FACTORS AFFECTING THE INSECTICIDE SUSCEPTIBILITY OF *AEDES NIGROMACULIS* LARVAE IN LABORATORY TESTS

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ABSTRACT. Several techniques, useful in handling *Aedes nigromaculis* (Ludlow) larvae in susceptibility testing programs, were evaluated: When larvae were reared from eggs which had been stored for 4 months, the average decrease in LC_{50} for several insecticides was 15 percent, compared to field-collected larvae. There was no significant difference in parathion susceptibility between those larvae which had been field-collected at an early age and subsequently lab-reared, and those larvae allowed to develop in the field. When larvae were held for 1 to 3 days at 15° C in order to retard their development, there was a significant but small increase in their fourth instar susceptibility to fenthion and Dursban®, but not to RE11775 or parathion. In general, early instars were more susceptible to the compounds tested than were late instars; resistance was higher in fourth instars than in second instars of an OP-resistant strain. When larval susceptibilities of field populations were measured over a 30-month period, it appeared that, in some cases, there was an increase in LC^{50} to mid-season, followed by a decline.

Aedes nigromaculis (Ludlow), the pasture mosquito, is of major pest and economic importance in the Central Valley of California. Because of its propensity to develop high levels of insecticide resistance, and since present and future ability to control this species is of constant concern, this mosquito is under intensive laboratory study, particularly as regards resistance levels of field populations and efficacy of candidate larvicides.

This paper describes several techniques which are useful in rearing and handling *A. nigromaculis* larvae for use in testing programs, and evaluates the effects of these techniques on larval vigor and consequent susceptibility in larvicide tests. Seasonal changes in the organophosphorus (OP) susceptibilities of several field populations also are reported.

SUSCEPTIBILITY TEST METHODS. Susceptibility tests were conducted as follows: Twenty fourth instar larvae were placed in 100 ml of unchlorinated tap water contained in 4-oz waxed paper cups. Acetone solutions of given larvicides were applied to the water surface; a graded series of duplicated or triplicated concentrations was selected to cover the range of mortality. Treated larvae were held at 21° C water temperature for 24 hours. Larvae which could not move or which behaved abnormally were scored as dead.

The data were analyzed by the approximate comparison method of Wadley (1967). This involved plotting the results of each replication on probit-mortality paper and estimating, by eye, the individual LC_{50} values; the logarithms of these were compared in a factorial analysis of variance as a completely randomized design. Significance was determined at the .05 probability level.

The OP larvicides, fenthion, Dursban® and parathion, and the carbamates, propoxur and RE11775 (m-sec-butylphenyl N-methyl-N-(phenylthio) carbamate) were used. Not all compounds were utilized in every test.

LARVAL REARING. Approximately 300 field-collected *A. nigromaculis* larvae were placed in 3 liters of a mixture of one part field water and one part unchlorinated tap water in an enamel pan. Alber's Spur, a livestock food supplement, was used as larval food; a handful of dry straw was also added to each pan to serve as "browse" for foraging larvae. The water temperature was maintained at 25° C and aeration was provided.

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VIGOR OF LARVAE REARED FROM STORED EGGS

Under proper conditions, *A. nigromaculis* eggs will remain viable for several months. This enables us to field-collect mated female adults, harvest the eggs, and store them for use at a later date. A series of three tests, utilizing OP-resistant populations, was performed to determine whether larvae reared from stored eggs suffered any loss of vigor which would influence their insecticide susceptibility.

METHODS. For each test, larvae and gravid females were collected from the same field. Both stages were not always available at the same time from a given field, but usually only a few days separated the two collections. Since the two stages were at the most only one or two generations apart, their inherent susceptibility levels were considered equal.

Field-collected larvae were lab-reared and tested as fourth instars. Adult females were netted as they approached the collector in search of a blood meal; they were maintained in 1 cubic foot rearing cages, and fed on mature roosters.

The eggs, deposited on wet, black terry cloth placed in the cages, were washed daily into enamel pans and then transferred onto filter paper circles. These "egg papers" were kept at least one week at approximately 23° C and 80 percent relative humidity to allow for embryonation, after which they were transferred to the refrigerator for storage at 4° C and 98 percent relative humidity. Prior to hatching, eggs were "reconditioned" for one week or longer by transferring the egg papers to an area maintained at 23° C and 98 percent relative humidity. Eggs were hatched by immersing the egg papers in water containing a dilute yeast infusion. Larvae were reared in enamel pans; yeast, livestock food pellets, and dry straw made up the rearing medium. Upon reaching the fourth instar the larvae were subjected to the insecticide susceptibility tests; each of the three tests was individually analyzed.

RESULTS. Table 1 shows the mean LC_{50} values for the three tests. Fenthion and Dursban were included in all three tests, while RE11775 and propoxur were each used only once. The analyses revealed that the differences due to the insecticide factor were significant, as expected, in all three tests. Differences due to the "method" factor were not significant in the second Costa test but were in the first Costa test as well as in the Sanchez test. The F ratio was much higher in the Sanchez test ($F=34$) than in the significant Costa test ($F=5.9$).

When larvae are reared from stored eggs, there are two major factors which can affect larval vigor. One factor is due not to egg storage as such, but to that which occurs as a result of the larvae being totally lab-reared. When larvae are collected as early instars and then lab-reared, they are about as vigorous as those allowed to mature in the field. These larvae, however, get a good "start" in the field before

TABLE 1.—Relative susceptibility of field-collected larvae compared to larvae reared from stored eggs—mean LC_{50} values (ppm).

Source	Method	Term of egg storage	Insecticide			
			Fenthion	Dursban	RE11775	Propoxur
Sanchez	Field-collected larvae	....	.020	.01	.....	...
	Larvae reared from stored eggs	6 mo.	.012	.0063	.....	...
Costa	Field-collected larvae	....	.018	.01	.01	...
	Larvae reared from stored eggs	4 mo.	.020	.0093	.0077	...
Costa	Field-collected larvae	....	.020	.014	.....	.26
	Larvae reared from stored eggs	4 mo.	.018	.01	.....	.21

being brought into the laboratory; and this apparently can make a considerable difference in their rearability. The other factor is due directly to storage of the eggs. Those eggs stored for longer periods are sometimes more difficult to rear to the later stages, and larval development is often more variable and mortality is greater. In the case of the Sanchez test, where the increase in susceptibility was the greatest (39 percent average decrease in LC_{50}), the eggs were stored for 6 months, whereas in the other two tests, where there was less susceptibility change (15 percent average decrease in LC_{50}), the eggs had been stored for only four months.

REARING-VIGOR TESTS

Mosquito larvicide susceptibility tests are routinely performed on fourth instar larvae. These larvae may be field-collected at any earlier instar and reared in the laboratory to the fourth instar for testing. Since laboratory-reared insects are often less vigorous than field-collected specimens, it seemed advisable to determine whether or not pasture mosquito larvae, which have been field-collected at an early instar and subsequently reared in the laboratory, suffer any loss of vigor which would affect their level of susceptibility to insecticides.

Methods. Larvae were collected from the same field population on consecutive days, beginning at the second instar. Since this species typically goes through one instar daily at summer temperatures, a series of second, third, and fourth instars was available. Those collected as early larvae spent most of their larval stage under laboratory conditions, while those collected as fourth instars spent practically all of their larval stage under natural conditions. Care was taken to make each daily collection from the same area in a given pasture to eliminate any variations caused by habitat or brood differences. Conveniently, for each test series, larval development in the laboratory always paralleled that in the field so that the series of larval samples reached the fourth instar on the same day, allowing for simultaneous testing of each collection. Parathion was used as the test insecticide. This experiment included larvae from three areas: Costa, a highly OP-resistant population; Morsehead, a susceptible population; and Livingston, a population having a low degree of parathion resistance.

Results. Table 2 shows the mean LC_{50} values of fourth instar *A. nigromaculis* collected as second, third, and fourth instars. The greatest difference in mean LC_{50} values was in the Morsehead test where larvae collected as second instars were most susceptible and those collected as fourths were least susceptible. The opposite trend prevailed in the Costa test where early-collected larvae were least susceptible and those collected as fourths were most susceptible. In the Livingston test those collected as thirds were most susceptible while those collected as fourths were least susceptible. These differences are minor, and reflect expected variations which occur in larvicide tests; they were not statistically significant at the .05 level. Differences due to source effect were significant, as expected, since these populations differ in their resistance levels.

In a study of OP susceptibility levels of *Anopheles freeborni,* Womeldorf *et al.* (1970) compared fourth instar levels of

Table 2.—Relative parathion susceptibility of fourth instar larvae collected at different instars.

Source of larvae	Instar collected	No. of days lab-reared	Fourth instar mean LC_{50} (ppm)
Costa	second	2	.089
	third	1	.080
	fourth	0	.077
Morsehead	second	2	.0025
	third	1	.0034
	fourth	0	.0044
Livingston	second	2	.0033
	third	1	.0030
	fourth	0	.0042

larvae collected as early instars, larvae collected as fourth instars, and lab-reared progeny of field-collected adults. They found that use of the different methods resulted in similar LC_{50} values.

LARVAL STORAGE TESTS

For laboratory insecticide susceptibility tests, pasture mosquito larvae are collected from the field, transported to the laboratory, and reared to the fourth instar at which time they are used; or, if they are already fourth instars when collected, then the tests are run upon arrival at the laboratory. This requires that a technician be available to test the larvae on the day they reach the fourth instar testing stage, and also 24 hours later when post-treatment mortality counts are made. It also limits the number of tests which can be made from a collection since all larvae must be utilized at the same time, assuming that they were all of the same stage when collected, which is usually the case with this floodwater species.

To introduce more flexibility into our larvicide testing regimen by reducing these limitations, a method for "storing" field-collected *A. nigromaculis* larvae at a lowered temperature, to slow their growth rate, has been evaluated. Larvae were field-collected and transported to the laboratory where they were divided into two groups. One group was reared normally at 25° C water temperature; the other group was placed in a cool chamber at 15° C water temperature for one or more days, then allowed to gradually warm to room temperature, after which they were reared to the fourth instar for testing. The parathion tests were run in duplicate, the others in triplicate.

RESULTS. When larvae were held at 15° C, their activity and growth rate were greatly reduced. If storage was limited to a maximum of 2 days, their activity, growth rate, and apparent vigor returned to normal after reacclimatization. When storage was extended beyond 2 days, however, results were variable. In some cases the stored larvae appeared normal while in others the larvae were obviously in a weakened state. In the tests herein described, only vigorous-appearing groups of stored larvae were used.

Susceptibility test results are shown in Table 3. In the fenthion and Dursban tests, the LC_{50} values of the stored larvae were significantly different from those of the unstored larvae, but in the parathion and RE11775 tests, there was no significant difference.

The average decrease in LC_{50} due to larval storage was 19 percent and 18 percent, respectively, for Dursban and fenthion. For the entire series of tests, in-

TABLE 3.—Relative susceptibility of stored and unstored fourth instar larvae—mean LC_{50} values (ppm).

Source	No. days Stored	Instar stored	Fenthion Unstored larvae	Fenthion Stored larvae	Dursban Unstored larvae	Dursban Stored larvae	RE11775 Unstored larvae	RE11775 Stored larvae	Parathion Unstored larvae	Parathion Stored larvae
Tarabini	2	4th	.....	.....	.001	.0074	.....	.....	....	....
Pacheco	2	3rd & 4th	.0016	.0015	.0087	.0082	.0095	.010	....	....
Hahsey	2	2nd & 3rd	.0016	.0013	.0073	.0074	.0099	.0078	....	....
Culp	3	2nd & 3rd	.....	.....	.....	......	.0088	.0099	....	....
Gilbert	2	3rd & 4th	.0015	.0012	.001	.00053	.0067	.0066	....	....
White	2	3rd & 4th	.0068	.0059	.0046	.0036	.0082	.014	....	....
Evans	2	2nd & 3rd	.010	.012	.0073	.0062	.....	.....	....	....
Sanchez	1	2nd	.011	.011	.0054	.0051	.....	.....	.20	.19
Nunez	3	3rd & 4th	.0039	.0032	.0015	.0013	.....	.....	.09	.064
Avila	2		.0068	.0047	.0011	.0020	.....	.....	.034	.032
Total Mean			.0075	.0063	.0030	.0026	.0095	.0093	.084	.074

cluding all four larvicides, the average decrease was 13 percent.

RELATIVE SUSCEPTIBILITY OF INSTARS

Differences in the insecticide susceptibility of larval instars of pasture mosquitoes are of interest from the standpoint of applied larval control as well as in laboratory investigations. In a study comparing the susceptibility of different instars of a laboratory strain of *Culex pipiens quinquefasciatus*, Mulla (1961) reported varying results, depending on the insecticide used. With 9 of 11 insecticides, the first instars were more susceptible than fourth instar larvae, but there was no difference with two insecticides; the second and third instars (mixed) were more susceptible than fourths with four insecticides, but there was no difference with the other seven insecticides.

To determine whether instar susceptibility differences exist in *A. nigromaculis*, a series of tests was performed to compare the response of second, third, and fourth instar larvae. First instar larvae were not utilized since their minute size makes them difficult to collect and handle.

METHODS. Larvae were gathered from a given field starting at the second instar, with collections thereafter as third and fourth instars. Each instar was tested against a series of concentrations for each insecticide. The OP larvicides, fenthion and Dursban, and the experimental carbamate, RE11775, were used. A duplicate series of concentrations was run for the Tarabini tests; for all others each series was triplicated. The LC_{50} values were analyzed in a factorial analysis of variance comparing instar and strain (pasture name) as the two main factors. Separate analyses were performed for each insecticide.

RESULTS. Mean LC_{50} values are shown in Table 4. The two main effects, strain and instar, as well as interaction, were significant for all three insecticides. Duncan's multiple-range test was used to determine significance between instars; and since interaction was significant, this was done for each strain as well as for the total means. For fenthion the total mean LC_{50} was significantly highest for the fourth instar, but second and third instars were not significantly different from each other. However, results of the four individual fenthion tests are not the same. For example, in both Gilbert tests third instar larvae were significantly more susceptible

TABLE 4.—Relative susceptibility of instars—mean LC_{50} values (ppm).[a]

Instar	Pasture				Total mean (avg. effect of instar)
	West Gilbert	East Gilbert	Costa	Tarabini	
			Fenthion		
2nd	.0019[b]	.0015[b]	.0075[a]	.00069[a]	.0021[a]
3rd	.0015[a]	.00094[a]	.015[b]	.00061[a]	.0020[a]
4th	.0019[b]	.0015[b]	.018[c]	.001[b]	.0029[b]
			Dursban		
2nd	.00035[a]	.00041[a]	.0030[a]	.00038[a]	.00067[a]
3rd	.00075[b]	.00076[b]	.0080[b]	.00041[a]	.0013[b]
4th	.00095[c]	.00069[b]	.011[c]	.00064[b]	.0016[c]
			RE11775		
2nd	.0025[a]	.0031[a]	.0043[a]	......	.0032[a]
3rd	.0053[b]	.0066[b]	.0067[b]	......	.0060[b]
4th	.0059[b]	.01[c]	.01[c]	......	.0084[c]

[a] Means followed by a common letter within each cell are not significantly different at the .05 probability level (Duncan's multiple-range test).

than seconds or fourths, and those two did not differ significantly from each other. In the Tarabini test, second and third instars were not different, but both differed significantly from the fourth instar LC_{50}. Only in the Costa test were all three instars significantly different, and their LC_{50} values were correlated with instar.

For Dursban, the total mean values show that, in general, susceptibility was correlated with instar, and there were significant differences between instars. This held true in the individual Costa and West Gilbert tests, but in the Tarabini test the fourth instars were significantly less susceptible than the seconds and thirds, which did not differ significantly from each other.

In the RE11775 tests, the total mean values were significantly different for each instar, and susceptibility was correlated with instar. In the individual tests this was also the case with Costa and East Gilbert. In the West Gilbert test, second instar larvae were most susceptible, but the third and fourth instars were not significantly different.

In the tests involving fenthion and parathion, the OP-resistant Costa strain was more resistant in the fourth instar than in the second instar; that is, the resistance ratio (obtained by dividing Costa LC_{50} by average LC_{50} of the three relatively nonresistant strains) of the second instar Costa larvae was less than that of the fourth instar Costa larvae. This is also indicated when comparing the ratio of the fourth-instar LC_{50} to the second instar LC_{50}; these were higher for the OP-resistant Costa strain than for the three other strains in the fenthion and Dursban tests.

SEASONAL CHANGES IN SUSCEPTIBILITY

In some areas, particularly where insecticide resistance is high, pasture mosquito control failures often occur in late summer. This is attributed to an increase in the resistance level of the population due to selection by intensive control efforts over the span of the breeding season, which may include 18 or more generations (Husbands, 1953). In evaluating candidate larvicides for use in California, their performance against resistant strains is a critical factor. Because *A. nigromaculis* are difficult to rear, field-collected larvae are used for larvicide evaluations; since seasonal changes in resistance levels could affect the apparent toxicity of candidate larvicides, the possibility of such changes was investigated.

To determine the variability in response to insecticides which can be expected within a population of pasture mosquitoes over a period of time, the fourth instar susceptibilities of three separate field populations of mosquitoes to three larvicides were measured over a period of 30 months. The three populations were: Tarabini, a susceptible population associated with several adjacent fields contained in a 1 square mile area located in an uncontrolled area of Madera County; Gilbert, a semi-resistant group of three small neighboring pastures all within a ¾ mile area in eastern Tulare County (an uncontrolled area but these mosquitoes have a low level of parathion resistance); and Costa, an OP-resistant population which has been under intensive control for several years, consisting of four neighboring fields within a 1 square mile area of eastern Kings County. Parathion, Dursban, and fenthion were used.

RESULTS. The LC_{50}'s of each population, plotted against time, are shown in Figure 1. In general the variability of response to fenthion was less than that for Dursban or parathion. With the susceptible Tarabini population, fenthion variability was practically nil over the study period, while it was greater for the semiresistant Gilbert strain and greatest for the resistant Costa strain. For Dursban the general variability was considerably greater than for fenthion, and it exists within each population. The variability to parathion was comparable to that to Dursban.

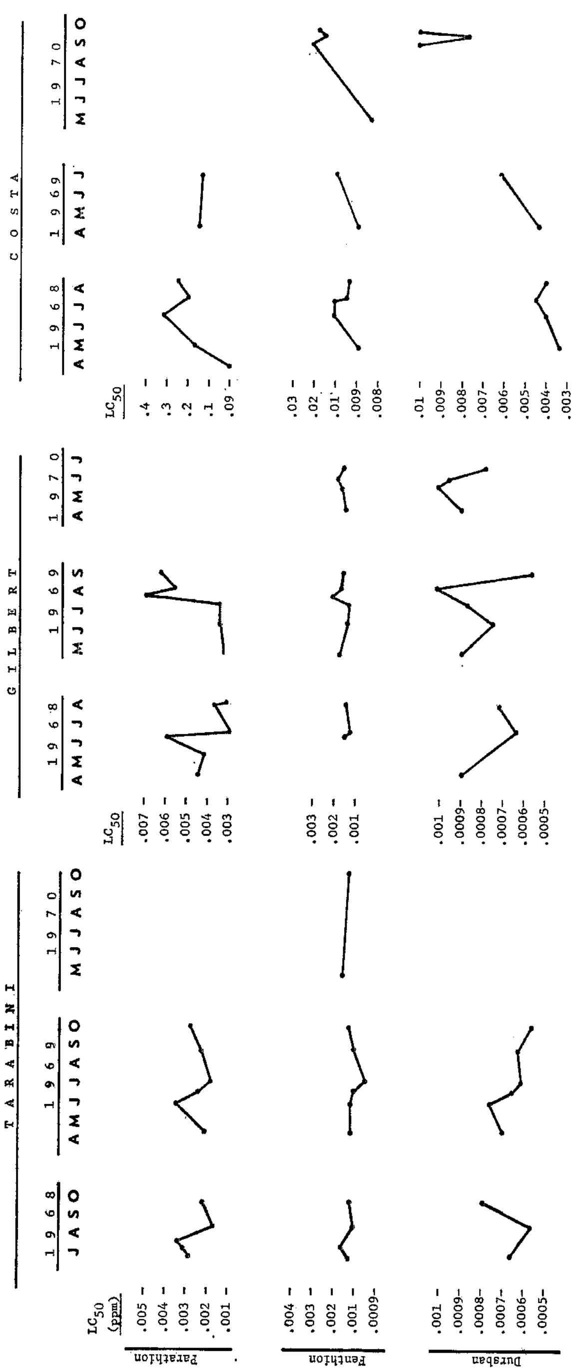

FIG. 1.—LC_{50} values plotted over a 30-month period.

With these limited data, the presence of a seasonal correlation cannot definitely be ascertained; however, a certain trend seems to exist, and this is most apparent with the Costa strain, where in 1968 the tolerance to all three compounds increased to a maximum at mid-season which was followed by a late-season decline. The seasonal direction is very unclear in 1969 and 1970 due to insufficient data; also, resistance to this population was on the increase during this period, especially to Dursban.

The response of the Gilbert population shows a similar pattern in some cases. With parathion in 1968 and 1969 there was a peak of tolerance in mid-summer followed by a decline. This also appears to be the case with Dursban in 1969 and 1970. With the Tarabini population, this trend is not very evident.

In a study of the seasonal susceptibility of larvae from two pastures in Kern County in 1964, Mulla (1968) found a slight decrease of OP tolerance in one pasture and a slight increase in the other. A trend toward progressively higher adult resistance was indicated from the data in another study (Wilder and Schaefer, 1970). In the present study, no trend is evident for the susceptible Tarabini population, but for the Gilbert and Costa populations, there appears to be a seasonal increase in LC_{50} up to mid-season, followed by a late-season decline. If this is the case, it could be due to an ethological factor; normally, adult pasture mosquitoes tend to remain in the vicinity of the breeding area, particularly when field conditions are satisfactory for continued host feeding and oviposition. However, later in the season, adults begin to migrate and are often found in foreign habitats, distant from the breeding area.

It has been suggested that this behavior is responsible for a return to normal resistance levels at the start of the following season (Wilder and Schaefer, 1970). It is possible that this decline in resistance, due to dilution of the resistant genotype by emigrating susceptible adults, would show up, not all at once the following season, but as a decline of resistance in late summer or fall. This does not explain the increased difficulty in controlling larvae as the season progresses, but other factors, such as slower water penetration and excessive vegetative canopy, may be involved (Wilder and Schaefer, *ibid.*).

SUMMARY

LARVAE REARED FROM STORED EGGS. Two of three tests showed significantly greater susceptibility of those larvae which were lab-reared from stored eggs. The greatest decrease in LC_{50} (39 percent) occurred when eggs had been stored for 6 months. In the two tests where eggs had been stored for only 4 months, the average decrease in LC_{50} was only 15 percent. This technique of storing *A. nigromaculis* eggs for lab use during the nonbreeding winter months is quite useful, but storage time should be limited to four months or less.

REARING-VIGOR TESTS. There was no significant susceptibility difference between those larvae which had been field-collected at an early age and subsequently lab-reared and those larvae allowed to complete their development in the field. *A. nigromaculis* larvae can be field-collected at whatever stage they are available, and then lab-reared to the fourth instar without suffering significant loss of vigor.

LARVAL STORAGE TESTS. When larvae were held for 1 to 3 days at 15° C in order to retard their development, there was a significant increase in their fourth instar susceptibility to fenthion and Dursban, but not to RE11775 or parathion. The greatest LC_{50} decrease (19 percent) was in the Dursban test; the average decrease for all tests was 13 percent. This is a useful technique which permits greater utilization of a single collection of pasture mosquito larvae. The loss of vigor experienced in these tests does not impose serious limitations on use of the technique, since a 20 percent or less decrease in LC_{50} would generally be of little consequence.

RELATIVE SUSCEPTIBILITY OF INSTARS. In general, the early instars were more

susceptible, but there was considerable variation between individual tests. Only in the Costa strain were there always significant differences between the LC_{50}'s of the instars, for each of the three insecticides employed. Not only strain, but insecticide, affected relative instar susceptibility. For all four strains tested the total average LC_{50} was correlated with instar for Dursban and RE11775, but for fenthion, the total mean LC_{50} values for second and third instars were not significantly different.

In the two tests involving Op insecticides, the fourth instar OP-resistant Costa larvae were more resistant than the second-instar larvae.

SEASONAL CHANGES IN THE SUSCEPTIBILITY OF *A. nigromaculis*. In some cases there was an apparent increase in LC_{50} to mid-season, followed by a late-season decline. These susceptibility differences amount to a maximum yearly variation of approximately threefold, and therefore could influence conclusions relating to degree of resistance or efficacy of candidate larvicides. However, since this is probably based on genetic, and perhaps nutritional and crowding factors, it is not controllable and we can only be aware that these variations seem to depend on the compound used, and the season.

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CORRECTION

"I would like to have entered in the next number of *Mosquito News* the following: In *Mosquito News* 32(2):161, line 15 of the first paragraph of the Introduction—strike the words 'which were no doubt needed.' " . . . James B. Hoy