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SIMPLIFICATION OF ADULT MOSQUITO BIOASSAYS THROUGH USE OF TIME–MORTALITY DETERMINATIONS IN GLASS BOTTLES

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ABSTRACT. A simple method is described for treating 250-ml glass Wheaton bottles with insecticide, and using them as test chambers for detecting insecticide resistance in mosquito and sandfly populations. The methods for treating bottles, obtaining baseline data, and applying this technique to insects from the field are described. Sample data are presented from tests run on different vector species using a variety of insecticides. Time–mortality data from the bottle bioassay are presented alongside results from biochemical detection methods applied to the same mosquito population. The potential role, advantages, and limitations of the time–mortality bottle method are discussed.

KEY WORDS Insecticide resistance, bioassay, mosquito, surveillance, Culicidae

INTRODUCTION

Bioassay detection of insecticide resistance in adult mosquitoes has been based on a standard method recommended by the World Health Organization (WHO 1981). In the original WHO test (WHO 1963, 1970), susceptible mosquitoes were exposed to a series of insecticide-impregnated papers, each with a different dose, for a defined period of time, after which the mosquitoes were held in the absence of insecticide for 24 h. Mortality was recorded and a probit dose–mortality graph was generated for the particular insecticide. The test was modified to use only papers impregnated with a single discriminating or diagnostic dose when it became evident that sufficient numbers of mosquitoes of the proper age and reproductive state were difficult to collect in the field (WHO 1981). The discriminating dosage selected was twice the experimentally derived 100% lethal concentration (LC_{100}) value.

The WHO discriminating dosage method (Shidrawi 1990) has drawbacks, particularly for initial detection of resistance by field personnel. The WHO resistance test kit has become expensive. Test papers are not available for some insecticides, such as dibrom or resmethrin, that are routinely used in vector control. The insecticide diagnostic dosages available are not applicable to all vector species. No provision is made in the WHO kit for using synergists to evaluate potential resistance mechanisms. Integrating test kit results with biochemical resistance detection methods is difficult. Finally, care must be exercised with pyrethroids to place the testing chambers on their sides to keep mosquitoes that have been knocked down in contact with the insecticide.

A more practical and timely approach to detection of resistance focuses on detecting resistance in individual mosquitoes. We have modified use of the WHO test kit over the last decade, especially in field studies where bioassay data have been collected on the same mosquito population pools as microplate-based biochemical assays for resistance mechanisms (Brogdon et al. 1988a, 1988b; Beach et al. 1989; Brogdon 1989; Cordon-Rosales et al. 1990). Most recently we have changed the original protocol through the use of insecticide-coated glass bottles and solutions of standards-grade insecticides or synergists. Data supporting this approach in detection of insecticide resistance in field populations are presented here. Characterization of susceptible populations is described, along with examples of how resistant individuals are identified through integration of these data with biochemical microassay data from the same resistant individuals and from individual mosquitoes in the same sample population. The methods described function in both laboratory and field situations.

MATERIALS AND METHODS

Mosquitoes

Mosquitoes were from insecticide-susceptible or -resistant strains or field isolates of *Anopheles albimanus* Wied., *An. gambiae* Giles, *An. dirus* Peyton and Harrison, *An. stephensi* Liston, *An. freeborni* Aitken, *Aedes aegypti* L., *Ae. albopictus* Skuse, *Culex restuans* Theobald, *Cx. pipiens* L., *Cx. nigripalpus* Theobald, *Cx. salinarius* Coq., and *Cx. territans* Walker maintained or reared at the Centers for Disease Control (CDC) and Prevention, Atlanta, GA. Mosquitoes used in assays were 3–4 days postecdysis and were not bloodfed prior to experiments. All resistant colonies contained both susceptible and resistant mosquitoes.

Bioassay materials

World Health Organization kit tubes fitted with insecticide-impregnated papers and bottles coated with pyrethroid solutions in acetone were used in the experiments. For the purposes of presenting the method, bottle-bioassay data were used from ex-

periments where a synthetic pyrethroid served as the toxicant.

For treatment (coating) of bottle interiors, technical grade solutions of pyrethroids (standards maintained at CDC, Atlanta, GA, or purchased from Chem Service, West Chester, PA) were diluted in acetone. A 2-ml portion of diluted toxicant was transferred to a 250-ml Wheaton bottle. The bottle was shaken, rolled, and inverted such that all surfaces were exposed to the solution. Once the liquid phase was evenly distributed, the volume of excess insecticide solution was determined before it was discarded into a waste container and the bottle (and cap) were inverted on paper toweling overnight in a dark cabinet. Matching bottles and caps should be identified with a label when a number of different insecticides or dosages are being prepared.

The concentration of insecticide remaining in the bottle is not accurately measured. In practice, when care is taken to prepare all bottles in an equivalent manner, the bottle method gives highly reproducible results. Alternatively, bottles may be dried on a bottle roller, in which case all of the insecticide remains in the bottle. Our experience has been that either method for insecticide application and solvent removal is acceptable.

A range of insecticide concentrations is tested against a susceptible sample population of mosquitoes for determination of the response baseline. An ideal concentration of insecticide is the lowest concentration that gives straight line (regressions of r^2 = 0.9 or better) time–mortality data from 0 to 100% mortality over a convenient test period; we recommend 1 h. This procedure also determines the range in time of survival at a particular insecticide dosage.

Bottles with incorporated synergist–insecticide combinations are prepared in the same way as the insecticide-impregnated bottles. A series of synergist concentrations is used to verify that the concentration chosen for use in experiments is below toxic levels. In this paper, the synergists piperonyl butoxide (PB; an oxidase synergist) and *S,S,S*-tributyl-phosphorotrithioate (DEF; an esterase synergist) were used.

Bioassay Data Collection

Mosquitoes were transferred into test chambers at time = 0, and mortality was scored at regular intervals until mortality was complete, or until the experiment was terminated; some highly resistant mosquitoes were observed to survive 24–48 h of continuous exposure. Our criterion for mortality was that mosquitoes could not right themselves or fly when the test chamber was slowly rotated. Care must be exercised to interpret this parameter consistently. In addition, the WHO test kit tubes were placed on their sides throughout the experiment to avoid having mosquitoes fall out of contact with the insecticide before receiving a lethal dose and subsequently reviving. We found it more convenient to keep the glass bottles on their sides during the experiments, but any orientation is acceptable, because all interior bottle and cap surfaces are treated.

Bioassay Data Interpretation

Percent mortality at each time point is plotted on semilogarithmic paper. Alternatively, values were plotted on probit logarithmic paper (of the type supplied in WHO test kits). Mosquitoes that survive beyond the upper range limit for time of survival in the reference susceptible population are scored as less susceptible.

Determination of Diagnostic Insecticide Concentrations

The appropriate concentration of insecticide used to treat bottles must be determined empirically with a susceptible population. Thus far, a number of insecticides have been adapted to the bottle format and the most useful concentration in all instances has fallen within a factor of 10. The criterion that we use is that the insecticide concentration should kill all susceptibles within 1 h, with a near linear probability plot of dose versus mortality. A dosage of 25–100 μg insecticide/bottle seems to work well for most insecticides, but baseline values should be prepared by anyone using these methods.

Biochemical Assay Methods

The procedures for the elevated esterase, glutathione *S*-transferase, oxidase, altered acetylcholinesterase, and protein assays have been reported elsewhere (Brogdon and Dickinson 1983; Brogdon 1984a, 1984b; Brogdon and Barber 1990; Brogdon et al. 1997), as has the hybrid bioassay/biochemical assay we call the target enzyme assay for organophosphate resistance (Brogdon et al. 1992).

RESULTS

Time–Mortality Baseline Values Using Impregnated Test Papers

Papers were impregnated with permethrin (WHO, 0.25%), lambda-cyhalothrin (ICI, 0.025%), malathion (WHO, 5%), chlorphoxim (WHO, 5%), and fenitrothion (WHO, 1%). The time required for mortality of susceptible *An. albimanus* to reach 100% at these dosages varied considerably for some compounds (Fig. 1). The rate at which the organophosphates inhibited their target enzyme, acetylcholinesterase, closely matched the time until mortality began to occur (Fig. 2). In comparing Figs. 1 and 2, note that, although the 3 organophosphates required varying lengths of time to kill the mosquitoes, mortality from each insecticide be-

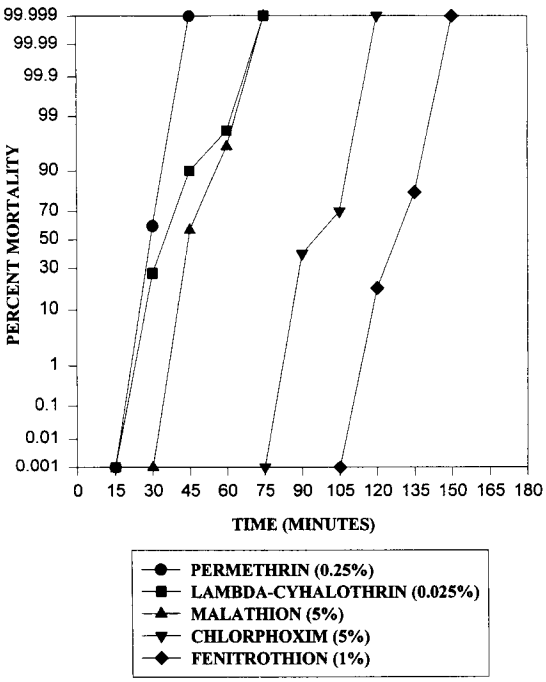

Fig. 1. Time–mortality data measured for 3–4-day-old adult female *Anopheles albimanus* exposed to 5 sets of insecticide treated test papers. Results are means for 6 replicates of 50 mosquitoes. Standard deviations were less than 5%.

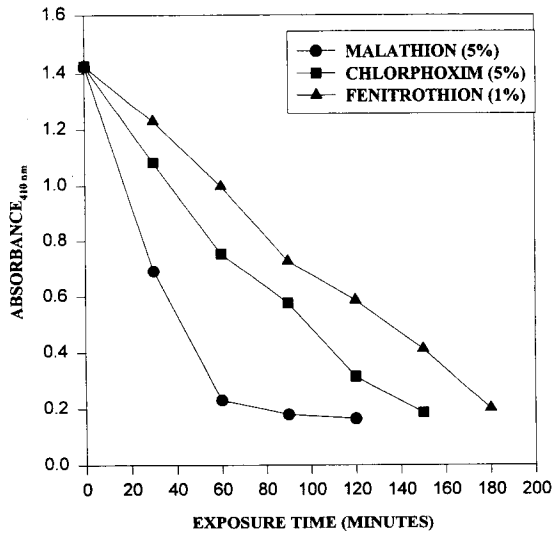

Fig. 2. Acetylcholinesterase levels in 3–4-day-old adult female *Anopheles albimanus* exposed to organophosphate-impregnated papers for varying lengths of time. Each data point represents means for 32 mosquitoes. Standard deviations were less than 8%. An absorbance of 0.1 corresponds to a hydrolysis rate of 1.13 nmol/min/assay or 11.3 nmol/min/mosquito.

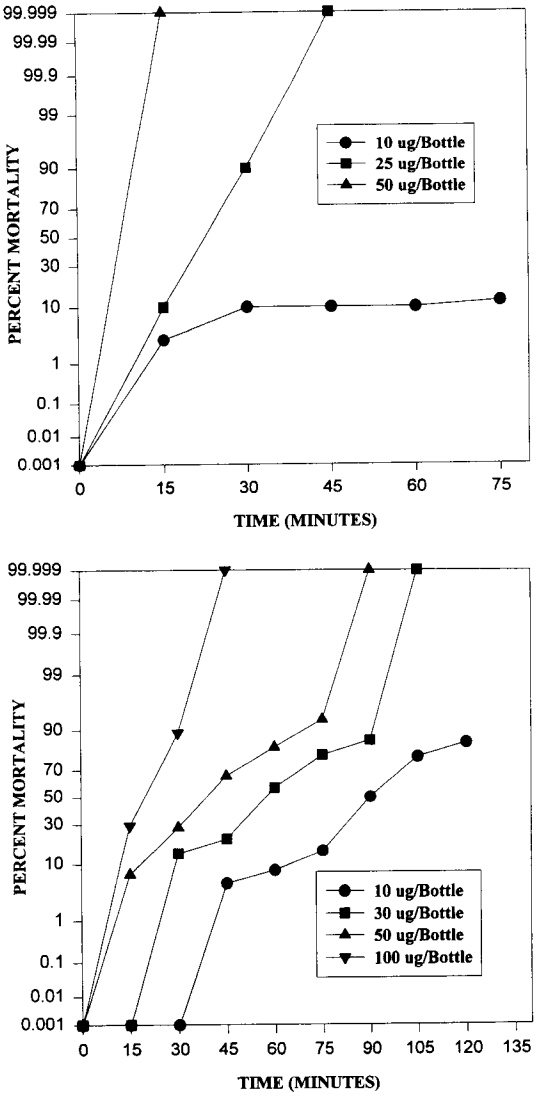

Fig. 3. Percent mortality of 3–4-day-old adult female *Anopheles freeborni* in bottles treated with dilutions of standard grade insecticide. (top) Bottles treated with dibrom (naled). (bottom) Bottles treated with resmethrin. n = 40 mosquitoes/replicate for each concentration of each insecticide with 6 replicates. Standard deviations were less than 5%.

gan to occur when mosquito average acetylcholinesterase activities had declined to approximately 40% of their original level. All mosquitoes were dead by the time the acetylcholinesterase activity had fallen to around 15% of its original level.

Obtaining Baseline Values in Insecticide-Treated Bottles

Data are shown for range-finding experiments on *An. freeborni* using varying concentrations of dibrom (naled) and resmethrin (Figs. 3 top, 3 bot-

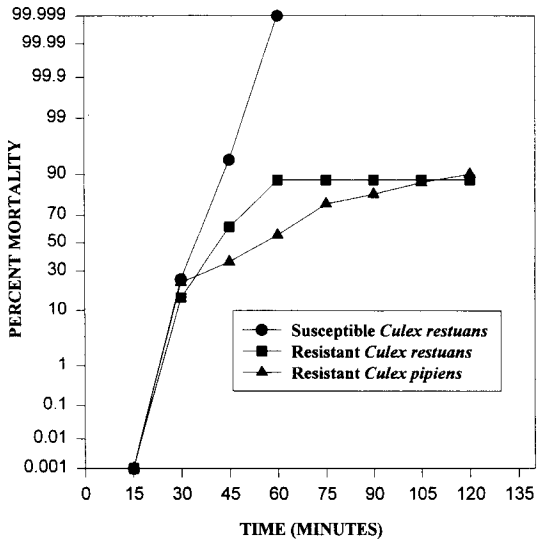

Fig. 4. Comparison of dibrom time–mortality data for susceptible and resistant 3–4-day-old adult *Culex restuans* and *Culex pipiens*. Data from susceptible *Cx. pipiens* were virtually identical to those from *Cx. restuans* and are not shown. n = 25 mosquitoes/replicate with 3 replicates. Standard deviations were less than 6%.

tom). Papers impregnated with these compounds are not available. Dosages selected for resistance detection were 50 μg/bottle for resmethrin and 25 μg/bottle for dibrom. These concentrations gave similar results for susceptible *An. albimanus, An. gambiae, An. stephensi, An. dirus, Ae. albopictus, Ae. aegypti, Cx. restuans, Cx. pipiens, Cx. nigripalpus, Cx. salinarius, Cx. territans,* and for the phlebotamine sandfly, *Lutzomyia youngi* Feliciangeli and Murillo (data not shown).

Detection of Resistance Frequency Using Bottle Bioassay

The time–mortality relationships for susceptible and resistant *Cx. restuans* and *Cx. pipiens* in dibrom-treated bottles are compared in Fig. 4. Similar responses, although at differing resistance frequencies, have been measured for organophosphate, dichlorodiphenyltrichloroethane (DDT), carbamate, and pyrethroid resistance in *An. albimanus* from Guatemala; organophosphate and pyrethroid resistance in *An. gambiae* from Kenya; organophosphate resistance in *Ae. albopictus* from Missouri; organophosphate and pyrethroid resistance in *Ae. aegypti* from Cali, Colombia; organophosphate and pyrethroid resistance in *Culex* species from the Midwest; and pyrethroid resistance in Colombian *L. youngi* (data not shown). Note that, based upon the percentage dead at 1 h in the susceptible population, the relative resistance frequency in the *Cx. restuans* population tested is 10% and that for *Cx. pipiens* is 55%.

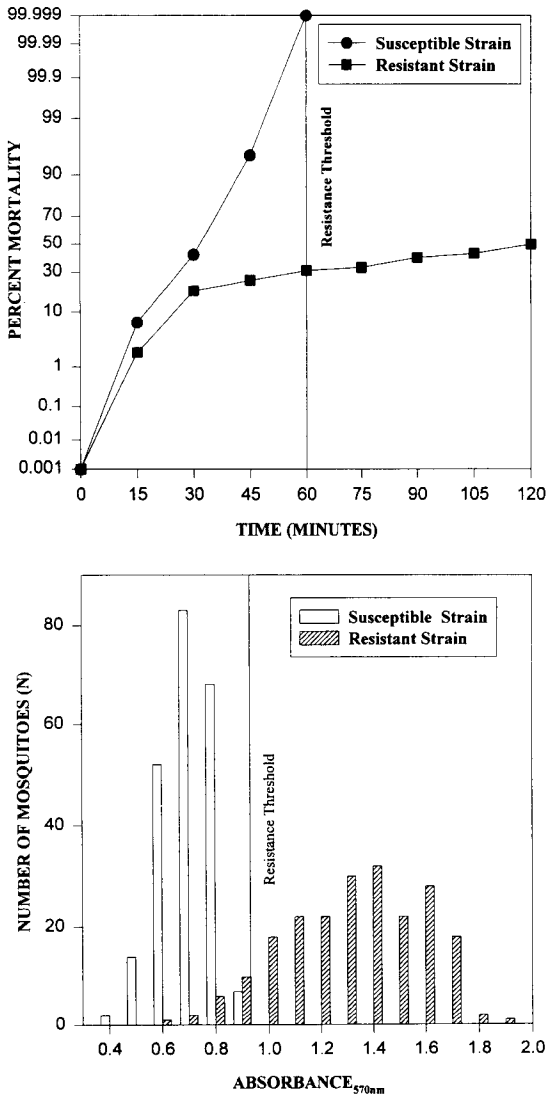

Fig. 5. Comparison of fenitrothion time–mortality data from (top) a bottle bioassay (100 μg/bottle), and (bottom) from a nonspecific esterase microplate assay on resistant and susceptible populations of *Anopheles albimanus*. In the bioassay, n = 25 mosquitoes/replicate with 6 replicates. Standard deviations were less than 8%. In the biochemical assay, the frequency distributions of mosquitoes at each 570-nm absorbance class are shown. The coefficient of variation was 0.07 in the biochemical assays.

Correlation of WHO Bioassay, Bottle Bioassay, and Biochemical Assay Data

Using the upper range limit method, the bottle bioassay and biochemical assay methods give similar results in the mosquito and sandfly populations we have tested. A resistance level to fenitrothion of approximately 70% was measured in an *An. albimanus* population (Fig. 5 top). In the biochemical test, the percentage of resistant mosquitoes is calculated as the

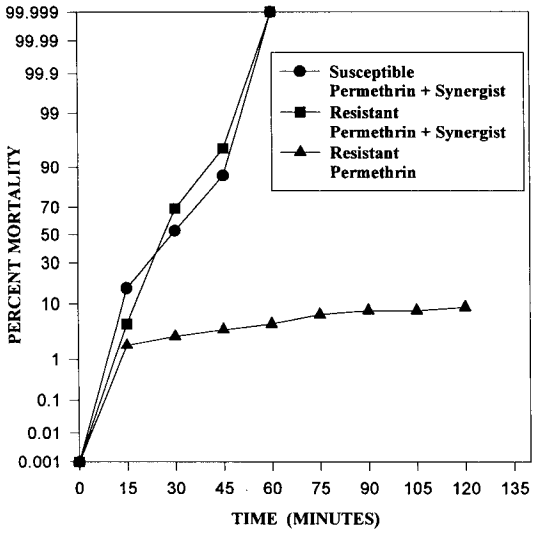

Fig. 6. Comparison of susceptible and resistant populations of 3–4-day-old adult *Anopheles albimanus* in permethrin-treated bottles and permethrin + piperonyl butoxide-treated bottles. n = 25 mosquitoes/replicate with 3 replicates. Standard deviations were less than 6%.

number of mosquitoes with esterase or oxidase levels above the threshold divided by the total number of mosquitoes assayed. Both the bottle assay and biochemical assay measured a fenitrothion resistance level of 70% in the same population (Fig. 5 bottom). The WHO assay method reported resistance levels 50% lower for organophosphates and 90% lower for pyrethroids than the other methods.

Use of Synergists in the Bottle Bioassay Format

The synergist PB was added to bottles containing the critical permethrin dosage. This combination was then used in a bottle bioassay to link permethrin resistance to an oxidative mechanism in a population of *An. albimanus* (Fig. 6). The mechanism was confirmed through an oxidase microplate assay (data not shown). The use of synergists such as PB or DEF requires prior experiments to determine a dosage of synergist that will not kill test insects.

DISCUSSION

We have discussed the use of the bottle-based bioassay for pyrethroid insecticides but the method has been applied with equal success using organophosphate and organochlorine insecticides. Our method is similar to the vial test of Plapp et al. (1987) in using a glass container as an insecticide-treated surface, but differs significantly in the method of data collection. Here, time–mortality data are collected at a fixed concentration until all test insects have died. The fixed concentration represents a predetermined discriminating dosage based upon data collected using a susceptible population of the test species. The use of time–mortality data takes advantage of the fact that mortality is proportional to insecticide uptake (Ariaratnam and Brown 1969) and its success in reaching its biochemical target. The larger container allows 50 or more mosquitoes to be assayed simultaneously, while preventing easy escape of individuals during loading.

In practical terms, the WHO bioassay format is a laboratory exercise, because obtaining sufficient mosquitoes in the field is frequently a problem. The bottle bioassay differs, in that 5 or fewer mosquitoes may be tested and results from a series of tests on a series of collections may be pooled. Probit analysis (Finney 1971) assumes a homogeneous population for testing, but field populations are rarely homogeneous and probit results obtained in the field or with field populations are usually nonlinear. Methods developed to deal with this lack of homogeneity and also with differences in time of action of insecticides are mathematically complex and, depending upon the specific data set, may require a choice to be made between competing statistical methods (Robertson et al. 1984; DeBanne and Haller 1985, Preisler 1988). Most importantly, these methods can only refine the accuracy of median lethal dose (LD_{50}) or median lethal time (LT_{50}) measurements, data that, in any case, cannot adequately allow detection of low levels of resistance (Roush and Miller 1986). The bottle bioassay avoids the problem by asking a simpler question: will the insecticide at a concentration that gives 100% mortality for a susceptible population kill the test mosquitoes at the same rate? Thus, the bottle procedure seems more suited to the needs of field personnel.

Mosquitoes can be brought into the laboratory for rearing and selection to produce homogeneous populations, but few programs have the resources to rear and select insects routinely. Even then, the inheritance of the resistance may be complex or multiple mechanisms for resistance may be present. In these instances, probit analysis will still not show a linear response. The selection of more highly resistant laboratory lines can be a useful research tool, but the populations produced have little practical relevance to those requiring control, showing levels of resistance enzymes or responses in bioassays that are not encountered in the field populations. Moreover, these methods cannot aid efforts to detect resistance at low frequency.

The tendency of the WHO bioassay to report lower resistance frequencies is, we believe, a reflection of the different phenomena measured by the tests. As originally envisaged, the WHO bioassay was intended to reproduce, as nearly as possible, the results of a mosquito resting on an insecticide-treated surface in a domicile after taking a blood meal (Fay et al. 1953). This goal reflects the original development of the method as a tool in malaria control. Resistance is now developing in

vectors of dengue and yellow fever viruses, and leishmaniasis. Updated methods are needed for vectors that are controlled by means other than wall spraying.

The goal of our bottle protocol is to directly measure the toxicologic response of an insect to a given dose of insecticide. Our method provides results considerably faster than the WHO test, identifies the mechanisms involved in changes in susceptibility, and does not limit the investigator to currently available test papers. Based upon our experience with various vector species in different field settings, the methods described here represent a more sensitive and versatile toxicologic measurement of changes in the susceptibility of populations than the WHO bioassay and are more realistic candidates for routine use by control personnel.

Neither method can unambiguously answer the operational question of whether a given insecticide application (especially in the case of aerial or ground-based application) will kill the target population. Our goal now is to correlate the results of more sensitive methods of resistance detection to the results of field bioassays. The final answer needed by disease control programs (and the answer must be timely) is whether an insecticide will reduce a specific vector population.

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