ON THE BIONOMICS OF BROMELIAD-INHABITING MOSQUITOES. V. A MARK-RELEASE-RECAPTURE TECHNIQUE FOR ESTIMATION OF POPULATION SIZE OF WYEOMYIA VANDUZEEI

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ABSTRACT. The mark-release-recapture technique used in a study of the adult population dynamics of Wyeomyia vanduzei Dyar and Knab in Florida is described. Reasons for selection of the various methods are given. The technique was used, during 1976, in a study of 42 weeks duration in which the percentage of recaptured adults exceeded 11%.

INTRODUCTION. A study of a natural population of Wyeomyia vanduzei Dyar and Knab was begun in 1973 near Vero Beach, Florida (Frank et al. 1976). Initial sampling of the population was designed to investigate the dynamics of the immature stages of the mosquito. In late 1975 attention was given to estimation of population size and survival of adults, with the objective of establishing a technique to be used during 1976 and subsequently.

A long-term study of this type has been undertaken previously (with Aedes aegypti (L.)) by Sheppard et al. (1969), and brief studies (with Ae. aegypti, females only) by Conway et al. (1974), (with Culex p. fatigans Wiedemann [quinquefasciatus Say] by Macdonald et al. (1968), and (with Toxorhynchites brevipalpis Theobald) by Trpis (1973).

CATCHING ADULTS. A method for capturing adults of both sexes, alive and undamaged, was needed. It was also necessary that the method should not use more than 4 man-hours per day, but that sufficient adults should be caught during this time to provide statistically meaningful data. The actual optimal number of adults needed could not be predicted in advance without a prior estimation of (a) adult population size and (b) percentage of marked adults recaptured; a preliminary investigation of these parameters would be necessary.

In 1975 we tried using traps baited with rabbits but found that these caught very few Wyeomyia adults and large numbers of adults of Ae. taeniorhynchus (Wiedemann). The few adult Wyeomyia caught were in poor condition (probably as a result of desiccation), had wings damaged, legs and scales missing (as a result of mechanical injury perhaps partly through contact with the more robust and larger adults of Ae. taeniorhynchus), and were preponderantly female. We made a number of "feeding stations," each of which consisted of a vial containing 5% sugar water with honey secured to a small piece of sheet aluminum, in turn secured to the limb of a tree, but no adult Wyeomyia was observed to use one of these despite repeated inspections.

We made a small, screen-covered cylinder in which we placed adult female Wyeomyia and hung from the limb of a tree in hope of attracting male Wyeomyia, but found that the female Wyeomyia did not survive long and no males were attracted.

Observation had indicated both male and female adult Wj. vanduzei to be found on the rough bark of buttonwood (Conocarpus erectus L.) trees in the study area, but our initial reaction was that these adults were well-camouflaged so that collecting them from the tree trunks would be slow and laborious. Adults of both sexes spend a considerable portion of time apparently resting. Casual observation indicated that males are more active and that they perform characteristic flights, for minutes at a time, up and down the tree trunks close to the bark. Encounter of a male in flight of this type, with an apparently resting female, often resulted in mat-
ing. The males, therefore, appeared to be searching for females when making this type of flight. We provided a number of additional "resting places" in the study area, consisting of pieces of plywood and small walls of concrete blocks (without mortar) whose plane surfaces should enable the mosquitoes to be seen readily in silhouette; although Wyeomyia adults did use these "resting places" they did not do so in sufficient numbers to make this method of assemblage practicable. Because the bark of buttonwood trees is fairly dark, we carried out a cage experiment in the laboratory in which we demonstrated \( P < 0.01 \) in a choice test that adult Wyeomyia preferred to "rest" on a dark surface rather than on a pale surface. We then painted some of the concrete blocks in our walls matte black, and after a few days to allow the paint to dry, inspected the walls several times per day for several days. There was some indication that the black blocks were preferred by the mosquitoes, but because their numbers "resting" on the walls were still small, we did not bother to pursue this approach.

Our only practicable method of collecting adults seemed to be to aspirate them directly from tree trunks. We made a number of vials, each consisting of a piece of Lucite® tubing, 9 cm long, 1.5 cm inside diameter, one end covered with a piece of fiberglass screening of the mesh size (16 x 18 strands per inch) readily available from building suppliers, the other end plugged with a cork. Each vial was labelled with a number. Adult Wyeomyia collected by aspirator were blown from the aspirator into vials. When the screened end of a vial was held upmost, the cork could be removed and a mosquito blown in, even if the vial already contained several mosquitoes, with little risk of escape after a certain dexterity was acquired. Three canvas aprons were made, each with 10 separate, narrow pockets to hold individual vials. These pockets not only held the vials securely, but maintained adequate humidity so that mosquitoes in vials inside them could survive for several hours. However, mos-quitoes blown into the vials were occasionally blown, or observed to escape through, the mesh, so that this was later replaced by a finer mesh size of 32 strands per inch (ca. 12 strands per cm.).

In October 1975, with the mean daily air temperature in the study area at about 25°C, we found that we could capture about 80 adults in 1 well under 2 hrs, and with the 2 sexes approximately equally represented. This number might be increased by experience of the collector in recognizing favorable "resting" sites and, we suspected, might conceivably provide figures large enough for statistical analysis if the percentage recovery of marked adults proved to be sufficiently high. We had the options of collecting for a longer period of time (up to 4 hrs) and increasing the population size by increasing the number of bromeliads (larval habitat) suspended from the trees (Frank et al. 1976).

**Immobilizing Adults.** Initial attempts to immobilize adults during marking were concentrated on cold anesthesia. Adults were placed in a refrigerator at ca. 6°C to chill them, then placed on a hollow metal block containing ice for inspection and marking under a binocular microscope. Adults placed in the refrigerator without subsequent placement on the metal block usually recovered too rapidly on the microscope stage and attempted to fly, thus defeating attempts to mark them. Although the chilled metal block helped to keep them anaesthetized, it produced beads of moisture condensed on its surface, so that the mosquitoes became wet and suffered high mortality. Attempts to immobilize adults under small pieces of screen cloth or by forceps alone were too cumbersome for use with these small insects and resulted in mechanical injury.

An attempt was then made to anaesthetize adults with chloroform vapor. After various unsuccessful initial attempts, it was found that an appropriate vapor concentration might be obtained by placing cotton wool in a plastic wash bottle and moistening it with liquid chloroform. When the wash bottle was squeezed the
vapor produced from its orifice would be of the right concentration. If the cotton wool was made too wet, the vapor would contain too high a concentration of chloroform droplets which would kill the mosquitoes.

Recovery of adults from anesthesia was variable until the importance was discovered of maintaining them under conditions of high humidity. This was achieved by placing them after anesthesia on leaves, picked green from shrubs outside the laboratory, in plastic containers. The cylindrical containers, with one end covered with screen material, the transparent lid at the other end fitted with a small rubber stopper, were placed in a bio-room maintained at 80% R.H., while the mosquitoes recovered. The green leaves and the high relative humidity of the bio-room appeared to produce 100% recovery from the effects of anesthesia provided that the cotton wool in the wash bottle was not too saturated with liquid chloroform.

**Marking of Adults.** Initial investigations of the longevity of newly-emerged adults under controlled conditions in a bio-room, indicated that females lived longer than males and that the mean life span of females was greater than 20 days. With planned daily marking of adults in a field experiment, it was necessary to have more than 20 distinguishable marks.

Therefore use of colored dyes was scarcely feasible and attention was given to the use of paints, based on various organic chemicals as well as on water.

It was found that the finest available artist’s camel hair brush produced a paint spot we considered to be too large and we eventually arrived at the following solution. A short piece of nylon monofilament of 6-lb. test strength, available from fishing equipment suppliers, was passed rapidly through a gas flame to form a minute bead at its end. The terminal 2.5 cm were cut from the monofilament and taped to a fine wooden applicator stick, so that the last 1 cm protruded beyond the end of the stick and with the bead distal. The bead provided a surface to which the paint could adhere and inhibited the paint from flowing toward the handle of the applicator.

Trials of the various types of paints available, at various dilutions, indicated that the best for the purpose was a type of water-based poster paint of high chroma, readily available from stationery suppliers. Paint of this type is available in a range of colors and is generally referred to as “glossing” paint or under the trade name of a manufacturer. We found that the paint is supplied in small glass jars, often packaged as a set of 6 different colors. In order to obtain 20 different colors we tried mixing colors, but found that we could not readily distinguish between more than about 8 different colors when the colors were applied as minute spots to the thorax of a mosquito. We finally reduced the number of different colors to 5 (2 of the original 6 colors appeared to us too similar), and with a 2-spot 2-position 5-color code system, arrived at 25 different combinations.

We altered the viscosity of the paint by adding a small amount of liquid detergent, ca. 1% by volume, to each jar of paint. This improved our ability to apply spots of paint with precision to the mosquitoes, probably because the detergent removed a small part of the waxy coating of the integument. Although the organic solvents of some of the other paints tried also produced this effect, we found the solvents to be harmful to the mosquitoes and/or these paints to take longer to dry than did the water-based paint.

Although Sheppard et al. (1969) applied paint spots to the wings of mosquitoes, we found it difficult to accept that paint spots so located would not interfere with flight, simply by their weight. We marked mosquitoes on the dorsum of the thorax using, for an initial marking, one anterior and one posterior spot.

Accordingly, chloroform vapor was blown into the Lucite® vials in which we brought the mosquitoes to the laboratory. When the mosquitoes in a vial were inert, they were shaken from the vial, one at a time, onto the microscope stage. Each was
handled with fine forceps while marking. A skilled operator could handle inert mosquitoes by the legs without apparent damage. After marking, each mosquito was placed on a leaf in a plastic container as described above. All the mosquitoes of one Lucite® vial were marked and placed inside one plastic container which was placed immediately in the bio-room. The waxy surfaces of the leaves, in addition to maintaining high air humidity, also prevented the drying paint spots on the thoraces of the mosquitoes from adhering to the plastic containers or to any other part of any mosquito. Recovery from anaesthesia and marking took only a few minutes. Each operator, with practice, was able to reduce mortality from the effects of marking to zero.

INITIAL TRIAL AND EXPERIMENTAL DESIGN. The initial trial of the technique began on 28 October 1975 and continued each of the subsequent weekdays (weekends excepted) until 7 November. During this time we captured 419 adult Wy. vanderzeei in the study area. In part because our operators were not skilled at that time and some of the mosquitoes did not recover after marking, in part because the adults captured on the final day (7 November) were not released, we released 330 marked adults; of these 143 were male, 187 female. We recaptured 92 marked individuals (14 males, 18 females) on days subsequent to the day of release, so that the percentage of marked adults recaptured was 9.7%. If we had continued to capture adults after 7 November our percentage of recaptured adults might have been expected to rise above 10%. Additional improvement might have been expected had we continued operations through the weekend of 1-2 November.

During the initial trial we standardized our collecting so that exactly 2 hrs were spent each morning searching for and capturing adults. The time period was also standardized at 9:30–11:30 a.m. Although the theoretical optimal mark-release-recapture technique demands no time lag between capture of a mosquito and its subsequent return to the population after marking, this condition is not feasible. Because of the strictly limited number of staff members of our project (3 at that time) and the expectation that we would be dealing with larger numbers of mosquitoes when we began replication. Our mark-release-recapture project, it was decided that we should attempt to standardize the time period between capture and release. Since the capture period itself extended over 2 hrs, then the time period should be 4 hrs, extending from the mid-point of the capture period to the time of release. This should provide a sufficient time interval for transport of all captured mosquitoes to the laboratory, their marking, recovery, return to the field and release, even if one of the 3 staff members were absent. This decision was fortunate because the staff was reduced to 1 during 1976, one of whom has other duties in addition to the project.

Because, in this initial trial, some of the mosquitoes had died during or soon after marking, and because one of the conditions of the project was that we should not alter the natural study population either by increasing it or by depleting it after the beginning of the study, we had to plan to replace any adults we might kill. Our simplest option was to use adult Wy. vanderzeei captured from an isolated population immediately outside our laboratory to make up any deficiency we might inadvertently cause by killing adults from the study area. Because this population outside the laboratory was apparently small and we might have difficulty finding sufficient adults on any given day, we increased it by planting several hundred exotic bromeliads of the genus Bilbergia (which we were given) in the woodland outside the laboratory, in order to increase the habitat available to Wyomyia larvae. Adults from this increased population would at least be wild (which we considered preferable to laboratory-reared individuals), would be of mixed ages (in the same way as those we captured from the study area), and would be separated geographically from those of the study area by only about 0.4 mile, even though they
were from a different type of habitat and their genetic composition might conceivably differ somewhat. The interval between capture of mosquitoes in the study area and their subsequent release marked should also allow sufficient time to capture and mark adults from the population outside the laboratory in the event that we killed any adults from the study area.

From the results of the initial trial, we believed we could recapture better than 10% of the marked adults we released in the study area, a figure considerably higher than that obtained by Sheppard et al. (1969) working with Aedes aegypti in Thailand. Application of a simple Lincoln Index to our preliminary data suggested a population size of about 500 adults. If we were to increase our population size by doubling the amount of larval habitat, maintain a percentage recapture of marked individuals of 10% and run the experiment over an extended time period, then our expected data should be adequate for statistical analysis to allow us to determine population size and survival rate as well as the change in these parameters possibly as short as 4 weeks for these parameters. Results of this nature should enable us to examine the effects of weather (rainfall, temperature, humidity) on adult population size and survival. An independent assessment of adult recruitment (newly-emerged adults, reported in a separate publication) should give us data at least as reliable as those obtained by Sheppard et al. (1979).

The following changes were made in the study area before the beginning of the 1976 experimentation. The area for a distance of approximately 500 m extending in all directions from our study area was searched for bromeliads large enough to support populations of Wyeomyia larvae. All such bromeliads were removed so that the 500 bromeliads we had hung in the study area (Frank et al. 1976) were the only ones supporting the Wyeomyia population. Because of the apparently very limited flight range of adult Wyeomyia larvae, immigration could thus be eliminated as a factor affecting the experimental results. Emigration could not thus be eliminated, and within the personnel limitations there seemed to be no way in which it could be assessed. However, adults emigrating may be considered simply as adults dying as far as the population in the study area is concerned. The number of bromeliads within the study area was increased to 400. This was achieved by hanging an additional 200 plants of standard size. All plants of the original 200 which had outgrown the standard size limitations were also replaced, and all 400 plants were randomized in position. The study area was considered to be subdivided into 10 contiguous sections of irregular shape, each of which contained 1 or more buttonwood trees whose trunks provided "resting" places for adult Wyeomyia. Within each section 1 or more trees were designated as "collecting stations." These "collecting stations" were not considered to be equal in the sense that it was realized that some would be more "attractive" to mosquitoes than would others, partly because of the different numbers of bromeliads hung from them. For this reason, the time to be spent collecting at each station was not equal, and more time would be spent at the "more productive" than at the "less productive" stations. Direct "between station" comparisons would not be possible in terms of number of mosquitoes collected at each, but attention would be paid to collecting the largest possible number of mosquitoes from the time available, and these mosquitoes would be taken from the whole of the study area.

Record sheets were prepared on which to tabulate each day's captured adults, noting number of males and number of females captured at each station and, for each adult recaptured, its sex and the day(s) on which it had been marked previously. Additional sheets were prepared in order to tabulate an account of the number of adults recaptured 1, 2, 3, etc., days after release. However, the probability of recapture of an adult depends in part upon the day on which it was captured, marked and released because we collected no data for Saturdays and Sun-
days. Thus, in preparing our tabulation, it is obvious that adults captured, marked and released on Mondays are available (subject to mortality and emigration) for recapture on Tuesdays (i.e., after 1 day), on Wednesdays (after 2 days), on Thursdays (after 3 days), on Fridays (after 4 days), but not on Saturdays (after 5 days) or Sundays (after 6 days); they are, however, available for recapture on any of the weekdays the following week. It is evident that all adults marked on any weekday will be available for recapture (subject to mortality and emigration) after any multiple of 7 days and that therefore the total number of adults available for recapture after 7 days (and multiples) will be higher than the total number of adults available for recapture after 6 days. With this limitation of different availability of adults for recapture dependent upon the day on which they were captured, marked and released and dependent upon the time interval being examined, we may only compare percentage recapture (not actual number) of adults between time intervals.

Because adults were to be marked only on weekdays, not at weekends, our 25-color-code system, which could be used to distinguish adults marked on any of 25 different days, would "stretch" over a longer time period; i.e., it could be used to recognize an adult marked as long ago as 5 weeks. It was mentioned above that, at first marking, an adult received 2 paint spots, one anterior and one posterior on the thorax. Adults recaptured were marked again with the color code of the day of recapture, but this time the paint spots were applied one at left and one at right of the thorax. In the case of adults captured twice, and having 4 paint spots on the dorsum of the thorax, a fifth paint spot was applied centrally, the color applied being the second color of the day of marking (i.e., that which would be applied posteriorly at first capture or at right at second capture). Because we never recaptured an adult 3 times we were not faced with the problem of how to mark one so many times recaptured. Even if one were to be recaptured so many times (a) the possibility of its being recaptured yet again would be so remote as not to be worth the trouble of devising a further marking system and (b) its 5-mark color pattern would already have been recorded and recognized as unusual (at 3rd recapture) so that the individual would be recognized at 4th recapture without any additional marks.

The color code system was not designed to detect movement by adults from station to station without the study area, and little in analysis of this movement is possible. Limited analysis can be made only from lack of adults at certain stations on certain days, e.g., if on day x no adults were collected at station 4, yet on day x + 1 or subsequently adults bearing the color code of day x were collected at station 4, then evidently some movement had taken place. On each day adults captured at any given station were marked, then released at the same station.

**Survival of Marked Adults.** Survival of marked adults vs. unmarked adults was determined in two small cage experiments. On 19 July 1976, 20 adults were captured by aspirator from the population outside the laboratory. Every alternate adult was anaesthetized and marked in the normal way, the remainder neither anaesthetized nor marked. All were placed in a cage in the bio-room at 27.5°C, with 80% R.H. and a light cycle of 12 hrs light. Five percent sugar water was supplied in glass vials with cotton wicks. The cage was examined daily and dead adults were removed and recorded. Three of the marked adults were males and survived respectively 4, 20 and 22 days; all other adults were female and their survival times are indicated in Table 1. The difference in survival times between marked and unmarked females in the cage proved not significant.

The test was repeated on 16 November 1976 with 22 wild-caught adult females, 11 of which were anaesthetized and marked, 11 neither anaesthetized nor marked (Table 1). Again there was no significant difference in survival times for marked and unmarked females.

However, we have not yet tested survival...
Table 1. Comparison of survival of anesthetized and marked wild-caught female Wv. 
vanduzei vs. individuals neither anesthetized nor marked in cage tests. The difference in 
survival times between marked and unmarked 
individuals was not significant.

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<thead>
<tr>
<th>Survival times in days</th>
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$t_1 = -0.4959$  
$n.s.$  
$t_2 = -1.443$  
n.s.

of marked vs. unmarked and unanesthetized males in similar tests. Nor have we 
tested marked vs. unmarked adults in the 
presence of possible predators. Since it is 
conceivable that paint spots may make the 
mosquitoes more readily visible to possible 
predators such as lizards, we should investi-
gate this possibility in large-cage tests. 
Paint spots of some colors on the thoraces 
of mosquitoes can be seen clearly by us in 
the study area, without any magnification. 
Various species of lizards, as well as jumping 
spiders are suspected to be predators 
of the mosquitoes in the study area. If the 
presence of paint spots partially negates 
the camouflage of the mosquitoes, then 
predation of the marked mosquitoes may 
be increased.

Results and Discussion. Results of 
continuous sampling (weekends excepted) 
for a period of 42 weeks beginning in Feb-
uary 1976 and ending in November 1976 
are presented in Table 2. During this time 
a total of 5,920 males and 3,836 females 
was collected in the study area, all by aspi-
ration. The percentage of recaptured males after 20 hrs is calculated on the basis that 4,947 males were available for recapture 1 day after capture, mark and release, that 241 were recaptured after this time interval, resulting in a percentage recapture of 4.87%. In contrast, 5,895 males were available for recapture 164 hrs after capture, mark and release, and that 6 were recaptured after this time interval, resulting in a percentage recapture of 0.10%. Only if we were able to operate the program 7 days per week would the number of adults available for recapture be the same after each time interval, subject only to a slight difference at the termination of the program caused by the fact that there must be a finite last day of marking.

In compiling Table 2 we have considered double recaptures in the following way: if an adult was captured, marked and released on day n, recaptured on day n + 1 and recaptured again on day n + 5, then the first time it was recaptured 1 adult was considered to have been recaptured after 1 day, but the second time it was recaptured, 1 adult was considered to have been recaptured after 4 days and 1 after 5 days. In total, we recaptured 765 adults once and only 37 adults twice.

We have calculated exponential regressions of the number of adults (each sex separately) vs. time, using the formula \( y = ae^{xt} \), as has been done in previous studies of mosquitoes (Service 1976). We plotted time in hours along the horizontal (x) axis and the natural logarithm of the percentage of recaptured adults along the vertical (y) axis. The values of the independent variate (x) were given as 20 hrs, 44 hrs, . . . etc. to allow for the fact that the interval of time between release and subsequent first recapture attempt was approximately 20 hrs rather than 1 day, but that subsequent recapture attempts took place at intervals of 24 hrs. We obtained high correlation coefficients of -0.9535 and -0.9330 respectively for the regressions of males and females respectively on time, indicating a good fit to the data points. However, just as in the case of a similar calculated regression by Gillies (1961) of adults of Anopheles gambiae Giles on time, we found that the first 2 data points (for both sexes of adults) lay above the calculated line. Therefore the calculated exponential regressions with slopes of -0.0176 (males) and -0.0108 (females) did not describe the relationship of the percentage recapture of adults to time ideally. Further, the one recaptured adult female of Wv. vonduezii after 20 days appears to represent what has been termed a wild observation (Kruskal 1960) because we have no recaptures of adults after 17, 18 or 19 days and because the calculated slope of the regression line with this value excluded was -0.0128 (instead of -0.0108) with an improved correlation coefficient of -0.9651.

It appears that the calculated slope of the exponential regression for percentage males of Wv. vonduezii recaptured vs. time is steeper than that for females. This suggests that males are shorter-lived than females in nature, but the precise slopes of the lines and thus the mortality rates of Wv. vonduezii adults are not yet determined. This appears not to be the result of inadequacy of data, but because the exponential regressions used for analysis of our data, and apparently that of Gillies (1961), are applied empirically and do not provide an ideal description of the data. For the further analysis of these data we shall attempt to find a formula which will fit the data better. We must also investigate more completely any alteration of longevity caused by marking adults and, if necessary, correct the regression equation in the light of these findings.

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FENTHION RESISTANCE IN Aedes aegypti FROM SELECTION PRESSURE ON LARVAE

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ABSTRACT. Selection pressure applied to larvae of a field population of Aedes aegypti from the Villa Palmeras, Santurce section of San Juan, Puerto Rico resulted in the development of resistance to fenthion in the laboratory. After 9 filial generations of selection pressure, resistance occurred by a factor of 11 compared with the parents, by a factor of 18 compared with the Arecibo, Puerto Rico laboratory strain, and by a factor of 37 compared with the susceptible Fort Detrick laboratory strain. The LC50 values in parts per million were as follows: Fort Detrick laboratory strain, 0.004; Arecibo laboratory strain, 0.008; Villa Palmeras parents, 0.015; and Villa Palmeras selection F10, 0.15.

Previous studies have shown that Aedes aegypti develops resistance to malathion in Puerto Rico (Fox 1973). In the search for a replacement, similar work needs to be done with candidate insecticides. The purpose of this research was to find out whether a Puerto Rican field strain would become resistant to fenthion after selection pressure on the larvae in the laboratory.

MATERIALS AND METHODS. In February, 1974, I collected larvae from water in cement flower vases in the "Cementerio San José" in the Villa Palmeras, Santurce section of the city of San Juan, Puerto Rico, reared them to adults, and designated this colony the "Villa Palmeras" field strain. Offspring of these specimens were used in the experiments. Survivors of the tests were saved for breeding and testing subsequent generations so that selection pressure with fenthion occurred on the parents and each of 9 filial generations. For comparing normal levels of fenthion susceptibility I used 2 laboratory colonies, the Fort Detrick strain and the Arecibo, Puerto Rico laboratory strain. Originally, the Fort Detrick strain came from the Communicable Disease Center, Savannah, Georgia in 1962, and the Arecibo, P.R. strain from specimens collected in 1969. To make the tests, I exposed for 24 hr about 20 4th stage larvae in 250 ml solutions in open half-pint cardboard containers and replicated the tests 4 times. The concentrations used, 0.005, 0.01, 0.025,