

Time in Concentration: A Simple Technique for the Accurate Detection of Resistance to Insecticides in Mosquito Larvae *

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Recently, criticism has been directed at the tests recommended by the WHO Expert Committee on Insecticides for the detection of resistance to insecticides in larval populations of mosquitos—by Doby & Corbeau ^a and Hamon & Garrett-Jones,^b for instance. These criticisms concern the reliability of the tests to detect resistance in a population, especially where the resistance is not pronounced or well developed, and question the actual validity of the results obtained by the WHO Expert Committee method.

Unfortunately, the alternative techniques or modifications suggested by the above authors are complex, and require observations on mortality to be made over a considerably longer period of time than the methods currently recommended by the WHO

highly efficient; in a population containing individuals homozygous for dieldrin-resistance, heterozygous, or homozygous for dieldrin-susceptibility, the phenotype of each individual may be accurately determined. Although the work to date has been primarily restricted to the determination of the genotypes for dieldrin- and gamma-HCH-resistance, preliminary tests indicate that comparable results may be expected using DDT.

The technique herein suggested consists in placing larvae in a single concentration of insecticide for a period of 24 hours. It has been determined that larvae of different genotypes will survive for differential lengths of time in a given concentration (8 ppm) of dieldrin, and that the percentages and curves of mortality reflect accurately the known genotypes

TABLE 1
CUMULATIVE MORTALITY OF LARVAE FROM FIVE FEMALES,
HOMOZYGOUS FOR DIELDRIN-SUSCEPTIBILITY ^a

Exp. No.	No. of larvae	Larvae dead at hour (cumulative totals):								
		2.0	2.25	2.50	2.75	3.0	3.25	3.50	3.75	4.0
S-10	21	0	0	3	7	14	—	—	21	
D-27	24	0	1	10	—	20	22	23	—	24
D-21	28	1	12	17	—	25	27	28		
S-2	30	1	8	16	23	26	29	30		
S-3	27	0	3	5	9	12	20	24	26	27

^a Solution: 8 ppm dieldrin.

Expert Committee. These suggested methods would be especially difficult in field operations.

The present paper presents a simple and reliable alternative technique for the detection of resistance in larval populations. The proposed technique should prove to be adequate for the detection of incipient resistance even at a low level. The test is

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^a Doby, J. M. & Corbeau, J. (1962) *Bull. Wld Hlth Org.*, 27, 189.

^b Hamon, J. & Garrett-Jones, C. (1963) *Bull. Wld Hlth Org.*, 28, 1.

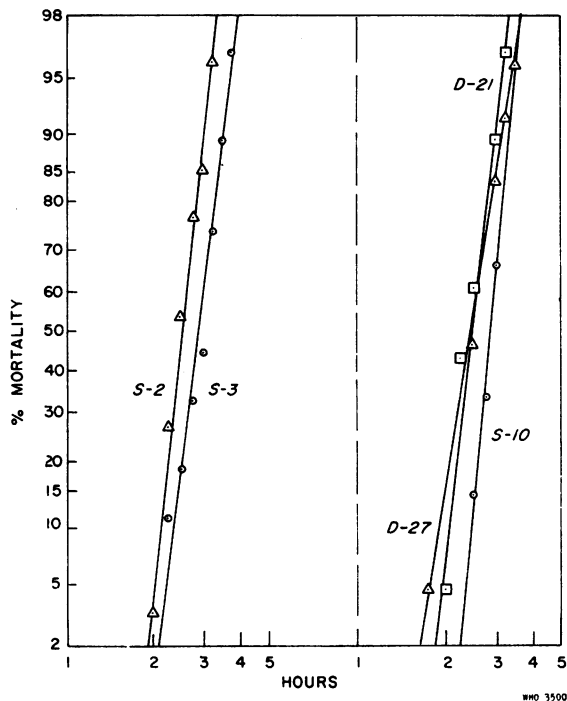
of the animals used in the tests. The descriptions of methods used have been presented elsewhere.^c

Results

Dieldrin tests on the progenies from single females. A group of larvae, homozygous for susceptibility to dieldrin, when placed in a test solution of 8 ppm dieldrin, will all die within a few hours. Data from five representative experiments are shown in Table 1. When these data are plotted

^c French, W. L. & Kitzmiller, J. B. (1963) *Proc. New Jers. Mosq. Exterm. Ass.*, 50, 241.

FIG. 1
PERCENTAGE MORTALITY IN TESTS WITH 8 ppm
DIELDRIN SOLUTION ON *A. QUADRIMACULATUS* LARVAE
FROM FIVE FEMALES, HOMOZYGOUS FOR DIELDRIN-
SUSCEPTIBILITY^a



^a For simplicity, the regression lines have been separated. Each set begins at hour one.

on a log-probit paper as an expression of time in concentration *versus* the cumulative percentage of mortality, the resulting curves are straight lines with steep slopes (Fig. 1).

These data indicate that homozygous susceptible

larvae are rapidly killed in a concentration of 8 ppm dieldrin. The steep slopes of the lines suggest relatively little influence of factors other than the toxic effects of the dieldrin.

Similar 24-hour tests with homozygous resistant larvae show no mortality during this period. In five representative experiments involving 162 larvae, only one larva died, and this one at the very end of the 24-hour period. Clearly, under our experimental conditions, homozygous resistant larvae survive the test for 24 hours.

Heterozygous resistant animals, when treated with dieldrin at 8 ppm, die considerably before the end of the 24-hour period. Representative data are presented in Table 2 for two groups of known heterozygotes; two separate samples of offspring from each of two females were tested at different times. The dual samples demonstrate the excellent reproducibility of the tests. The regression lines for cumulative mortality *versus* time are straight, with steep slopes. It can be seen by comparing the regression lines in Fig. 2 (for heterozygotes) with the regression lines in Fig. 1 (for homozygous susceptibles) that the two phenotypes can be clearly distinguished by the time-in-concentration method. Mortality is complete in the homozygous susceptible animals for some time before mortality first appears in the heterozygotes. The heterozygous resistant animals all die in the test concentration within a few hours and are thus clearly separated from the resistant animals which survive the 24-hour test period.

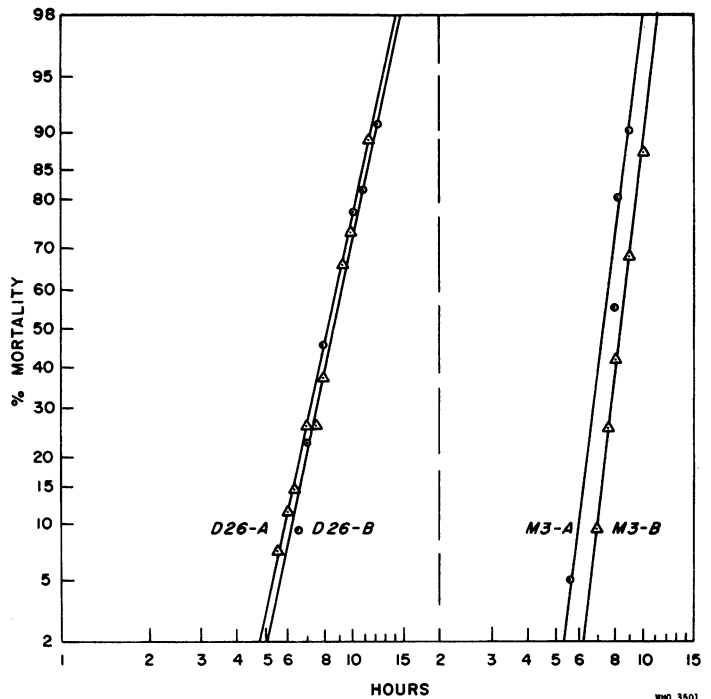
The clear separation of phenotypes can best be demonstrated by an examination of the progeny from crosses involving heterozygous animals. The progeny from such crosses should show the typical (F_2) 1:2:1 genetic ratios if resistance to dieldrin is due to an incompletely dominant gene (Table 3). Fig. 3

TABLE 2
CUMULATIVE MORTALITY OF HETEROZYGOUS LARVAE FROM TWO FEMALES
(TWO SAMPLES FROM EACH FEMALE)^a

Exp. No.	No. of larvae	Larvae dead at hour:																	
		5.5	6	6.5	7	7.5	8	8.25	9	9.25	10	10.25	11	11.25	12.5	14.25	21	22	24
D-26 A (74°F, 23.3°C)	27	2	3	4	7	7	10	—	—	18	20	—	—	24	—	—	—	—	27
D-26 B (74°F, 23.3°C)	22	0	0	2	5	—	10	—	—	—	—	17	18	—	20	22	—	—	—
M-3 A (76.5°F, 24.7°C)	20	1	—	—	—	—	11	16	18	—	—	—	—	—	—	—	—	20	—
M-3 B (74.5°F, 23.6°C)	31	0	0	0	3	8	13	—	21	—	27	—	—	—	—	—	—	—	31

^a Solution: 8 ppm dieldrin.

FIG. 2
PERCENTAGE MORTALITY IN TESTS
WITH 8 ppm DIELDRIN SOLUTION ON
TWO SAMPLES OF HETEROZYGOUS
A. QUADRIMACULATUS LARVAE FROM
EACH OF TWO FEMALES^a



^a Each set begins at hour one.

shows typical F₂ data where the expected phenotypes are sharply distinguished in the proper proportions by absolute plateaux: one between the homozygous susceptible animals and the heterozygotes, and a second plateau identifying the homozygous resistant larvae. It is clear from Fig. 3 that the expected phenotypes are present in classical genetic ratios.

Similar results which clearly show the expected phenotypes are presented in Fig. 4. These data resulted from the testing of progeny of crosses between heterozygotes and homozygous susceptibles (curve 4A) and between heterozygotes and homo-

zygous resistant animals (curve 4B). Typical 1 : 1 ratios are obtained and clear plateaux are present separating or identifying the appropriate genotypes.

Gamma-HCH tests on progenies from single females. In general the results of tests with gamma-HCH at 2.5 ppm closely parallel those with dieldrin. The absolute plateau between the homozygous susceptible animals and the heterozygotes is not present, and this concentration of gamma-HCH will eventually kill the homozygous resistant animals. A pronounced inflexion of the regression line, however,

TABLE 3
CUMULATIVE MORTALITY OF F₂ PROGENIES FROM SINGLE FEMALES^a

Female	No. of larvae	Larvae dead at hour:														
		2.0	2.25	2.5	2.75	3.0	3.25	3.5	3.75	4.5	5.5	8.0	8.25	9.0	21.0	24.0
M-A	179	13	35	39	41	42	42	42	45	63	91	122	—	128	134	134
M-B	96	6	19	25	27	27	27	27	28	33	48	63	65	66	71	71

^a Solution: 8 ppm dieldrin.

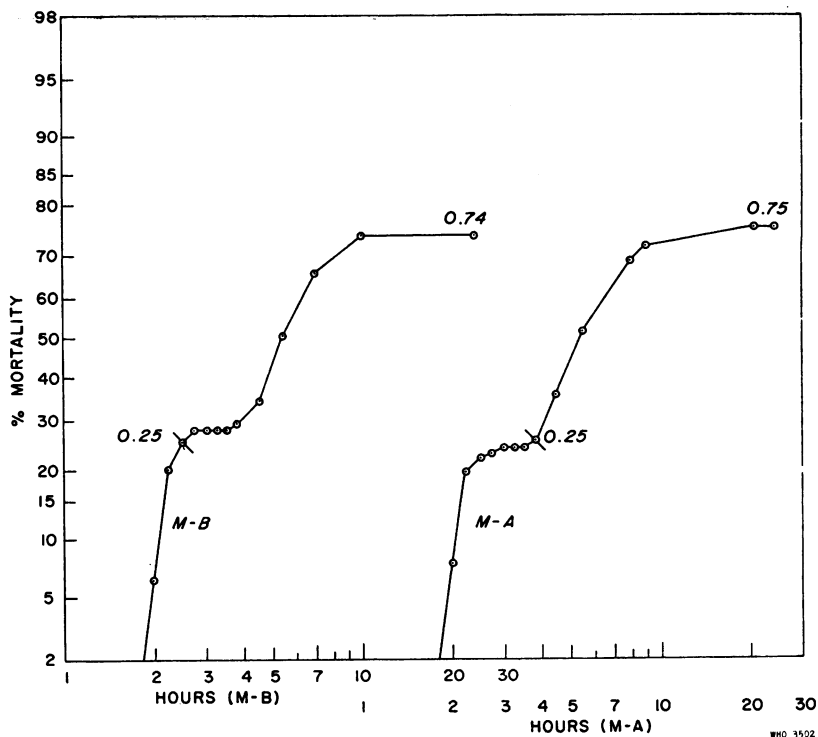


FIG. 3
PERCENTAGE MORTALITY IN
TESTS WITH 8 ppm DIELDRIN
SOLUTION ON F₁ PROGENIES
FROM SINGLE
A. QUADRIMACULATUS
FEMALES^a

^a For clarity, the curves have been separated. Note the overlapping time scales, and the inflexions at 0.25 and 0.75 levels.

clearly indicates the presence of the susceptible and heterozygous genotypes. An absolute plateau distinguishes the heterozygotes from the homozygous resistant individuals. Typical regression lines for homozygous susceptible larvae (curve 5A) and for homozygous resistant animals (curve 5D) are shown in Fig. 5. It should be noted that the homozygous susceptible larvae begin dying immediately and, at 74°F (23.3°C) are all dead within the first hour. The homozygous resistant larvae begin dying considerably later and are all dead at the conclusion of the test period.

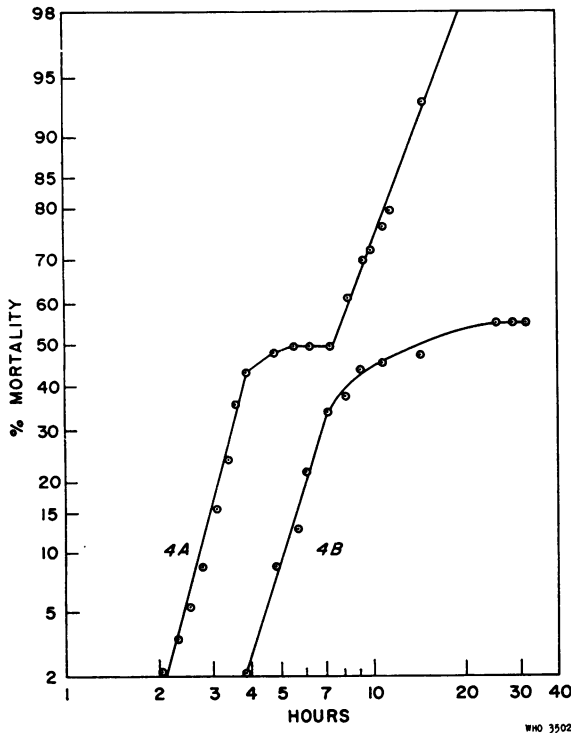
The discrimination between the homozygous susceptible animals and the heterozygotes can best be illustrated by the progeny of a backcross between a heterozygote and a homozygous susceptible. The progeny from such a cross should be approximately one-half heterozygotes and one-half homozygous susceptible animals. The data clearly show the presence of these two genotypes (curve 5B).

In the same way, the distinction between the homozygous resistant larvae and heterozygotes can best be demonstrated by a backcross of a heterozygote and a homozygous resistant animal. The

progeny from such a cross should be one-half heterozygotes and one-half homozygous resistant animals. In this case the heterozygotes are clearly distinguished from the homozygous resistant animals by an extensive plateau (curve 5C).

It can be seen from the data presented in Fig. 1 and Fig. 5 that there is some variability in the response to dieldrin and gamma-HCH among the progenies from different females, even when they are of the same genotype and when tested under identical conditions. This variability in response between families (a family being defined as the progeny obtained from a single female) can be considered to be due to the various environmental and genetic components generally grouped under the collective term "vigour tolerance". Though the present test methods are considered precise enough to study the various components of "vigour tolerance", this aspect of the problem is not of major concern in the present paper. What is of major concern, however, is whether vigour tolerance can be of a sufficient magnitude to make a precise determination of phenotypes impossible. In the study of progeny from single females the only effect of this tolerance

FIG. 4
PERCENTAGE MORTALITY IN TESTS WITH 8 ppm
DIELDRIN SOLUTION ON *A. QUADRIMACULATUS*
BACKCROSSES^a



^a 4A: Heterozygotes \times homozygous dieldrin-susceptibles (Rr \times rr); 4B: Heterozygotes \times homozygous dieldrin-resistants (Rr \times RR).

appears to be a shifting of the positions of the regression lines to a somewhat earlier or later time without any appreciable effect on the slope of the line or on the discrimination between the various genotypes.

Tests of larvae from mass cultures. The larvae used for these tests were reared from eggs taken at random from large stock maintenance cages and were probably, therefore, the progeny of several females. The stocks tested were known, from data obtained during previous tests, to possess the dieldrin-resistant gene. These larvae were reared as stock animals, but were taken from the mass population in the late fourth instar and tested. A random sample might be expected to contain homozygous susceptible, heterozygous, and homozygous resistant animals.

It can be seen from the data presented in Table 4A and Fig. 6 (curve 6B) that for dieldrin-resistance, absolute plateaux separate the phenotypes in the same way as in the progeny from single females.

Data presented in Table 4B and curve 6A show that for gamma-HCH the distinction between the homozygous susceptibles and the heterozygotes is not clear-cut; however, the change in the slope of the regression line clearly indicates the presence of these two phenotypes. The distinction between the heterozygotes and the homozygous resistant animals is clear and unambiguous, with an absolute plateau present.

It is also clear that the effects of vigour tolerance under the given test conditions are not of sufficient magnitude to obscure the accurate determination of phenotypes for dieldrin-resistance of larvae obtained from mass cultures.

To check the possibility that the plateaux might appear at positions which did not clearly and correctly separate the phenotypes, three groups of late fourth instar larvae whose genotypes were known were mixed together in common test vessels. One-third of the larvae were known to be homozygous resistant, one-third were known to be heterozygotes and the final third were known homozygous susceptibles. The results of testing this population of mixed genotypes are given in Table 5 and Fig. 7. These data show that, when mixed together in the test containers, the phenotypes can be clearly and precisely separated and that the phenotype of each larva can be determined. One larva was killed during pre-test handling after the larvae of various genotypes had been placed together. From the data, it can be seen that this animal was homozygous for dieldrin-resistance.

Tests on Anopheles albimanus. The work so far described in this paper has been conducted on *Anopheles quadrimaculatus*. To determine the effectiveness of this technique on another anopheline species, late fourth instar larvae were taken from our stock culture of *Anopheles albimanus* and tested in exactly the same manner as that described for *A. quadrimaculatus*. The results of larval testing of a random sample of mass-cultured *A. albimanus*, known to possess the gene for dieldrin-resistance, are presented in Table 6 and Fig. 8. The regression line shows two plateaux, one clearly distinguishing the homozygous susceptibles from the heterozygotes, and the second identifying the homozygous resistant larvae. It should be noted that other methods of larval testing have been unable to distinguish clearly

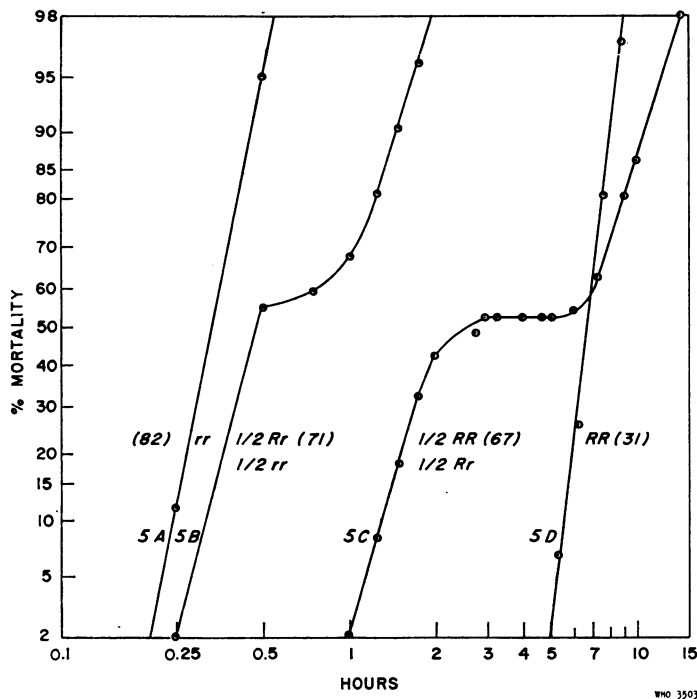


FIG. 5
PERCENTAGE MORTALITY IN TESTS
WITH 2.5 ppm GAMMA-HCH ON
HOMOZYGOUS DIELDRIN-SUSCEPTIBLE
AND DIELDRIN-RESISTANT
A. QUADRIMACULATUS LARVAE
AND BACKCROSSES ^a

^a 5A: Homozygous for dieldrin-susceptibility; 5B: Backcross, heterozygotes × homozygous susceptibles; 5C: Backcross, heterozygotes × homozygous resistants; 5D: Homozygous for dieldrin-resistance. Tests start at hour 0.1.

TABLE 4
CUMULATIVE MORTALITY OF A SAMPLE OF LARVAE FROM STOCKS KNOWN TO CONTAIN
THE DIELDRIN-RESISTANCE GENE

A. Larvae treated with 8 ppm dieldrin solution

No. of larvae	Larvae dead at hour:															
	3	3.25	3.5	3.75	4.0	4.25	4.5	5.0	5.5	6.0	6.5	7.0	7.5	11.0	14.25	24.0
43	13	24	29	32	33	34	34	35	35	35	35	36	36	39	39	39

B. Larvae treated with 2.5 ppm gamma-HCH

No. of larvae	Larvae dead at hour:																					
	0.5	0.75	1.0	1.25	1.5	1.75	2.0	2.25	2.5	3.0	3.25	3.5	3.75	4.0	4.25	4.5	5.0	5.5	6.0	6.5	7.0	11.0
40	6	14	19	24	26	27	32	33	33	33	33	33	34	34	34	36	37	38	38	38	39	40

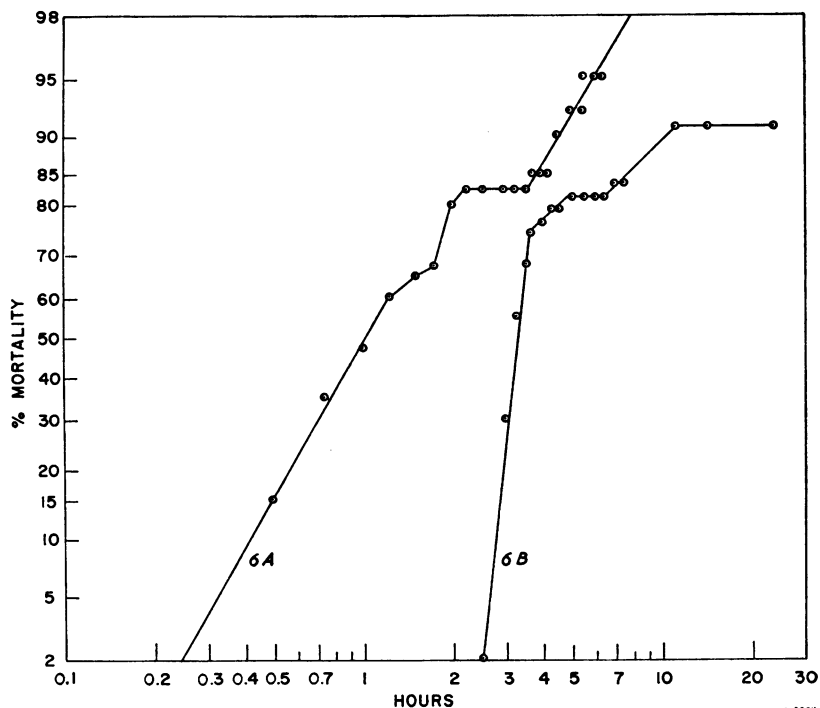
the heterozygotes from the homozygous susceptible larvae.^{d, e} The above data show that with the

^d Rozeboom, L. E. & Johnson, R. (1961) *Amer. J. trop. med. Hyg.*, **10**, 775.

^e Davidson, G. (1963) *Bull. Wld Hlth Org.*, **28**, 25.

proposed test method, the distinction between the heterozygotes and the homozygous dieldrin-susceptibles is clear even in mass populations reared without any of the special attention that is generally given to experimental animals.

FIG. 6
PERCENTAGE MORTALITY IN TESTS WITH 2.5 ppm GAMMA-HCH AND 8 ppm DIELDRIN SOLUTIONS ON A MASS POPULATION OF *A. QUADRIMACULATUS* LARVAE KNOWN TO CONTAIN THE DIELDRIN-RESISTANCE GENE^a



^a 6A: 2.5 ppm gamma-HCH; 6B: 8 ppm dieldrin solution. Both curves show presence of all three genotypes. Note the early action of gamma-HCH.

TABLE 5
CUMULATIVE MORTALITY OF EQUAL NUMBERS OF LARVAE OF THREE KNOWN GENOTYPES^a

No. of larvae	Larvae dead at hour:															
	2.5	3.0	3.25	3.5	4.5	5.5	5.75	6.25	6.5	7.0	8.0	10.25	11.0	12.5	14.25	24.0
65	5	15	22	22	22	22	22	22	24	27	32	39	40	42	44	44

^a Solution: 8 ppm dieldrin.

Preliminary tests with other insecticides. Larvae were removed from the stock cultures of a strain known to be homozygous for dieldrin susceptibility and tested concurrently with dieldrin, heptachlor and heptachlor epoxide, all at 8 ppm. The results of these tests are presented in Table 7 and Fig. 9. These data show that heptachlor and heptachlor epoxide begin to kill the animals more quickly than dieldrin, but show the same general effect in killing susceptible animals within a short time. The straight regression lines indicate the similar effect of all three insecticides upon the susceptible genotype.

Preliminary tests were also undertaken using DDT at 2.5 ppm on a mixed population containing the

gene for dieldrin-resistance. The data are presented in Table 8 and Fig. 10. The straight log-probit regression line indicates a uniformity of response of these animals to this chemical, suggesting that the tested population is genetically uniform with respect to this trait. Although DDT-resistant animals are not present in this laboratory for testing, the reported survival of DDT-resistant larvae for 24 hours at 2.5 ppm DDT^f suggests that the proposed test method would be adequate to determine the genotypes for DDT-resistance.

FIG. 7

PERCENTAGE MORTALITY IN TESTS WITH 8 ppm DIELDRIN SOLUTION ON A KNOWN SAMPLE OF *A. QUADRIMACULATUS* LARVAE, ONE-THIRD DIELDRIN-SUSCEPTIBLE (*rr*), ONE-THIRD HETEROZYGOTES (*Rr*), AND ONE-THIRD HOMOZYGOTES (*RR*)

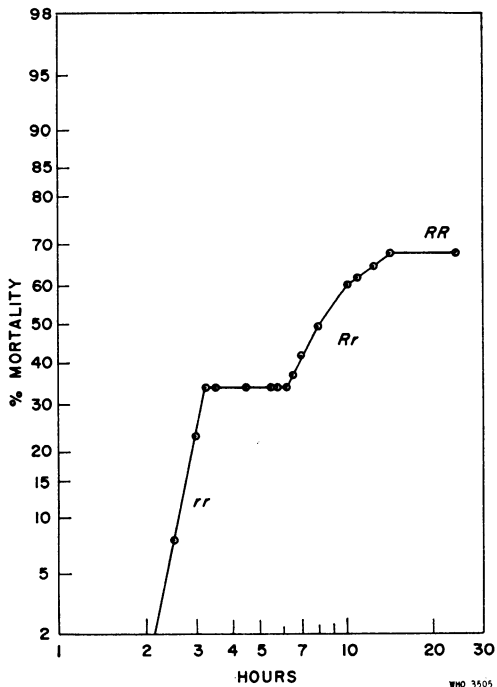
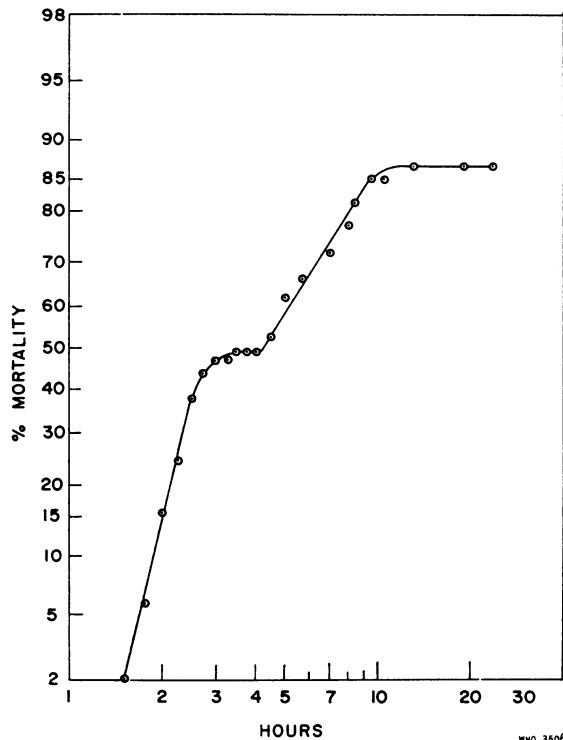


FIG. 8

PERCENTAGE MORTALITY IN TESTS WITH 8 ppm DIELDRIN SOLUTION ON A MASS POPULATION OF *A. ALBIMANUS* LARVAE^a



^a Curve shows presence of all three genotypes.

Discussion

The proposed test method has been designed to investigate the first-order effects of insecticides on the genetic constitution of mosquito larvae. The total final mortality resulting from dieldrin intoxication and the number of larvae which pupate and/or subsequently emerge as viable adults has not been

followed, since much of the subsequent mortality appears to result from secondary environmental effects on larvae weakened by the insecticidal challenge. The immediate mortality as assayed by the proposed test method shows a first order effect of the insecticide and precisely reflects the genetic constitution of the larvae tested.

TABLE 6
CUMULATIVE MORTALITY OF A MIXED POPULATION OF *ANOPHELES ALBIMANUS*^a

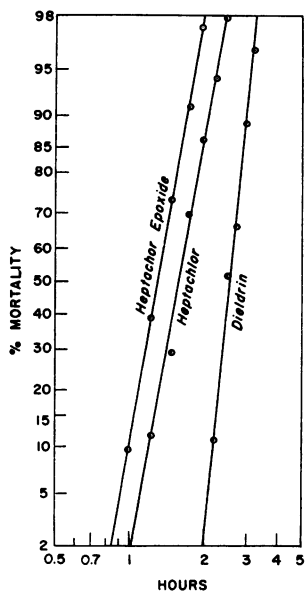
No. of larvae	Larvae dead at hour:																				
	1.5	1.75	2	2.25	2.5	2.75	3	3.25	3.5	3.75	4	4.25	5	5.75	7	8	8.5	9.75	10.5	13	24
53	1	3	8	13	20	23	25	25	26	26	26	28	33	35	38	41	43	45	45	46	46

^a Solution: 8 ppm dieldrin.

TABLE 7
CUMULATIVE MORTALITY OF LARVAE HOMOZYGOUS FOR DIELDRIN-SUSCEPTIBILITY TREATED WITH VARIOUS INSECTICIDES AT 8 ppm

Insecticide	No. of larvae	Larvae dead at hour :										
		1	1.25	1.5	1.75	2.0	2.25	2.5	2.75	3.0	3.25	3.5
Dieldrin	27						3	14	18	24	26	27
Heptachlor	51		6	15	36	44	48	50	51			
Heptachlor epoxide	46	4	18	34	42	45	46					

FIG. 9
PERCENTAGE MORTALITY IN TESTS WITH 8 ppm SOLUTIONS OF HEPTACHLOR EPOXIDE, HEPTACHLOR AND DIELDRIN ON HOMOZYGOUS, DIELDRIN-SUSCEPTIBLE LARVAE OF *A. QUADRIMACULATUS*



Although the technique for larval testing has been described in detail elsewhere, three factors which are of some importance will be stressed at the present time. Jones⁹ showed that in *A. quadrimaculatus* the degree of tolerance to DDT increases as the larvae grow older and that the most pronounced larval tolerance is developed in the late fourth instar. Similar results in this laboratory show that the most clear and precise separation of

the dieldrin phenotypes can be obtained if late fourth instar larvae are used. For clear and unambiguous results, therefore, late fourth instar larvae are recommended as the test animals.

Nutrition is quite important, since only larvae well fed for several hours before testing will uniformly survive the 24-hour test period either as homozygous resistant larvae or as controls.

Perhaps the most crucial factor in this test procedure is the proper determination of mortality. To determine an accurate "mortality end-point" (the stage of intoxication from which no larva recovers) it is necessary to cause the larvae to dive to the bottom of the test container. The larvae are then excited, and those which respond by gross body tremors or by undulatory, snakelike, in-place movements are classified as moribund. Such larvae never regain the surface when pipetted into fresh water, and never survive.

Most of our tests have been carried out with larvae. Preliminary and incomplete results indicate that the time-in-concentration method may also be of value in the detection of resistance in adults.

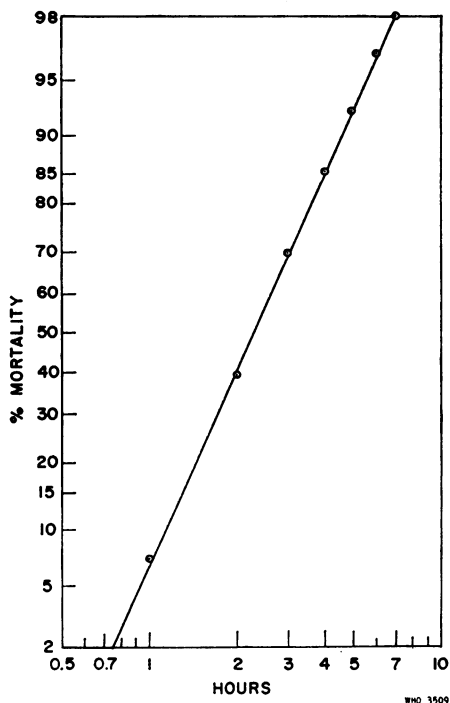
There are several advantages to the larval test method herein proposed. It is simple in that only one test concentration is required and mortality is easily determined. It is efficient in that the phenotype

TABLE 8
CUMULATIVE MORTALITY OF A MIXED POPULATION OF LARVAE TREATED WITH DDT AT 2.5 ppm

No. of larvae	Larvae dead at hour :							
	1	2	3	4	5	6	7	8
144	10	56	101	123	133	139	143	144

⁹ Jones, J. C. (1957) *Mosquito News*, 17, 1.

FIG. 10
 PERCENTAGE MORTALITY IN TESTS WITH 2.5 ppm DDT
 SOLUTION ON A MASS POPULATION OF *A. QUADRI-
 MACULATUS* LARVAE KNOWN TO CONTAIN
 THE DIELDRIN-RESISTANCE GENE^a



^a Regression line indicates genetically uniform population with respect to DDT genotype.

of each individual larva in the test series can be accurately determined. This efficiency, which can give one determination for each larva, may be compared with other test methods which give one determination for every 20-25 larvae. With the increased efficiency of the proposed test method, the log-probit regression lines can be determined much more accurately because of the increased number of points specifying their locations. Or, on the other hand, the positions and slopes of the regression lines and even phenotypes, can be accurately determined with a considerably reduced number of larvae.

In assaying the susceptibility of larval populations, it is an extremely rapid method, in that a test using dieldrin on a susceptible population is complete with accurate and precise data within four or five hours. Since mosquito larvae that are resistant to dieldrin also show a cross-resistance to gamma-HCH, the latter insecticide has been used in this laboratory to screen populations for dieldrin-resistance phenotypes. Tests with gamma-HCH on susceptible populations give accurate and complete data in less than one hour with this method.

It has been possible to detect resistance to the dieldrin group of insecticides in a population of *Culex restuans* collected locally (unpublished data). These animals were tested in the same way as described for the anophelines. Heptachlor (8 ppm) was used as the screening insecticide. This chemical acts more slowly than gamma-HCH but more rapidly than dieldrin. Within one hour after the initiation of the test, it was obvious that individuals carrying genes for dieldrin-resistance were present in the *Culex* population. The regression line showed a sharp inflexion, indicating the presence of heterozygotes, and later, an absolute plateau, indicating the presence of homozygous resistance. Further tests with dieldrin are expected to give precise gene frequencies. The test, therefore, can be extremely rapid for screening susceptible populations, and at the same time very precise when exact gene frequencies are required, for example, when resistance has been discovered in a population.

The tests are reproducible in that replicate tests run at different times on the same population and at the same temperature give log-probit regression lines that are essentially identical.

Furthermore, the tests are reliable in that the results obtained from testing various populations follow very accurately the best theoretical genetic expectations, and can distinguish with certainty the various phenotypes of every larva in a mixed population.

Finally, the tests are relatively independent of the factors generally grouped under the collective term "vigour tolerance". Vigour tolerance does not obscure the genetic results, but simply shifts somewhat the position of the regression lines without appreciably altering the slope.