ARTICLES

A NEW STANDARD FOR THE RAPID DETECTION OF DDT TOLERANCE IN *ANOPHELES QUADRIMACULATUS* LARVAE AND PUPAE

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This paper is concerned with defining the levels of tolerance to DDT in larvae and pupae of known sexes, stadial ages, and nutritional and rearing status of a laboratory-reared strain of *Anopheles quadrimaculatus,* as measured by a simple exposure test. This information is compared with data on randomly collected larvae reared under uniform conditions. These data furnish a simple method and a new standard for rapidly and accurately assessing acquired tolerance to DDT. The primary advantage of the test described is that it permits repetitive estimations on the same population. Experiments now in progress indicate that the method can be adapted for other insecticides.

METHODS. The LTD strain of *Anopheles quadrimaculatus* Say (Diptera, Culicidae) was used for all studies. This strain has been bred in the laboratory at the National Institutes of Health for approximately 10 years. For most of this time the mosquitoes were reared under unstandardized conditions. The strain was never knowingly exposed to DDT or any other toxicant.

1. *Rearing conditions.* One hundred newly hatched larvae were counted into each of 4 enamel photographic trays (8″ x 16″ x 2″) and fed daily on finely ground dogfood and brewer's U.S.P. yeast (1:1) until ready for use.

Two hundred twenty pupae were isolated daily for subsequent emergence as adults within 1-cubic-foot, copper-wire-screened, wooden cages. Those remaining were used for non-breeding purposes. Adults were given continuous access to 4 percent dextrose. Four-day-old females were given one hour offerings of male guinea pig blood six days a week until they were discarded at the end of two weeks. Eggs were collected daily from a series of cages, and newly hatched larvae selected usually from a single egg dish.

The insectary was held at 25 ± 2° C. and 70% ± 5% relative humidity.

2. *Sexing and aging of material.* Fourth-stage larvae of known ages were obtained by selecting immediately after their molt from the third stage while the head capsule was still undarkened. They were sexed with 100 percent accuracy by simple microscopic examination of their imaginal antennal discs as previously described by the author in another paper (1957). Male larvae begin to molt to the fourth stage predominantly during the morning on the seventh day after egg deposition. Females molt mainly in the afternoon and evening of that day. Fourth instars of known ages and sex will be referred to hereafter as "regulated" larvae. Those larvae whose sex was not determined and whose ages were only approximately known (± 1 day) will be referred to as "random" larvae.

Third-stage larvae of unknown chronological ages were sexed by differences in external pigmentation as described by Jones (in press), black larvae being about 90 percent females, and light pigmented larvae being 70 to 90 percent males.

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Unless stated otherwise, larvae had access to abundant food prior to and immediately following tests at all times.

Pupae were sexed by examination of their external genitalia. Since pupations occur throughout a day, pupae were picked hourly for precise determinations of age.

The ages of regulated fourth-stage larvae and pupae used in this study are accurate to ± 30 minutes. Each regulated animal was briefly examined microscopically to make sure that it appeared externally normal. Those with any observable abnormality were discarded. Only brief macroscopic observations were made on the appearance of random larvae.

3. *Preparation of the toxicant.* Pure, recrystallized, p,p'DDT was made up in a 1 percent stock solution in pure acetone (Fisher, certified reagent). For each test the stock was syringed directly into distilled water to give a final concentration of 1:10,000.[2] This concentration was employed, because it was desirable to reduce the exposure time as much as possible and still compare larval and pupal tolerance levels. Suspensions were used in preference to emulsions because larvae did not feed on suspensions and the DDT could be more easily washed off treated specimens.

DDT suspensions were never more than 30 minutes old at the beginning of any test. Microscopic examination of such suspensions showed extremely uniform particles which were evenly distributed.

4. *Testing procedure.* Larvae and pupae were rinsed once in distilled water just before placing single individuals in 30 ml. glass beakers containing 10 ml. of DDT 10^{-4}. From 5 to 10 specimens were used in a given test. Larvae were kept under fasting conditions only during the exposure to DDT and subsequent washing. Depending upon their age, larvae were exposed 1, 2½, 5, 10, 15, 20, 30, 45, 60, and 75 minutes. Pupae were exposed 30, 45, 60, 120, 180, and 360 minutes. Exposure times are accurate to ± 2 to 5 seconds. From 5 to 10 replicates were made at each exposure time for each age and sex. After exposure, all animals used in a test were rapidly pooled in a liter of distilled water and given an additional rinse in another liter of water before placing them individually in numbered, 50 ml. glass beakers containing food and water. The condition of the larvae and pupae was checked at 2 hours and daily thereafter until they died, pupated, or emerged, individual records being kept for each animal. Successful emergence terminated the tests and served as check on the accuracy of sexing. Approximately 5,000 specimens were used in this study. All tests were performed in a special insectary held at 25 ± 2° C. and 70 ± 5 percent relative humidity by a single operator.

5. *Statistical procedures.* MLT's, standard errors, 95 percent confidence limits, regression coefficients of probit fit were determined by Karber's method (Finney, 1947).

The data obtained in this study fitted an arithmetical better than a log probit model. The MLT's given are somewhat higher than they would be with a log scale; nevertheless, the statistical relationships are not changed in any important way.

RESULTS. 1. *Data on "regulated" material.* About 87 percent of those larvae which survive 24 hours after exposure to DDT 10^{-4} are able to pupate and successfully emerge as adults as shown in Table 1. The percentage tends to increase for the older fourth instars (trend at the 0.007 level), but there is no trend with length of exposure. The important fact in these data is that an accurate measure of adult survival can be calculated from the 24-hour readings alone.

In tolerance studies, the primary concern is with the number of organisms able to survive a given stress. The data in Table 2 present the percentages of larvae and pupae of different ages and sexes able to survive 24 hours and/or capable

[2] Equals 100 p.p.m.

TABLE 1.—Observed proportions of *Anopheles quadrimaculatus* larvae surviving 24 hours after DDT exposure which were able to pupate and successfully emerge as adults

Sex and age in 4th stage when exposed		% 24-hour survivors able to pupate and emerge as adults Minutes exposed to DDT									
		1*	2-1/2*	5*	10*	15*	20*	30*	45*	60*	Combined times
1 hr.	♂	77.8	82.1	85.7							80.8
	♀	80.0	83.9	55.5							77.8
24 hr.	♂	78.1	89.3	89.3	94.1	72.7	87.0				84.8
	♀	92.9	96.8	96.3	88.2	75.0	95.8				89.7
48 hr.	♂				93.5	96.7	90.5	75.0			92.2
	♀				94.6	100.0	92.6	70.6	100		92.4
70 hr.	♂							96.6	100	85.7	96.2
	♀							100.0	100	100	100.0
											87.0

TABLE 2.—Mean data on percentage of DDT-exposed *Anopheles* surviving at 24 hours and percentage able to develop into adults.

Stage and age when tested	Point of survival	Minutes exposed to DDT 10^{-4}											
		1'		2-1/2'		5'		10'		15'		20'	
		♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Third stage	24 hr S 1/	92.0	94.2	97.1	97.5	85.0	92.3	63.4	74.6	44.4	53.6	28.6	26.0
	Adult 2/	81.3	87.5	94.3	90.0	75.0	87.2	73.2	59.3	33.3	48.7	22.4	15.0
Fourth stage													
1 hr. old	24 S	97.3	83.3	77.8	96.8	28.6	33.3	2.6	5.6				
	Adult	75.7	66.7	63.9	81.3	24.5	18.2	2.6	2.7				
24 hr. old	24 S	97.0	96.6	100.0	100.0	90.3	75.0	97.1	81.0	60.0	90.9	51.1	63.2
	Adult	75.7	89.7	89.3	96.8	80.6	72.2	91.5	71.4	43.6	68.2	44.4	60.5
48 hr. old	24 S							98.9		90.9	87.5	75.0	87.1
	Adult							92.0	89.7	87.9	87.5	67.9	80.6

1/ Percent surviving 24 hours after exposure.

2/ Percent surviving to adult stage.

Table 2.—(Continued)

Stage and age when tested	Point of survival	Minutes exposed to DDT 10^{-4}													
		30'		45'		60'		75'		120'		180'		360'	
		♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Third stage	24 hr S[1]	.7	.7												
	Adult[2]	0	.7												
Fourth stage															
24 hr. old	24 S	5.4	9.1	0	0										
	Adult	3.6	9.0	0	0										
48 hr. old	24 S	28.6	54.8	7.5	23.7	1.1	18.2								
	Adult	21.4	38.7	8.7	23.7	1.1	7.7								
70 hr. old	24 S	82.9	93.8	29.3	48.4	9.9	29.0	6.8	0						
	Adult	80.0	93.8	29.3	48.4	8.5	29.0	6.8	0						
Pupal stage															
1 hr. old	Adult	68.2	91.7	64.0	72.9	52.5	44.1			12.0	30.0	2.9	5.6	2.2	0
24 hr. old	Adult	91.0	97.4	94.6	78.6	75.8	65.9			70.4	63.4	32.5	72.9	13.2	12.5

1/ Percent surviving 24 hours after exposure.

2/ Percent surviving to adult stage.

TABLE 3

Stage and/or age when exposed	Median lethal time of exposure to DDT 10^{-4}, with 95% confidence limits							
	In 24 hours				Before adult emergence			
	♂		♀		♂		♀	
	MLT	95% limits	MLT	95% limits	MLT	95% limits	MLT	95% limits
3rd stage	14	13-16	16	14-17	13	12-15	12	11-13
4th stage								
1 hour	4.5	3.7-5.3	5	4.2-5.8	3.8	3.1-4.4	3.8	3.3-4.2
24 hour	19.5	17-21	21	19-23	17	15-19	19	17-21
48 hour	27.5	25-30	36	32-40	26	23-28	32	28-36
70 hour	42	39-45	48	44-52	43	40-45	48	44-52
Pupal stage								
1 hour	-	-	-	-	76	74-79	87	84-89
24 hour	-	-	-	-	184	181-187	220	216-223

of emerging as adults after exposure to fresh DDT acetone suspensions.

From the data in Table 2, median lethal times of exposure (MLT) (in minutes) with 95 percent confidence limits were calculated as found in Table 3. The standard errors multiplied by the square root of n (n = number of larvae in a test series at each exposure time) and probit line data for the MLT's are given in Table 4.

there is a four- to fivefold increase within 24 hours in the fourth stadium and that as these larvae grow older they progressively increase in their ability to withstand DDT exposures until just before pupation they have developed a nine- to twelvefold increase in tolerance. As soon as they pupate, there is an additional 1.8 fold increase and within 24 hours some pupae are able to withstand a 6-hour exposure to DDT 10^{-4}. Once pupation occurs, the

TABLE 4.—Further calculations on 24-hour MLT data (see Tables 2 and 3)

Stage	SE $\sqrt{n-1}$ (n = number in group)		Probit line			
			intercept		slope of line	
	♂	♀	♂	♀	♂	♀
3rd stage	5.058	4.122	5.55	5.67	.038	.043
4th stage						
1 hour	2.522	2.187	6.83	6.77	.409	.354
24 hour	5.883	5.480	7.41	7.16	.124	.103
48 hour	7.904	11.109	7.53	7.02	.092	.056
70 hour	10.154	10.564	9.10	8.45	.097	.071

One of the major facts brought out in these tables is that the sex of the larvae does not significantly affect the tests. In other words, male and female larvae of the same ages are essentially equal in their responses to DDT exposure tests.

Relative tolerance levels of different preimaginal stages of *Anopheles quadrimaculatus* to DDT 10^{-4} may now be estimated from Table 3 so that an over-all picture may be obtained. By comparing the MLT's of 1-hour-old fourth instars with others in that stadium and with other stages, it may be seen in Table 5 that females are significantly more tolerant than males, but the percentage sexual differences do *not* grow significantly more pronounced with age of the pupae.

The data that have been presented provide a uniform and simple standard for detecting with considerable accuracy the levels of DDT tolerance in larvae and pupae of *Anopheles quadrimaculatus*. Levels outside the 95 percent confidence levels given in Table 3 would constitute significant alterations in tolerance to DDT.

2. *Data on random larvae.* It seemed especially desirable to perform these tests

TABLE 5.—Relative DDT tolerances in larval and pupal stages of *Anopheles quadrimaculatus*

Stage	Degrees of tolerance relative to 1-hour old fourth stage larvae			
	♂		♀	
	at 24 hrs.	at adult emergence	at 24 hrs.	at adult emergence
3rd larval	3.1	3.4	3.2	3.2
4th larval				
24 hours	4.2	4.5	4.2	5.0
48 hours	6.0	6.8	7.2	8.4
70 hours	9.3	11.3	9.6	12.6
Pupal stage				
1 hour	...	20.0	...	22.9
24 hours	...	48.4	...	57.9

with randomly selected larvae (grown under uniform conditions) whose ages were only roughly known (± 1 day) and whose sex was not determined, for then exposure testing might well be valuable in practical field tests for measuring tolerance in preimaginal stages of *Anopheles* mosquitoes.

On the eighth and ninth day after egg deposition, fourth instars of undetermined sexes were subjected to the exposure test and their MLT's calculated. These determinations were corrected by Abbott's formula for the percentage normally failing to pupate. The MLT for 8-day-old random larvae (approximately 12-hour-old fourth instars) is approximately 13 minutes (95 percent confidence limits 10–16). This value matches reasonably well the mean MLT of 10.9 minutes obtained from 1- to 24-hour-old fourth instars in Table 3. The MLT for 9-day-old random larvae (approximately 36-hour-old fourth instars) is 27 minutes (95 percent limits 23–30), which compares favorably with the mean MLT of 23.5 of 24- to 48-hour-old fourth instars of Table 3.

Two preliminary tests were made with 8- and 9-day-old random fourth instars to see whether feeding and overcrowding during exposure to DDT would affect the MLT. As Table 6 shows, neither of these factors significantly alters this value.

TABLE 6.—MLT's when 8- and 9-day-old random larvae were either fed or overcrowded during DDT exposure tests

Treatment		MLT minutes	95% confidence limits
8-day-old	Feeding during exposure	11	8–14
	Fasting during exposure	13	10–16
9-day-old	Crowded (5/beaker)	23	22–26
	Not crowded (1/beaker)	27	23–30

To determine what effect starvation would have, newly molted fourth-stage larvae of known sexes were made to fast for 24 hours and were then exposed individually to DDT. Twenty-four-hour-old starved larvae had strikingly lowered tolerances (MLT of 3 minutes, 95 percent limits 1.2–4.8). Starvation thus reduced tolerance levels to that of 1-hour-old fourth instars.

DISCUSSION. The data presented show (1) that DDT tolerance in *Anopheles* larvae reaches its lowest level after the molt, (2) that as larvae grow within the fourth stadium these levels significantly rise day by day, (3) that sexual differences in the larval stages are of no significance, (4) that newly metamorphosed pupae are approximately twice as tolerant to DDT as the most tolerant larvae, (5) that significant sexual differences appear first in the pupal stage, and (6) that while pupal tolerance also significantly increases with age, the differences between sexes do not grow greater.

The DDT exposure test described in this paper is rapid, easy to perform, requires a minimum of equipment, and can be used to provide laboratory standards of considerably wider range and greater accuracy than are now available with continuous exposure testing methods. Tests on random larvae reared under uniform conditions give sufficiently rapid and accurate data to suggest that it may have value in practical field testing for tolerance. The test has the special advantage of permitting as many as 6 laboratory determinations and at least 4 field determinations on the same population.

SUMMARY. 1. A simple exposure test is described for rapidly and accurately measuring DDT tolerance in larvae and pupae of *Anopheles quadrimaculatus*. This technique permits as many as 6 laboratory and 4 field determinations of resistance on a given population and may have considerable practical importance.

2. Different levels of tolerance to DDT in third and fourth stage larvae and pupae of *A. quadrimaculatus* are defined according to exact stadium age and sex.

3. Newly molted fourth-stage larvae obtain lethal doses of DDT significantly more rapidly than either third or older fourth instars.

4. A nine- to twelvefold increase in DDT tolerance occurs during the fourth stadium.

5. With pupation there is a further increase in tolerance and 1-day-old pupae are 48 to 58 times more tolerant than 1-hour-old fourth instars.

6. Female and male larvae of the same chronological ages have essentially equal levels of DDT tolerance, but once pupation occurs females are significantly more tolerant than males.

7. Valid data may be obtained with randomly collected larvae provided their stadium ages are known to roughly 1 day.

8. Tests on random larvae indicate that feeding or overcrowding during exposure testing do not significantly alter results. Starvation of larvae for 24 hours greatly reduces their tolerance.

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