ARTICLES

EFFECT OF CERTAIN CHANGES IN TESTING TECHNIQUE ON MORTALITY OF MOSQUITO LARVAE [1]

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The use of mosquito larvae in laboratory tests to compare toxicity of chemicals and to assay resistance has focused attention on the variation in mortality obtained with different testing techniques. The available data do not completely account for this variation. Kruse *et al.* (1952) concluded from tests against *Anopheles quadrimaculatus* Say that the number of larvae, the volume of liquid per test, the mass-surface area per larva, the formulation, and the type of surface of the container will influence the results obtained with DDT. Hawkins and Kearns (1956) reported on the ionic environment and zeta potential in relation to suspension stability of DDT. Hawkins (1956) reported that the volume of ethanol solvent used to prepare suspensions of DDT in water affected the mortality of *quadrimaculatus* in tests in which the solvent content was varied by diluting a stock suspension rather than preparing new suspensions at each concentration. In tests with acetone-water suspensions or solutions of three radioactive insecticides, Schmidt and Weidhaas (1958) showed that increasing the number of *quadrimaculatus* larvae per test decreased the mortality and the absorbed dosage of the insecticide in tests with DDT, but not with Bayer 21/199 or Am. Cyanamid 12880. They concluded that the importance of this factor varies with the chemical used and the percentage removed by the larvae.

Experiments were conducted at Orlando, Fla., to determine the effect of the more obvious variables on the mortality of laboratory-reared larvae of *quadrimaculatus* and/or *Aedes taeniorhynchus* (Wied.) when exposed to acetone-water suspensions of DDT. The variables studied with both species were susceptibility of different instars and volume of test suspension. Those studied with *quadrimaculatus* only were quantity of acetone per test container, method of preparing DDT suspensions, and feeding or starving larvae prior to test.

TEST METHODS. The variables were introduced one at a time into the standard test method used at Orlando, and comparisons were made with that method. In the Orlando method, with laboratory-reared mosquitoes, larvae are removed from the rearing pans on the day of testing after they have fed 3 to 4 hours on fresh food and are pooled in clean water. Twenty-five early fourth instar larvae are placed in 25 ml. of distilled water in a 50-ml. beaker. Suspensions are prepared by pipetting an appropriate amount of an acetone solution of the material to be tested (in these tests DDT) into 225 ml. of distilled water in a glass jar and stirring. The water containing the larvae is poured into the test suspension; this is done 2 to 3 hours after the removal of the larvae from the rearing pans and 4 hours after preparation of the test suspension.

In the tests here reported duplicate jars or pans of five or six concentrations of DDT ranging from 0.0010 to 0.050 p.p.m. for *quadrimaculatus* and 0.0050 to 0.10 p.p.m. for *taeniorhynchus* were used. Each test was replicated three times. There would have been less variability in mortality results if all tests had been run

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TABLE 1.—Susceptibility of different instars of *Anopheles quadrimaculatus* and *Aedes taeniorhynchus* larvae to acetone-water suspensions of DDT

Instar	24-hour exposure			48-hour exposure		
	LC–50 (p.p.m.)	LC–90 (p.p.m.)	Slope	LC–50 (p.p.m.)	LC–90 (p.p.m.)	Slope
			Anopheles quadrimaculatus			
Second	0.0021±.0005	0.0048±.0006	3.58±.55	—[a]	—[a]	—[a]
Third	.0033±.0003	.0068±.0009	4.35±.45	0.0022±.0002	0.0049±.0003	3.58±.19
Fourth	.0052±.0003	.0110±.0010	3.98±.33	.0042±.0005	.0110±.0009	3.33±.24
			Aedes taeniorhynchus			
Second	0.0027±.0002	0.0055±.0003	4.19±.25	0.0026±.0003	0.0051±.0004	4.35±.36
Third	.025 ±.0029	.059 ±.0090	3.61±.24	.0084±.0005	.024 ±.0035	3.16±.34
Fourth	.047 ±.005	—[b]	1.33±.33	.036 ±.0085	—[b]	1.45±.26

[a] Percent mortality too high for analysis.
[b] Percent mortality too low to obtain a valid LC–90.

TABLE 2.—Toxicity of acetone-water suspensions of DDT to mosquito larvae in different test volumes and containers. (250 ml. in glass jars, 1,000 ml. in enamel pans.)

Test volume (milliliters)	24-hour exposure			48-hour exposure		
	LC–50 (p.p.m.)	LC–90 (p.p.m.)	Slope	LC–50 (p.p.m.)	LC–90 (p.p.m.)	Slope
			Anopheles quadrimaculatus			
250	0.0079±.0008	0.015±.002	4.43±.19	0.0046±.0007	0.010±.0019	3.90±.44
1,000	.0088±.0013	.019±.002	4.16±.02	.0063±.0009	.016±.0018	3.28±.31
			Aedes taeniorhynchus			
250	0.038±.004	0.163±.028	2.20±.25	0.013±.006	0.040±.010	2.85±.29
1,000	.021±.004	.076±.012	2.35±.23	.014±.002	.035±.004	3.42±.32

on one batch of larvae, but these replicates were run on different days with different batches to approximate the variability that would be inherent in the use of this method for resistance studies. Mortality readings were taken at 24 and 48 hours, and the LC–50, LC–90, and slope were calculated. Since mortalities were sometimes too low at 24 hours or too high at 48 hours, the calculations were based on three to five significant concentrations.

In the first series of tests this method was used to determine the susceptibility of second, third, and fourth instar larvae. A second series was run to compare the mortality obtained in 1 liter of suspension in enamel pans with that in 250 ml. in glass jars. The pans were 8½ inches in diameter and the water was 1⅛ inches deep, whereas the jars were 3 inches in diameter and the water was 2⅛ inches deep. In a third series larvae were exposed by the standard method except that sufficient acetone was added to each jar to give a total of 1, 2, and 5 ml. of acetone. Since some acetone may have evaporated during the 4 hours between preparation of the suspensions and introduction of the larvae, this test was repeated and the larvae were introduced immediately after preparation of the suspensions. A comparison also was made of the effect of adding various concentrations of DDT in a uniform volume of acetone (1.25 ml.) and of adding the same concentrations in various volumes of acetone (0.31 to 1.25 ml.) as is done in the standard method. Finally, the susceptibility of larvae fed up to the time of testing was compared with that of others starved for 4 hours.

SUSCEPTIBILITY OF DIFFERENT INSTARS. With both species the susceptibility to DDT decreased with the age of the larvae, as shown in table 1. With *quadrimaculatus* the LC–50 and LC–90 increased 1.6 and 1.4 times from the second to the third instar after 24 hours and 1.6 times from the third to the fourth instar after 24 hours and 1.9 to 2.2 times after 48 hours. With *taeniorhynchus* the respective increases from the second to the third instar were 9.3 to 10.7 times at 24 hours and 3.2 to 4.7 times at 48 hours, and from the third to the fourth instars 1.9 times at 24 hours and 4.3 times at 48 hours. The *taeniorhynchus* larvae, which were from a colony resistant to DDT, showed a much greater increase between the second and fourth instars than did *quadrimaculatus*. After 24 hours second instar larvae of both species were approximately equal in susceptibility, whereas the LC–50 of fourth instar *taeniorhynchus* was about nine times that for fourth instar *quadrimaculatus*. With *quadrimaculatus* the slopes of the regression lines do not show significant differences between instars; however, with *taeniorhynchus* they are significantly flatter for each succeeding instar.

VOLUME OF TEST SUSPENSION. The results with 250 ml. of suspension in glass jars compared with those with 1 liter in enamel pans are given in table 2. With *quadrimaculatus* the LC–50 and LC–90 were not significantly different at 24 hours, but at 48 hours they were significantly lower with 250 ml. With *taeniorhynchus* the LC–50 and LC–90 were significantly lower at 24 hours with 1 liter, but at 48 hours there were no significant differences. Since the total available DDT per larva was four times as great in the tests with 1 liter as in those with 250 ml., it can be concluded that the total available DDT was not the single factor controlling mortality.

QUANTITY OF ACETONE. The results of tests in which the content of acetone was varied are given in table 3. There was no significant difference in mortality in suspensions containing 1, 2, or 5 ml. of acetone when larvae were added immediately after the acetone and when they were added after 4 hours the only significant difference occurred in the LC–90 with 5 ml. of acetone.

METHOD OF PREPARING DDT SUSPENSIONS. The results of tests to compare two methods of preparing a range of DDT suspensions are given in table 4. When the concentration of DDT in the acetone was kept constant, and different volumes

TABLE 3.—Effect of different volumes of acetone on the toxicity of DDT to fourth-instar larvae of *Anopheles quadrimaculatus*

Volume of acetone (ml.)	24-hour exposure			48-hour exposure		
	LC–50 (p.p.m.)	LC–90 (p.p.m.)	Slope	LC–50 (p.p.m.)	LC–90 (p.p.m.)	Slope
			Larvae introduced 4 hours later			
1	0.0052±.0007	0.013 ±.0030	3.67±.40	0.0029±.0004	0.011 ±.0011	2.69± .47
2	.0052±.0005	.010 ±.0010	4.71±.45	.0036±.0007	.0092±.0037	3.34± .43
5	.0044±.0004	.0075±.0010	5.86±.42	.0027±.0003	.0066±.0007	3.37± .27
			Larvae introduced immediately			
1	.0055±.0002	.011 ±.0013	4.58±.81	.0029±.0006	.0089±.0011	2.56± .16
2	.0059±.0006	.012 ±.0018	4.54±.63	.0027±.0006	.0086±.0017	2.53± .13
5	.0049±.0005	.010 ±.0009	4.02±.08	.0036±.0012	.0066±.0019	3.79±1.28

TABLE 4.—Comparison of effects of two methods of preparing DDT suspensions on toxicity to fourth-instar *Anopheles quadrimaculatus* larvae

Method of preparation	24-hour exposure			48-hour exposure		
	LC–50 (p.p.m.)	LC–90 (p.p.m.)	Slope	LC–50 (p.p.m.)	LC–90 (p.p.m.)	Slope
Varying volume of acetone, constant concentration	0.0051±.0003	0.0098±.0010	4.76±.32	0.0034±.0003	0.0081±.0008	3.45±.20
Constant volume of acetone, varying concentration	.0067±.0008	.013 ±.002	4.60±.30	.0048±.0003	.010 ±.0012	3.96±.59

TABLE 5.—Toxicity of acetone-water suspensions of DDT to fourth-instar *Anopheles quadrimaculatus* larvae when they were starved for 4 hours or fed up to the time of testing

	24-hour exposure			48-hour exposure		
	LC–50 (p.p.m.)	LC–90 (p.p.m.)	Slope	LC–50 (p.p.m.)	LC–90 (p.p.m.)	Slope
Fed	0.0065±.0013	0.013±.0010	4.31±.24	0.0044±.0010	0.010±.0007	3.72±.55
Unfed	0.0065±.0008	0.012±.0010	4.38±.56	0.0047±.0005	0.011±.0010	3.74±.39

of acetone were added to the water, the LC–50 and LC–90 were not as high as when the volume of acetone was kept constant and the concentration varied. However, the slopes of the regression lines were not significantly different, and the LC–50 and LC–90 obtained at a constant volume were no higher than those in some other tests with varying volumes, such as those given in the first line of table 2. It is therefore concluded that when acetone is used as the solvent the two methods give equal results.

EFFECT OF FEEDING LARVAE. As shown in table 5, there was no significant difference in mortality between larvae fed up to the time of testing and those starved for 4 hours.

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THE STRATIFICATION OF MOSQUITOES

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INTRODUCTION. As part of studies on the bionomics of mosquitoes in southwestern Georgia, collections were made at six elevations from 3 feet to 50 feet in an effort to determine the extent of natural stratification among mosquito species.

Previous studies on the vertical distribution of mosquitoes have been made in this country (MacCreary, 1941; Gjullin *et al.*, 1950; Snow, 1955) and in other parts of the world (Davis, 1944; Bates, 1944; Haddow *et al.*, 1945 a and b, 1947, 1948, 1949; Garnham *et al.*, 1946; Kumm *et al.*, 1946, 1951; Galindo *et al.*, 1951), but in these investigations attractants were employed. Studies conducted in this area of Georgia on the stratification of mosquitoes at various times throughout the night as measured by light traps are reported elsewhere (Love *et al.*, manuscript). The present report gives the results of mosquito collections made without attractants.

MATERIALS AND METHODS. From May 1954 to July 1956 collections of mosquitoes were made one night each week with mechanical sweeping devices at elevations of 3-, 6-, 15-, 25-, 40-, and 50-foot elevations in a wooded area adjacent to a breed-