericus* product for only one season (Su et al. 2001). Under the field conditions, if expected efficacy according to product label and previous field experience was not achieved post-treatment (control failure) when there were no other factors attributable to reduced efficacy existed such as improper product handling (Su et al. 2018a), substantial habitat dilution, extremely high organic levels, inappropriate application, and sampling, etc., the possibility of reduced susceptibility even resistance in the target population should be considered and bioassay is recommended against the product of interest. Mostly, a simple rotation or mixing (if technically feasible) of pesticides with different modes of action by IRAC can be a practice to minimize resistance development, even though rotation of *B. t. israelensis* and *B. sphaericus* with intention to prevent *B. sphaericus* resistance could be an exception where resistance to *B. sphaericus* developed faster in rotation than in mixture of both (Zahiri and Mulla 2003).

Among the other pesticides tested, the *B. sphaericus*-resistant population was as susceptible as a laboratory reference colony to *B. t. israelensis*, another microbial mosquito larvicide with the same mode of action (IRAC Group 11—microbial disruptors of insect midgut membranes and derived toxins). This microbial larvicide appears to possess an intrinsic mechanism of prevention of resistance development, as resistance to intact natural *B. t. israelensis* is rare regardless of mosquitoes' resistance status to other pesticides (Su 2016b). Reduced susceptibility or tolerance to the combination of *B. t. israelensis* and *B. sphaericus* was detected at LC_{50} level (RR = 2.36) in this *B. sphaericus*-resistant population, which indicated that high-level resistance to *B. sphaericus* may have compromised its susceptibility to the mixture of *B. t. israelensis* and *B. sphaericus*. The known synergism of *B. t. israelensis* to *B. sphaericus* (Su and Mulla 2004, Wirth et al. 2004, Sreshty et al. 2011) did not overcome the resistance to *B. sphaericus* completely. For other pesticides against larval stages tested, widely ranging from ones with microbial and IGR origins to newly developed neonicotinoid, phenylpyrazole, oxadiazine, as well as a conventional organophosphate, no noticeable shift in susceptibility was observed. Therefore, these pesticides can be candidates, depending on registration status, to combat *B. sphaericus*-resistant mosquitoes. These pesticides, with exception of temephos, are all categorized as reduced risk pesticides by the U.S. Environment Protection Agency (U.S. EPA), and some of them have already been registered for larval mosquito control in the United States, such as spinosad (IRAC Group 5—nicotinic acetylcholine receptor allosteric modulator), methoprene (IRAC Group 7A—juvenile hormone analog), and novaluron (IRAC Group 15—chitin synthesis inhibitor). Others such as diflubenzuron (IRAC Group 15—inhibitor of chitin biosynthesis) and pyriproxyfen (IRAC Group 7—juvenile hormone mimic) do have promising potential for application in mosquito control operations. The former

Table 3. Susceptibility (LC or LT with 95% CI) to other commonly used pesticides in F_6 of a natural population of *Culex pipiens* L. from Salt Lake City, UT, in comparison with a laboratory reference colony

	Laboratory colony				Field collection			
Pesticides tested	LC_{50} (ppm) or LT_{50} (min)[a] (95% CI)	LC_{90} (ppm) or LT_{90} (min)[a] (95% CI)	Slope	*H* value[b]	LC_{50} (ppm) or LT_{50} (min)[a] (95% CI)	LC_{90} (ppm) or LT_{90} (min)[a] (95% CI)	Slope	*H* value[b]
Bacillus thuringiensis israelensis (VectoBac WDG, 37.4%)	0.002 (0.001 to 0.004)	0.013 (0.006 to 0.019)	1.55 ± 0.46	0.33	0.002 (0.001 to 0.004)	0.0013 (0.006 to 0.020)	1.43 ± 0.41	0.86
B. t. israelensis + *Lysinibacillus sphaericus* (VectoMax FG, 4.5% + 2.7%)	0.114[c] (0.067 to 0.154)	0.591 (0.451 to 0.931)	1.79 ± 0.31	0.23	0.269[c] (0.239 to 0.301)	0.597 (0.505 to 0.756)	3.70 ± 0.39	0.36
Spinosad (Natular G30, 2.5% spinosyns A & D)	9.75×10^{-3} (5.70×10^{-3} to 1.87×10^{-2})	2.65×10^{-2} (1.52 to 9.95×10^{-2})	2.95 ± 0.30	2.38	9.20×10^{-3} (6.08×10^{-3} to 1.46×10^{-2})	1.90×10^{-2} (1.30×10^{-2} to 0.70×10^{-1})	4.11 ± 0.38	2.36
Spinetoram (Radiant, 11.7% spinosyns J & L)	1.29×10^{-3} (1.05 to 1.40×10^{-3})	3.98×10^{-3} (3.16 to 5.15×10^{-3})	2.55 ± 0.27	0.15	1.52×10^{-3} (8.70×10^{-4} to 2.20×10^{-3})	3.24×10^{-3} (2.25 to 7.73×10^{-3})	3.88 ± 0.37	1.01
Abamectin (Advance 375A, 0.011%)	0.024 (0.018 to 0.036)	0.107 (0.061 to 0.287)	1.97 ± 0.30	0.13	0.014 (0.011 to 0.020)	0.109 (0.056 to 0.383)	1.43 ± 0.24	0.46
Pyripro × yfen (NyGuard, 10%)	5.90×10^{-5} (4.50 to 7.40×10^{-5})	3.12×10^{-4} (2.35 to 4.61×10^{-4})	1.78 ± 0.19	0.40	8.40×10^{-5} (3.00×10^{-5} to 1.67×10^{-4})	3.13×10^{-4} (1.85×10^{-4} to 3.38×10^{-3})	1.98 ± 0.20	2.12
Methoprene (Altosid LL, 5%)	2.08×10^{-3} (1.58 to 2.76×10^{-3})	1.62×10^{-2} (1.04 to 3.06×10^{-2})	1.44 ± 0.15	0.42	1.92×10^{-3} (1.39 to 2.64×10^{-3})	2.31×10^{-2} (1.37 to 4.98×10^{-2})	1.19 ± 0.13	0.18
Diflubenzuron (Dimilin 25 WP, 25%)	6.72×10^{-4} (2.85×10^{-4} to 1.10×10^{-3})	1.02×10^{-2} (6.55×10^{-3} to 2.09×10^{-2})	1.09 ± 0.18	0.64	4.72×10^{-4} (1.75 to 8.04×10^{-3})	5.21×10^{-3} (3.57 to 9.27×10^{-3})	1.23 ± 0.22	0.10
Novaluron (Mosquiron 0.12CRD, 0.12%)	1.08×10^{-3} (8.58×10^{-4} to 1.42×10^{-3})	7.43×10^{-3} (4.40×10^{-3} to 1.82×10^{-2})	1.53 ± 0.22	0.85	8.12×10^{-4} (6.98 to 9.50×10^{-4})	2.62×10^{-3} (2.04 to 3.73×10^{-3})	2.52 ± 0.26	0.72
Temephos (Skeeter Abate, 5%)	3.75×10^{-3} (2.17 to 4.64×10^{-3})	6.50×10^{-3} (4.79 to 8.92×10^{-3})	3.12 ± 0.40	0.92	7.32×10^{-4} (4.47 to 9.81×10^{-4})	2.83×10^{-3} (2.26 to 3.89×10^{-3})	2.18 ± 0.35	0.74
Imidacloprid (ImadPro 4SC, 40.7%)	0.075 (0.052 to 0.108)	0.242 (0.158 to 0.498)	2.52 ± 0.22	1.71	0.075 (0.040 to 0.101)	0.265 (0.142 to 0.466)	3.51 ± 0.21	1.54
Fipronil (Taurus SC, 9.1%)	1.02×10^{-3} (8.59×10^{-4} to 1.2×10^{-3})	3.44×10^{-3} (2.74 to 4.70×10^{-3})	2.44 ± 0.25	0.47	3.03×10^{-4} (1.49 to 4.07×10^{-4})	8.36×10^{-4} (6.89×10^{-4} to 1.14×10^{-3})	2.91 ± 0.67	0.29
Indoxacarb (Advion RIFA bait, 0.045%)	0.170 (0.43 to 0.202)	0.519 (0.413 to 0.702)	2.65 ± 0.25	0.50	0.223 (0.108 to 0.472)	0.671 (0.357 to 1.282)	2.68 ± 0.29	2.15
Permethrin	3.70 (2.06 to 5.47)	46.88[b] (35.68 to 67.78)	1.16 ± 0.14	0.84	8.30 (5.26 to 11.39)	193.87[b] (123.81 to 387.75)	0.94 ± 0.11	0.21

[a]Bottle bioassay.

[b]$H = \chi^2/df$.

[c]Significances in LCs or LTs were indicated by separated 95% CI ranges ($P < 0.05$).

Table 4. RRs to other commonly used pesticides at LC_{50} and LC_{90} or LT_{50} and LT_{90} in F_6 of a natural population of *Culex pipiens* L. from Salt Lake City, UT, in comparison with a laboratory reference colony

Pesticides tested	At LC_{50} or LT_{50}[a]	At LC_{90} or LT_{90}[a]
Bacillus thuringiensis israelensis	1.00	1.00
B. t. israelensis + *Lysinibacillus sphaericus*	2.36[b]	1.01
Spinosad	0.94	0.70
Spinetoram	1.18	0.81
Abamectin	0.58	1.02
Pyriproxyfen	1.42	1.20
Methoprene	0.92	1.42
Diflubenzuron	0.70	0.51
Novaluron	0.75	0.35
Temephos	0.20	0.47
Imidacloprid	1.00	1.10
Fipronil	0.30	0.24
Indoxacarb	1.31	1.29
Permethrin	2.24	4.14[b]

[a]Bottle bioassay.

[b]Significances in RR were indicated by separated 95% CI of the LC or LT ($P < 0.05$).

used to have U.S. EPA label (Dimilin 25W) to control Chironomid midges, a closely related nematoceran group to mosquitoes, whereas the latter was formulated and labeled (NyGuard IGR) to control a wide variety of flying and crawling arthropods of urban, household, and public health importance, including mosquitoes. The remaining pesticides such as imidacloprid (IRAC Group 4—nAChR agonist), fipronil (IRAC Group 2B—GABA-gated chloride channel antagonist), and indoxacarb (IRAC Group 22—voltage-dependent sodium channel blocker) do not currently have product labels for mosquito control; however, they are commonly used to manage urban and household pests because of their U.S. EPA-reduced risk recognition. Last, this *B. sphaericus*-resistant population did show some tolerance to permethrin (IRAC Group 3A—sodium channel modulator) as an adulticide; however, this tolerance is well expected when one considers the extensive use of pyrethroids in the urban environment to mitigate various arthropod pests including mosquitoes (Zhu et al. 2016). Mosquitoes can be exposed at larval and/or adult stages to pyrethroids of urban uses intentionally or unintentionally, which leads to complicated resistance evolution. Considering the similar mode of action in all pyrethroids, the detected tolerance to permethrin can be indicative to the existence of resistance to other pyrethroids that have been applied in urban environment.

While facing the spread of mosquitoes and mosquito-borne diseases, biorational pesticides based on microbial organisms and IGRs are some of the few available tools we can rely on. Resistance development due to lack of rotation of different modes of action and improper use of products has historically dampened the value of the insecticides and made mosquito control more challenging and complicated. The most effective way of resistance management is risk assessment based on mode of action, strategic monitoring of susceptibility, and implementation of mitigation measures. Among the historic cases of *B. sphaericus* resistance worldwide, the relationship of treatment regimens (rates and durations) and level of resistance seems unpredictable. The safe approach that can be recommended is to measure the *B. sphaericus* susceptibility of the target populations and compare it with that of a susceptible population, before, during, and after continuous treatment. The test material can be technical powder or water-dispersible granules with high potency, which ideally contains the same *B. sphaericus* strain as that in the products intended for field application, even though the susceptibility to various *B. sphaericus* strains can be cross-referenced. Insecticide resistance management should be an important component of all integrated mosquito control programs. Given the increasing global burdens posed by mosquitoes and the pathogens that they vector, in addition to a limited arsenal of available insecticide classes for mosquito control, we highly urge the implementation of an insecticide management program within public health and vector control organizations.

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GUIDELINES FOR LABORATORY AND FIELD TESTING OF MOSQUITO LARVICIDES

WORLD HEALTH ORGANIZATION
COMMUNICABLE DISEASE CONTROL, PREVENTION AND ERADICATION
WHO PESTICIDE EVALUATION SCHEME

CONTENTS

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1. INTRODUCTION

The purpose of this document is to provide specific and standardized procedures and guidelines for testing larvicides, including bacterial larvicides and insect growth regulators (IGRs), against mosquitoes. Its aim is to harmonize the testing procedures carried out in different laboratories and institutions to generate data for the registration and labelling of larvicides by national authorities.

The document is an expanded and updated version of the guidelines recommended by the WHO Pesticide Evaluation Scheme (WHOPES) Informal Consultation on the evaluation and testing of insecticides, held at WHO headquarters (HQ), Geneva, 7–11 October 1996 (*1*). The guidelines were reviewed and recommended by the Eighth WHOPES Working Group Meeting, held at WHO-HQ, Geneva, 1–3 December 2004 (*2*).

The document provides guidance on laboratory studies and small-scale and large-scale field trials to determine the efficacy, field application rates and operational feasibility and acceptability of a mosquito larvicide. The table below summarizes the sequence and objectives of the studies and trials. The procedures provide some information on the safety and toxicity of the larvicides for non-target organisms, but it is presumed that preliminary eco-toxicity and human assessments have been undertaken before any field study is carried out – detailed treatment and analysis of these extra data are beyond the scope of this document.

Table 1.1
Sequence of the stages of evaluation of mosquito larvicides

Phase	Type of study	Aim
Phase I	Laboratory studies	• Biopotency and activity • Diagnostic concentration and assessment of cross-resistance
Phase II	Small-scale field trials	• Efficacy under different ecological settings • Method and rate of application • Initial and residual activity • Effect on non-target organisms
Phase III	Large-scale field trials	• Efficacy and residual activity • Operational and community acceptance • Effect on non-target organisms

2. PHASE I: LABORATORY STUDIES

The objective of laboratory testing is to determine the inherent biopotency of the technical material or, in the case of formulated larvicides, their activity. It is assumed that the compound's mode of action has already been established. Information on the speed of activity is important, as this will determine the type of testing procedures to be employed.

To evaluate the biological activity of a mosquito larvicide, laboratory-reared mosquito larvae of known age or instar (reference strains or F1 of field-collected mosquitoes) are exposed for 24 h to 48 h or longer in water treated with the larvicide at various concentrations within its activity range, and mortality is recorded. For IGRs and other materials with delayed activity, mortality should be assessed until the emergence of adults. It is important to use homogenous populations of mosquito larvae or a given instar. These are obtained using standardized rearing methods (see Annex 1).

The aims of the tests are:

- to establish dose–response line(s) against susceptible vector species;
- to determine the lethal concentration (LC) of the larvicide for 50% and 90% mortality (LC_{50} and LC_{90}) or for 50% and 90% inhibition of adult emergence (IE_{50} and IE_{90});
- to establish a diagnostic concentration for monitoring susceptibility to the mosquito larvicide in the field; and
- to assess cross-resistance with commonly used insecticides.

2.1 Determination of biological activity

2.1.1 Larvicides other than bacterial products and insect growth regulators

2.1.1.1 Materials required for testing

- One pipette delivering 100–1000 µl.
- Disposable tips (100 µl, 500 µl) for measuring aliquots of dilute solutions.
- Five 1 ml pipettes for insecticides and one for the control.
- Three droppers with rubber suction bulbs.
- The following materials to make a strainer: two wire loops, one piece of nylon netting (30 cm^2) and one tube of cement. It is suggested that two pieces of netting be cut and cemented to opposite sides of the larger end of the wire loops. More cement should then be applied around the edges of the loops to join the two pieces of netting. When dry, the netting may be trimmed with scissors.
  If a strainer is not available, a loop of plastic screen may be used to transfer test larvae into test cups or vessels.
- Data recording forms (see Annex 4).
- Disposable cups (preferred as they avoid contamination) or, if not available, glass bowls or beakers of two capacities: 120 ml (holding 100 ml) and 250 ml (holding 200 ml).
- Graduated measuring cylinder.
- Log–probit software or paper.

2.1.1.2 Preparation of stock solutions or suspensions and test concentrations

The technical materials of many organic compounds are insoluble in water. These materials have to be dissolved in appropriate organic solvents such as acetone or ethanol (the manufacturer should be consulted) in order to prepare dilute solutions for laboratory testing. The formulated materials are, however, miscible with water. Suspending or mixing these formulations in water requires no special equipment – homogeneous suspensions can be obtained by gentle shaking or stirring.

The volume of stock solution should be 20 ml of 1%, obtained by weighing 200 mg of the technical material and adding 20 ml solvent to it. It should be kept in a screw-cap vial, with aluminium foil over the mouth of the vial. Shake vigorously to dissolve or disperse the material in the solvent. The stock solution is then serially diluted (ten-fold) in ethanol or other solvents (2 ml solution to 18 ml solvent). Test concentrations are then obtained by adding 0.1–1.0 ml (100–1000 µl) of the appropriate dilution to 100 ml or 200 ml chlorine-free or distilled water (see Table A2.1). For other volumes of test water, aliquots of dilutions added should be adjusted according to Table A2.1. When making a series of concentrations, the lowest concentration should be prepared first. Small volumes of dilutions should be transferred to test cups by means of pipettes with disposable tips. The addition of small volumes of solution to 100 ml, 200 ml or greater volumes of water will not cause noticeable variability in the final concentration.

When a test is carried out using formulated materials, distilled water is used in the preparation of the 1% stock solution or suspension and in subsequent serial dilutions, according to the content of the active ingredient.

2.1.1.3 Bioassays

Initially, the mosquito larvae are exposed to a wide range of test concentrations and a control to find out the activity range of the materials under test. After determining the mortality of larvae in this wide range of concentrations, a narrower range (of 4–5 concentrations, yielding between 10% and 95% mortality in 24 h or 48 h) is used to determine LC_{50} and LC_{90} values.

Batches of 25 third or fourth instar larvae are transferred by means of strainers, screen loops or droppers to small disposable test cups or vessels, each containing 100–200 ml of water. Small, unhealthy or damaged larvae should be removed and replaced. The depth of the water in the cups or vessels should remain between 5 cm and 10 cm; deeper levels may cause undue mortality.

The appropriate volume of dilution is added (see Table A2.1) to 100 ml or 200 ml water in the cups to obtain the desired target dosage, starting with the lowest concentration. Four or more replicates are set up for each concentration and an equal number of controls are set up simultaneously with tap water, to which 1 ml alcohol (or the organic solvent used) is added. Each test should be run three times on different days. For long exposures, larval food should be added to each test cup, particularly if high mortality is noted in control. The test containers are held at 25–28 ^{o}C and preferably a photoperiod of 12 h light followed by 12 h dark (12L:12D).

After 24 h exposure, larval mortality is recorded. For slow-acting insecticides, 48 h reading may be required. Moribund larvae are counted and added to dead larvae for calculating percentage mortality. Dead larvae are those that cannot be induced to move

when they are probed with a needle in the siphon or the cervical region. Moribund larvae are those incapable of rising to the surface or not showing the characteristic diving reaction when the water is disturbed. The results are recorded on the form provided (Fig. A4.1), where the LC_{50}, LC_{90} and LC_{99} values, and slope and heterogeneity analysis are also noted. The form will accommodate three separate tests of six concentrations, each of four replicates.

Larvae that have pupated during the test period will negate the test. If more than 10% of the control larvae pupate in the course of the experiment, the test should be discarded and repeated. If the control mortality is between 5% and 20%, the mortalities of treated groups should be corrected according to Abbott's formula (*3*):

$$\textit{Mortality}\ (\%) = \frac{X - Y}{X}\ 100\ ,$$

where X = percentage survival in the untreated control and Y = percentage survival in the treated sample.

2.1.1.4 Data analysis

Data from all replicates should be pooled for analysis. LC_{50} and LC_{90} values are calculated from a log dosage–probit mortality regression line using computer software programs, or estimated using log–probit paper. Bioassays should be repeated at least three times, using new solutions or suspensions and different batches of larvae each time. Standard deviation or confidence intervals of the means of LC_{50} values are calculated and recorded on a form (Fig. A4.1). A test series is valid if the relative standard deviation (or coefficient of variation) is less than 25% or if confidence limits of

LC_{50} overlap (significant level at $P < 0.05$). The potency of the chemical against the larvae of a particular vector and strain can then be compared with the LC_{50} or LC_{90} values of other insecticides.

2.1.2 Insect growth regulators

Testing methods for the juvenile hormone (JH) analogues (juvenoids) and the chitin synthesis inhibitors differ. JH analogues interfere with the transformation of late instar larvae to pupae and then to adult, whereas chitin synthesis inhibitors inhibit cuticle formation and affect all instars and immature stages of the mosquito. The delayed action of IGRs on treated larvae means that mortality is assessed every other day or every three days until the completion of adult emergence. The effect of both types of IGR on mosquito larvae is expressed in terms of the percentage of larvae that do not develop into successfully emerging adults, or adult emergence inhibition (IE%).

2.1.2.1 Preparation of stock solutions or suspensions and test concentrations

The preparation of the test solutions or suspensions and bioassay set-ups are the same as for the fast-acting compounds (see Sections 2.1.1.1 and 2.1.1.2). Technical materials are generally soluble in organic solvents and stock solution (1%) should be made by dissolving 200 mg in 20 ml. Formulated materials should be diluted with water and serial dilutions made in the same manner.

2.1.2.2 Bioassays

Third instar larvae are used for testing JH analogues and chitin synthesis inhibitors. The accurate initial count of larvae is essential because of the cannibalistic or scavenging behaviour of larvae during the long exposure period. The long duration of the test also means that the larvae have to be provided with a small amount of food (finely ground yeast extract, rabbit pellets, or ground fish or mouse food) at a concentration of 10 mg/l at two-day intervals until mortality counts are made. The food powder should be suspended in water and one or two drops added per cup. The larvae in the control are fed in the same manner as those in the treated batches. If necessary, all the test and control cups should be covered with netting to prevent successfully emerged adults from escaping into the environment. Mortality or survival is counted every other day or every three days until the complete emergence of adults. The test containers are held at 25–28 ^{o}C and preferably for a photoperiod of 12L:12D.

At the end of the observation period, the impact is expressed as IE% based on the number of larvae that do not develop successfully into viable adults. In recording IE% for each concentration, moribund and dead larvae and pupae, as well as adult mosquitoes not completely separated from the pupal case, are considered as "affected". The number of successfully emerged adults may also be counted from the empty pupal cases. The experiment stops when all the larvae or pupae in the controls have died or emerged as adults. Data are entered on a form (Fig. A4.2). Any deformities or morphogenetic effects that occur in either the moulting immature mosquitoes or the emerging adults are also recorded.

2.1.2.3 Data analysis

The data from all replicates of each concentration should be combined. Total or mean emergence inhibition can be calculated on the basis of the number of third stage larvae exposed. The overall emergence of adults reflects activity. IE% is calculated using the following formula (*4*):

$$IE(\%) = 100 - \left(\frac{T \times 100}{C} \right) ,$$

where T = percentage survival or emergence in treated batches and C = percentage survival or emergence in the control.

If adult emergence in the control is less than 80%, the test should be discarded and repeated. Where the percentage is between 80% and 95%, the data are corrected using Abbott's formula (see Section 2.1.1.3). IE values obtained at each concentration should be subjected to probit regression analysis to determine IE_{50} and IE_{90} values (using computer software programs or estimated from log–probit paper). The data analysis procedures stated in Section 2.1.1.4 should be followed.

2.1.3 Bacterial larvicides

The laboratory bioassay procedures for bacterial products are the same as those for chemical larvicides, except in the preparation of stock suspensions.

2.1.3.1 Principles

The biopotency of the material is first examined by comparing mosquito larval mortality produced by the product under test with the mortality produced by the corresponding reference standard or other technical or formulated product. The toxicity of preparations based on *Bacillus thuringiensis* subsp. *israelensis (B. thuringiensis* subsp. *israelensis)* can be determined against a standard product that has been calibrated using *Aedes aegypti (A. aegypti)* larvae. The potency of products tested is determined by the following formula:

$$\text{Potency of product "X"} = \frac{\text{Potency standard (ITU)} \times LC_{50}\ \text{(mg/1) standard}}{LC_{50}\ \text{(mg/l) of "X"}}$$

When the international reference standard is used, potency is expressed in International Toxic Units per milligram (ITU/mg). The biopotency of products based on *B. thuringiensis* subsp. *israelensis* is compared with a lyophilized reference powder (IPS82, strain 1884) of this bacterial species using early fourth instar larvae of *A. aegypti* (strain Bora Bora). The potency of IPS82 has been arbitrarily designated as 15 000 ITU/mg powder against this strain of mosquito larva.

The biopotency of products based on *Bacillus sphaericus* (*B. sphaericus)* is determined against a lyophilized reference powder (SPH88, strain 2362) of this bacterial species using early fourth instar larvae of *Culex pipiens pipiens (C. pipiens pipiens)* or *Culex quinquefasciatus*. The potency of SPH88 has been arbitrarily set at 1700 ITU/mg of powder against this mosquito strain.

The use of other bacterial larvicide reference powders and/or alternative strains of mosquito in this test is possible but must be approached warily, because it is inevitable that different results will

obtain. Such alternatives must be the subject of careful cross-calibration with the reference powders and strains identified above. Ideally, such cross-calibration should be conducted by a group of independent expert laboratories. The alternative powders or strains, and the cross-calibration data that support them, should be made available to anyone who wishes to use, or check, the test.

In general, it is not necessary to calibrate with or test against the standard if comparing the activity of a bacterial product with other larvicide products. Bioassay results providing LC_{50} and LC_{90} values of products are sufficient to enable comparison among different products.

2.1.3.2 Additional materials required for testing

- Top-drive homogenizer or stirrer for lyophilized products
- Ice bath (container of crushed ice) for grinding or sonication
- Micropipette
- 10 ml pipette
- 12 ml plastic tubes with stoppers or caps
- 120 ml or 250 ml plastic or wax-coated paper cups to hold 100 ml or 200 ml water

2.1.3.3 Preparation of reference standard suspensions for calibration of the bioassays

To prepare a “stock suspension”, weigh 200 mg or 1000 mg of the solid product, place in a vial (30 ml) or volumetric flask, and add 20 ml or 100 ml distilled water, yielding 1% stock suspension, or 10 mg/l. Most powders do not need blending or sonication. Vigorous shaking or stirring will facilitate suspension. If placed in

tubes, the stock suspension can be frozen for future bioassays. Frozen aliquots must be homogenized thoroughly before use, because particles agglomerate during freezing.

From the “stock suspension”, any necessary subsequent dilutions (see Table A2.1) are prepared by serial dilution. Plastic or paper cups are filled with 100 ml deionized water. Twenty-five late third or early fourth instar larvae of *A. aegypti* or *C. pipiens* (depending on the bacterial species to be tested: *Aedes* larvae for *B. thuringiensis* subsp. *israelensis* and *Culex* larvae for *B. sphaericus*) are added to each cup. Using micropipettes, 400 µl, 300 µl, 200 µl, 100 µl, 80 µl and 50 µl of a given suspension (see Table A2.1) are added to the cups and the solutions mixed to produce final concentrations of 0.04 mg/l, 0.03 mg/l, 0.02 mg/l, 0.01 mg/l, 0.008 mg/l and 0.005 mg/l, respectively, of the reference standard powder. Four or more replicate cups are used for each concentration and the control, which is 100 ml deionized water.

2.1.3.4 Preparation of suspensions of the product to be tested

For bioassays of technical (solid or liquid) products of unknown potency, an initial homogenate is made simply by mixing without reducing particle size. For assays of liquid formulations, 20 ml water is added to 200 mg in a vial. Serial dilutions are made and cups and larvae are prepared as described in the previous section.

Range-finding bioassays are performed using a wide range of concentrations of the product to determine its approximate toxicity. The results are then used to determine a narrower and more refined range of concentrations for precise bioassay.

2.1.3.5 Bioassays

To prepare a valid dose–response curve, only concentrations giving values between 10% and 95% mortality should be used. A minimum of two concentrations above and two below the LC_{50} level must be used. Each bioassay series should involve at least four concentrations; and each concentration should be tested in four replicates of 25 late third or early fourth instar larvae per replicate.

No food is added to larval vessels when the exposure period is 24 h. Food may be required if the exposure period is longer. Finely ground yeast extract or ground mouse or rabbit pellets suspended in water (1.5 mg) is added to the water in test vessels at 10 mg/l. Mortality is determined at 24 h for *B. thuringiensis* subsp. *israelensis* and 48 h for *B. sphaericus* by counting the live larvae remaining. The results of the tests at different concentrations (including LC values) are entered on the form (Fig. A4.1). If more than 10% of larvae pupate, the test is invalidated because late instar larvae do not ingest 24 h before pupation and too many larvae may have survived simply because they are too old. All tests should be conducted at 25–28 °C, preferably with a 12L:12D photoperiod.

2.1.3.6 Data analysis

If the control mortality is between 5% and 20%, the mortalities of treated groups should be corrected according to Abbott's formula (see Section 2.1.1.3). Tests with control mortality greater than 20% or pupation greater than 10% should be discarded. A mortality–concentration regression is made using log–probit analysis software or log–probit paper. Bioassays should be carried out at least three times and the validity of the results assessed as for the other

larvicides. LC values (Fig. A4.1) are determined and compared to examine the activity of one product versus another.

2.2 Determination of the diagnostic concentration

The diagnostic or discriminating concentration is determined from the dose–response regression lines of testing a technical material against susceptible vector species according to the procedures outlined in Section 2.1. The diagnostic concentration is double that of the estimated $LC_{99..9}$ value.

2.3 Cross-resistance assessment

New, candidate larvicides are tested simultaneously against a small number of distinct, multi-resistant mosquito strains and a susceptible strain, according to the procedures outlined in Section 2.1. If cross-resistance is detected, its exact nature will be determined by testing the larvicide against strains that each possess a single resistance mechanism. The mechanism of resistance may be assessed following the procedures outlined in the WHO document *Techniques to detect insecticide resistance mechanisms (field and laboratory manual)* (*5*).

Susceptible strains of some mosquito species are kept in laboratories. Otherwise, any susceptible strains should be collected in the field (if truly susceptible populations still exist). If not, susceptible strains may be artificially selected using bioassays, assays for individual resistance mechanisms and selection between lines derived from individually mated females.

The resistant strains should be identified using well established assay techniques. The strains should preferably be homozygous for one or more known resistance mechanisms. If homozygosity cannot be achieved, periodic selection is usually necessary to prevent natural selection favouring the susceptible at the expense of the resistant. Established reference strains should be regularly monitored by bioassays and biochemical and/or molecular assays so that any changes in resistance or underlying mechanisms can be assessed and rectified by selection.

3. PHASE II: SMALL-SCALE FIELD TRIALS

Larvicides that show promise in laboratory studies (Phase I) may be subjected to small-scale field testing (Phase II). In Phase II, field trials of formulated products are performed on a small scale against target mosquitoes, preferably in representative natural breeding sites or, where such trials are not feasible, under simulated field conditions (see Section 3.2).

Evaluation procedures should be selected on the basis of the breeding sites and the behaviour of mosquitoes. The formulations are tested at three–five concentrations and the Phase I studies will guide the dosages chosen for use in the Phase II trials. Usually, this will be multiple concentrations of LC_{90} for the target species. Treatment concentrations are calculated on the basis of the amount of active ingredient per volume of water (if known or measurable) or surface area of the habitat.

The objectives of small-scale field trials are:

- to determine efficacy, including residual activity, against different mosquito vectors in different breeding sites and ecological settings;
- to determine the optimum field application dosage(s);
- to monitor abiotic parameters that may influence the efficacy of the product; and
- to record qualitative observations on the non-target biota cohabiting with mosquito larvae, especially predators.

3.1 Trials in natural breeding sites

The field efficacy of the larvicide under various ecological conditions is determined by selecting representative natural breeding habitats of the target species. These include stagnant drains (cement lined and unlined), soakage pits, cesspits, cesspools, domestic service tanks collecting sewage water, pools, wetlands, irrigated fields and unused wells for *Culex* spp.; cement tanks, drums, cisterns, water storage containers and air coolers for *A. aegypti*; and disused wells, garden pits, ponds, curing yards, rice plots, stream pools, wetlands, marshes, irrigated fields and seepages for *Anopheles*spp.

A minimum of three replicates of each type of habitat should be randomly selected for each dosage of the formulation, with an equal number of controls. The size of the plot should be recorded, taking account of surface area and depth. As far as possible, the plots selected should be similar and comparable. Each of the confined breeding sources or containers can be considered as a discrete plot or replicate. Habitats such as drains, irrigation canals, irrigated fields, rice fields, streams and seepages may be divided into discrete areas of 4–50 m^2 and replicated for treatment and control.

Pretreatment immature abundance (first and second instar larvae, third and fourth instar larvae, and pupae) should be recorded in both experimental and control sites (minimum of two observations at equal intervals). The sampling method should be appropriate to the type of breeding habitat, and the appropriate number of samples should be taken from each habitat based on the type and size of the habitat. Larval instars and pupae from each sample are counted and recorded. At least three different dosages of the larvicide should be applied to the breeding habitats. These can be applied using small atomizers, compression sprayers or, in most cases, plastic squeeze bottles. Granules, pellets, tablets and briquettes can be manually broadcast or thrown in the water.

Post-treatment immature abundance (all stages) should be monitored on day 2 and then weekly until the density of fourth instar larvae (or pupae in the case of IGRs) in the treated habitats reaches a level comparable to that in the control. Data are recorded on the form (Fig. A4.3).

Characterization of the habitats in terms of abiotic and biotic factors aids the interpretation of results. Rainfall and any change in water level or other parameters, such as algal bloom or predators in the habitats, should be recorded.

The efficacy and residual activity of the larvicide at different dosages are determined from the post-treatment counts of live larvae and pupae in treated and control sites compared with the pretreatment counts or the control, taking into consideration the dynamics of change occurring in the treated and the control batches (see below).

The assessment of an IGR's efficacy is based on the level of inhibition of emergence of adults and the percentage reduction in larval and pupal densities. Larvae and pupae are sampled as described above. Adult emergence can be monitored directly in the field by floating sentinel emergence traps in treated and untreated habitats (see Fig. A4.4), by pupal isolation, or by sampling and counting pupal skins. Adult emergence may also be assessed by collecting pupae (20–40 per replicate) and bringing them to the laboratory in glass containers with the water from the respective habitats, then transferring them to small cups inside the holding cages. Dead larvae and pupae found in the cups should be removed and any morphological abnormalities recorded.

When monitored directly in the field, the pretreatment and post-treatment data on adult emergence in treated and untreated habitats are analysed for IE%. The following expression (*6*) is used to calculate IE% values:

$$IE(\%) = 100 - \left(\frac{C1}{T1}\right) \times \left(\frac{T2}{C2}\right) \times 100 \quad ,$$

where $C1$ is the number of adults emerged in control habitats before treatment, $C2$ the number of adults emerged in control habitats at a given interval after treatment, $T1$ the number of adults emerged in treated habitats before treatment and $T2$ the number of adults emerged in treated habitats after treatment.

When adult emergence is monitored in the laboratory using pupae collected from treated and untreated habitats, IE% is calculated using the following formula, on the basis of determining adult emergence from the number of pupae isolated (see also Section 2.1.1.3):

$$IE(\%) = \left(\frac{C - T}{C} \right) \times 100 \ ,$$

where C = percentage emerging or living in control habitats and T = percentage emerging or living in treated habitats.

3.1.1 Data analysis

The mean number of pupae or larvae collected per dip for each replicate of each treatment and the control is calculated for each day of observation. The percentage reduction in larval and pupal densities, or the IE% on post-treatment days, will be estimated for each replicate of each treatment using Mulla's formula. The difference between treatments treatments can be compared by two-way analysis of variance (ANOVA) with treatment and number of days as independent factors. The ANOVA should be carried out after transforming the percentage reduction to arcsine values.

The post-treatment day up to which 80% or 90% reduction is observed for each treatment or dosage will then be compared to determine the residual effect and optimum application dosage (see Section 3.3).

3.2 Simulated field trials

In these trials, multiple artificial containers (jars, bucket, tubs, cylinders, etc.) of water are placed in the field or under simulated field conditions and the materials are tested against laboratory-reared or field-collected larvae. The type and size of the container will depend on the natural larval habitat of the target mosquito species. The water-filled containers are given at least 24 h for

conditioning or ageing. A batch of 25–100 laboratory-reared third instar larvae of the mosquito species to be tested is released into each container or replicate and larval food is added. After 2–3 h of larval acclimation, the containers are treated with selected dosages in a randomized manner using pipettes or appropriate hand atomizer sprays, or by broadcasting solid materials over the water surface. The containers are covered with nylon mesh screen or solid covers to prevent other mosquitoes or other insects from laying eggs and to protect the water from falling debris. The water level in the containers must be sustained. A minimum of four replicates of each dosage and four controls are to be used. For fast-acting agents all the containers are examined after 48 h and live larvae are counted to score post-treatment larval mortality. For slow-acting materials, such as IGRs, the survival of larvae, pupae and pupal skins is assessed seven days or more after treatment, by which time all larvae would have pupated and emerged as adults. The pupal skins provide the best gauge of final or overall effectiveness. To test residual activity, a new batch of laboratory-reared, late third instar larvae of the same mosquito species is introduced to each container, and mosquito larval food is added on alternate days or weekly. Larvae survival is assessed 48 h post addition, and pupal skins are counted seven days or more after addition. This process continues until no mortality is noted.

Data are recorded on the form in Fig. A4.2. For the IGRs under test, pupae are removed from the treated and control containers every other day and put into vials or cups with water from the respective containers, then placed in cages and adult emergence is recorded. Another precise method of assessing emergence is to count and remove pupal skins from containers (Fig. A4.4). Adults not freed from pupal skins are considered dead. The test is terminated when there is no statistically significant residual activity in terms of larval mortality or inhibition of adult emergence when comparing the

treated (at the highest dosage tested) batches and the untreated controls. Values of pH and water temperature are recorded throughout the evaluation.

Alternatively, tests can be conducted by exposing third instar larvae in small natural breeding sites to selected dosages of larvicides using screened floating cages (minimum of three replicates, two cages per replicate). These cages should have screened portholes to allow the movement of water and food into the cage from outside. For each dosage, at least three treated and three untreated control habitats are selected. The habitats are treated with the selected dosages of the material to be tested. Twenty-five laboratory-reared or, preferably, field-collected third instar larvae are placed in each cage. The number surviving is counted every other or every third day until all larvae have pupated and emerged. Percentage mortality or IE% is calculated. To test residual activity, 25 third instars are set weekly in treated and untreated control cages. As with the initial batches of larvae, assessments of mortality should be made every other or every third day post introduction. The weekly settings of larvae continue until no difference in mortality is recorded between untreated controls and treated batches.

3.2.1 Data analysis

The method given in Section 3.1.1 can also be used to analyse data collected under simulated trials. However, since the denominator is known for simulated trials, a probit or logistic regression analysis is more suitable than ANOVA and is described below.

The data on the number of live and dead larvae and pupae from all replicates of each dosage on one day should be combined and percentage mortality or IE% calculated. Logistic or probit

regression of the percentage mortality or IE% on dosage and number of post-treatment days can be used to determine the post-treatment day (and its 95% CI) up to which 80% or 90% (the desired level of control) is achieved for a given dosage. This analysis can be done using appropriate statistical software packages.

3.3 Selection of optimum field application dosage

From the dosages tested against a target species in the small-scale or simulated field trials, the minimum dosage at which the maximum effect (immediate as well as residual) is achieved should be selected as the optimum field application dosage for each type of habitat. The frequency of larvicidal treatment is determined based on the reappearance of fourth instar larvae or pupae, in the case of common larvicides and bacterial larvicide products, or the day reduction in inhibition of emergence falls below 90% for IGRs.

4. PHASE III: LARGE-SCALE FIELD TRIALS

The efficacy of larvicides found to be suitable in small-scale field trials (Phase II) should be validated in larger scale field trials against natural vector populations in natural breeding habitats. In this phase, the larvicide is applied to the breeding sites of the target mosquito at the optimum field dosage(s) selected in the small-scale field trials using appropriate application equipment, depending on the formulation.

The objectives of the trial are:

- to confirm the efficacy of the larvicide at the selected field application dosage(s) against the target vector when applied to large-scale plots in natural breeding sites;
- to confirm residual activity and application intervals;
- to record observations on the ease of application and dispersal of the insecticide;
- to observe community acceptance;
- to record any perceived side-effects on operators; and
- to observe the effect of the treatment on non-target organisms.

4.1 Selection of study sites

The experimental plots selected will depend on the type of larval habitat and the environment. Care should be taken that all the representative habitats of the target vector species are included in the trial. A minimum of 25–30 replicates or plots of each type of larval habitat of the target species should be selected for control and then again for treatment. Just as for the small-scale trials, each confined habitat can be considered as an individual replicate; larger habitats can be subdivided into replicates of about 10 m^2.

4.2 Assessment of pretreatment density

Pretreatment larval and pupal abundance (and adult emergence in the case of IGRs) in the treatment and control habitats should be carried out for a week on at least two occasions before treatment. The immature population and adult emergence should be estimated in different types of larval habitat by using appropriate sampling devices (as in the small-scale field trials with natural populations).

4.3 Application of larvicide

All the breeding sites within the unit should be treated at the optimum field application dosage determined in Phase II, using equipment that is appropriate to the formulation and its operational use. The optimum dosage for the major or most important larval habitat of the target species in the area can be used for all the habitats. Where small-scale trials found wide variation between optimum dosages for each type of habitat, the specific optimum dosage should be applied to each type of habitat.

4.4 Assessment of post-treatment density

The impact of larvicidal treatments on the larvae and pupae of mosquitoes (and the inhibition of adult emergence) should be evaluated by sample collection at 48 h and then at weekly intervals using a fixed number of dips or sentinel cages. Sampling procedures are similar to those followed for small-scale trials conducted in natural breeding habitats. Data should be recorded on the relevant form (Figs. A4.3 or A4.4).

4.5 Effect on non-target organisms

Specific, separate trials have to be carried out to assess the impact of larvicides on non-target organisms. However, during the large-scale trial, and where appropriate, non-target organisms cohabiting with mosquito larvae can be counted and examined for impact of treatments while sampling mosquito larvae. Larvivorous fish, snails, polychaetes, shrimps, cray fish, crabs, mayfly naiads, copepods, dragonfly naiads, coleopterans and heteropterans,

ostracods and amphipods are some of the non-target organisms that coexist with mosquito fauna.

4.6 Operational and community acceptability

During the trial, observations should be made on the ease of storage, handling and application of the insecticide formulation on the breeding sites, and of the effects of the insecticide formulation on the proper functioning of application equipment such as nozzle tips and gaskets, rotors, blowers, etc.

Observations are also recorded on the acceptability of the insecticide treatments to the residents of the area, particularly on domestic and peridomestic breeding sites.

4.7 Data analysis

The mean number of pupae or larvae or non-target organisms collected per dip on each day of observation is calculated for each replicate in treatment and control. The statistical analysis to determine residual efficacy – including the number of post-treatment days over which the desired level of control is achieved at the selected dosage – is carried out following the method described in Section 3.1.1.

REFERENCES

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ANNEX 1
PRODUCTION OF TEST LARVAE

Use of homogenous batches of mosquito larvae is of prime importance in laboratory studies and is crucial in determining the activity and biopotency of synthetic larvicides, IGRs, bacterial larvicides and natural products. The following standard procedure is proposed for rearing *A. aegypti* and *Culex* spp. Other species may be reared according to these procedures, subject to any modifications necessary to fit the biological requisites of the test species.

For *A. aegypti*, eggs are laid in a cup lined with filter paper strips and one third filled with deionized or tap water. About one third of the paper strip should be in water. This will keep the strips moist where the eggs are laid above the water line. The paper strips are dried at room temperature and stored at room temperature for several months in a sealed plastic bag. When larvae are needed, the paper strip is immersed in de-chlorinated or distilled water. To synchronize and promote hatching, add larval food to the water 24 h before adding the eggs. The bacterial growth will de-oxygenate the water and this triggers egg hatching. This process usually induces the first instars to hatch within 12 h of hydration. The hatched larvae are then transferred to shallow pans or trays containing 2 l de-chlorinated water. The aim is to create a population of 500 to 700 larvae per container. Larval food may be flakes of protein as used for aquarium fish, rabbit pellets, chicken mash or powdered cat biscuit. The containers are held at 25 $\pm$ 2 °C. It is important that the amount of food is kept low to avoid strong bacterial growth (which kills the larvae), increasing food provision as the larvae grow. Several feeds at intervals of one or two days and daily observation of the larvae are optimal. Provision of solid pellets (chicken mash or rabbit pellets) prevents turbidity and scum. If the

water becomes turbid (in the case of powdered food), replace all water by filtering out the larvae and then transferring them to a clean container with clean water and food, a process that may result in larval mortality. A homogenous population of late third or early fourth instars (5 days old and 4–5 mm in length) should be obtained five to seven days later.

The materials and procedures necessary to rear *Culex* larvae, especially those that are severe pests or vectors of disease, are essentially the same as for *A. aegypti*, except that *Culex* eggs are deposited on water as egg rafts and will hatch 1–2 days after deposition. They require no conditioning and cannot be dried. If they do not hatch in two days they will die. It is more difficult to obtain a homogenous population of third or fourth instars of *Culex* spp. larvae. First, a large number of egg rafts must be laid and collected on the same day. These can be stored at 15–18 °C in order to accumulate more eggs for hatching over a day or two. The first instars are fragile and thus should not be handled. Development to the second instar usually takes 3–4 days at 25 $\pm$ 2 °C after the eggs are hatched. In trays containing 2–3 l de-chlorinated water at 4–6 cm depth, 400–600 larvae per tray are reared. Food (see above) is provided as needed. Early fourth instars suitable for testing are usually obtained within 7 days, although sometimes 8 or 9 days are required.

ANNEX 2
DILUTIONS AND CONCENTRATIONS

Table A2.1
Aliquots of various strength solutions added to 100 ml water to yield final concentration

Initial solution		Aliquot (ml)[a]	Final concentration (PPM) in 100 ml
%	PPM		
1.0	10 000.0	1.0	100.0
		0.5	50.0
		0.1	10.0
0.1	1 000.0	1.0	10.0
		0.5	5.0
		0.1	1.0
0.01	100.0	1.0	1.0
		0.5	0.5
		0.1	0.1
0.001	10.0	1.0	0.1
		0.5	0.05
		0.1	0.01
0.0001	1.0	1.0	0.01
		0.5	0.005
		0.1	0.001
0.00001	0.1	1.0	0.001
		0.5	0.0005
		0.1	0.0001

[a] For 200 ml double the volume of aliquots.

ANNEX 3
MEASUREMENTS AND CONVERSIONS

Volume
l l = 1000 ml
1 ml = 1000 µl
1 cubic foot = 7.5 gallons = 28 l
1 gallon = 4 quarts = 8 pints = 128 ounces = 3785 ml

Surface
1 ha = 10 000 m^2 = 2.2 acres
1 acre = 43 560 square feet
1 square foot = 0.111 square yard = 0.105 m^2

Length
1 km = 0.62 miles = 1093 yards
1 m = 39.7 inches
1 inch = 2.54 cm = 0.0254 m
1 foot = 0.333 yards = 0.3048 m
1 yard = 91.44 cm = 0.9144 m
1 mile (statute) = 1760 yards = 5280 ft = 1609.3 m

Weight
1 pound = 0.454 kg
1 kg = 2.2 pounds
1 g = 0.035 ounces

Conversion factors
Square inches to square centimetres, multiply by 6.5.
Square yards to square metres, multiply by 0.8.
Square feet to square metres, multiply by 0.09.
Acres to hectares, multiply by 0.4.
Square miles to square kilometres, multiply by 2.6.

ANNEX 4
DATA RECORDING FORMS

Fig. A4.1

Laboratory evaluation of the efficacy of larvicides against mosquito larvae

Experiment No: _____ Investigator: _____ Location: _____ Treatment date: _____

Material: _____ Formulation: _____ Temp: _____ Lighting: _____

Species: _____ Larval instar: _____ Larvae/cup or vessel: _____

Water: Tap/Distilled Volume of water: _____ ml Food: _____ Date stock solution made: _____

		No of dead larvae at various conc. (mg/L) post exposure (hr.)													
		24 hr							48 hr						
Date	Replicate	0.00							0.00						
	1														
	2														
	3														
	4														
	5														
	6														
	7														
	8														
	9														
	10														
	11														
	12														
	Total														
	Ave.														
	% mortality														

LC50 (CL 95%): _____ LC50 (CL 95%): _____

LC90 (CL 95%): _____ LC90 (CL 95%): _____

LC99: _____ LC99: _____

Slope: _____ Heterogeneity: _____ Slope: _____ Heterogeneity: _____

Fig. A4.2

Laboratory evaluation of the efficacy of insect growth regulators against mosquito larvae

Experiment No: _____ Investigator: _____ Location: _____ Treatment date: _____

Material:_____ Formulation:_____ Sampling technique: _____

Species: _____ Larval instar: _____ No. of larvae released/exposed: _____ Setting date: _____

Cumulative number of dead / alive mosquitoes after treatment (date or days pre or posttreatment or setting) L=larvae, P=pupae, A=adults

	Date:																Grand total			
Conc.		Alive			Dead			Alive			Dead			Alive			Dead			Alive			Dead		
(mg/L)	Rep	L	P	A	L	P	A	L	P	A	L	P	A	L	P	A	L	P	A	L	P	A	L	P	A	L	P	A	L	P	A	L	P	A	L	P	A	L	P	A	L	P	A	L	P	A	L	P	A
0.0	1																																																	
	2																																																		
	3																																																		
	4																																																		
	Total																																																		
	Mean																																																		
T1	1																																																		
	2																																																		
	3																																																		
	4																																																		
	Total																																																		
	Mean																																																		
T2	1																																																		
	2																																																		
	3																																																		
	4																																																		
	Total																																																		
	Mean																																																		
T3	1																																																		
	2																																																		
	3																																																		
	4																																																		
	Total																																																		
	Mean																																																		
T4	1																																																		
	2																																																		
	3																																																		
	4																																																		
	Total																																																		
	Mean																																																		
T5	1																																																		
	2																																																		
	3																																																		
	4																																																		
	Total																																																		
	Mean																																																		

Fig. A4.3

Small-scale field testing and evaluation of larvicides against mosquito larvae

Experiment No: _____ Starting date: _____ Location: _____ Investigator: _____
Assessment date: _____ Pre or days posttreatment: _____ Types of Habitat:_____Species _____

Live larvae (L3-4) and pupae (P)/sample							
Treatments		Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Grand total
Dosage ( )	Sample	L3-4* P*	L3-4 P	L3-4 P	L3-4 P	L3-4 P	L3-4 P
Control	1						
	2						
	3						
	4						
	5						
	Total						
	Mean						
	%red						
T1	1						
	2						
	3						
	4						
	5						
	Total						
	Mean						
	%red						
T2	1						
	2						
	3						
	4						
	5						
	Total						
	Mean						
	%red						
T3	1						
	2						
	3						
	4						
	5						
	Total						
	Mean						
	%red						
T4	1						
	2						
	3						
	4						
	5						
	Total						
	Mean						
	%red						
T5	1						
	2						
	3						
	4						
	5						
	Total						
	Mean						
	%red						

Fig. A4.4

Small-scale field testing and evaluation of insect growth regulators against mosquito larvae

Experiment No: _____ Starting date: _____ Location: _____ Investigator: _____
Assessment date: _____ Pre or days posttreatment: _____ Type of Habitat: _____ Species:_____

		Live larvae (L3-4), pupae (P) and adult emergence (A) /sample or cage or trap												
Treatments		Rep 1			Rep 2			Rep 3			Rep 4			Rep 5			Grand total			Visual count	
Dosage ( )	Sample	L3-4*	P*	A*	L3-4	P	A	L3-4	P	A	L3-4	P	A	L3-4	P	A	L3-4	P	A	Pupae	Pupal skins
Control	1																				
	2																				
	3																				
	4																				
	5																				
	Total																				
	Mean																				
	%red																			IE%	
T1	1																				
	2																				
	3																				
	4																				
	5																				
	Total																				
	Mean																				
	%red																			IE%	
T2	1																				
	2																				
	3																				
	4																				
	5																				
	Total																				
	Mean																				
	%red																			IE%	
T3	1																				
	2																				
	3																				
	4																				
	5																				
	Total																				
	Mean																				
	%red																			IE%	
T4	1																				
	2																				
	3																				
	4																				
	5																				
	Total																				
	Mean																				
	%red																			IE%	
T5	1																				
	2																				
	3																				
	4																				
	5																				
	Total																				
	Mean																				
	%red																			IE%	