THE EFFECTS ON MOSQUITOES OF SUBLETHAL EXPOSURE TO INSECTICIDES. I. DDT, DIELDRIN, MALATHION AND THE BASAL FOLLICLES OF *AEDES AEGYPTI* (L.)

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In contrast to the great segment of research dealing with insecticide mode of action and resistance, little attention has been given to the effect of sublethal amounts of insecticides on insects. Research on this aspect should indicate not only important side-effects on insect survivors but also, by using the insect as an indicator organism, possible effects on non-target organisms within the insecticide-treated environment. The purpose of the present study is to determine the effect on the mosquito, which, in the process of reinfestation concomitant with the disappearance of efficient insecticide residues under field conditions, is exposed to sublethal amounts of insecticides.

The term "sublethal" can be given to any dosage of insecticide which does not cause mortality. However, since effects will generally, but not always, be proportional to dosage, it is necessary to further define the sublethal level and its units of expression in order that the measurement of an observed effect be meaningful. Since insecticides vary in toxicity, it is reasonable that sublethal dosages be expressed with reference to some measurable lethal level. Some authors have termed certain lethal levels as also sublethal (Knutson, 1955; Kuipers, 1962). The LC50 per unit time, for example, is also sublethal to 50 percent of the population for that time. However, effects observed in survivors at the LC50 level may not be due to the insecticide but instead may be metabolically related to or be the cause for survival. For example, the increased progeny from adult *Drosophila* surviving (66–99% mortality) dieldrin exposure (Knutson, 1955) is probably due not directly to the insecticide but only indirectly through selection of survivors having a longer life span and consequently producing more eggs. Conceivably the increased amounts of yolk associated with increased numbers of eggs might serve in the survivors as innocuous storage sites for DDT. Therefore, although sublethal effects may be observed in survivors of levels toxic to a proportion of the population, studies on the effect of sublethal dosages must be based on levels sublethal to the entire population.

Fractions of the LC50, e.g. 0.1 LC50, might serve as means of expressing dosages at the truly sublethal levels. However, the slopes of the dosage mortality regression lines vary between insecticides, and 0.1 LC50 of an insecticide with a steep regression line would be more sublethal than that of an insecticide with a flat regression line. This would also be true to a lesser degree with LC30 and LC10 values. While the use of the LC0 value would eliminate the influence of the slope of the regression line, this value cannot be extrapolated from the line. There remains the intermediate value LC0.1 which is extrapolatable, minimally influenced by the regression slope and, therefore, appropriate as a base for expressing units in the sublethal range. Since the LC0.1 is close to

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the LCo, it can be termed more suitably for sublethal studies as the sublethal maximum, SL max, and be defined as the maximum concentration at which there occurs mortality no greater than 0.1 percent above that experienced in control populations. With units based on SL max, levels below this can be expressed as fractions thereof, e.g. 0.5 SL max. On this basis comparisons between insecticides and their sublethal effects can be made. The measurement of sublethal dosages is, of course, dependent upon length of exposure (continuous, discontinuous, periodic, etc.) and other factors influencing toxicity.

MATERIALS AND METHODS. The strain of *Aedes aegypti* was obtained from Dr. Trager of the Rockefeller Institute in Princeton, New Jersey in 1936. In 1942 it was strengthened by sample populations from the U.S.D.A. strain at Orlando, Florida. Since that time during a period of over twenty years the strain has not been subjected to selection by any insecticide. Toxicological research on the strain has been reported elsewhere (Sutherland, 1964).

The method for exposing larvae to sublethal amounts of insecticide was a modification of the World Health Organization method for insecticide resistance assay (Brown, 1958). One milliliter of the insecticide solution in 95 percent ethanol was added under the water surface (225 ml. distilled water in 250 ml. Pyrex low-form beakers), and mixing was accomplished by blowing air through pipette while stirring. Twenty-five mosquito larvae, 3 to 4 hours after hatching, in 25 ml. of water were added. Across the mouth of the beaker was placed a strip of nylon screen (1.5 x 17 cm.) to which was pinned a dental wick (4 cm.) to be moistened subsequently at adult emergence with 15 percent sucrose solution. Pint plastic bags were inverted over the mouth of the beaker and held in place by elastic bands. A temperature of 30.5° C. was maintained throughout the experiments.

On hatching and again on the third day of larval life, 0.2 ml. of a suspension of finely ground laboratory food (less than 177 microns, 0.12 gm./ml. water) was added to each beaker. Addition of food or sucrose was made through the plastic bag by means of a syringe. Mortality, pupation and emergence were recorded at approximately 12-hour intervals. At 8 to 12 hours after emergence, female adults were transferred to fresh plastic bags containing a cotton wick saturated with 15 percent sucrose solution. At 72 to 96 hours after emergence they were frozen and subsequently examined for numbers of basal follicles.

The numbers of basal follicles (termed "the ovariole number" by van den Heuvel, 1963) were determined in the following manner in 0.9 isotonic saline. The intersegmental membrane anterior to the penultimate abdominal segment was cut, the posterior segments held firmly, and the anterior of the body pulled away thereby exposing the ovaries. The ovaries were transferred to a microscope slide, adhering tissues were removed, and a portion of a cover slip affixed. In this manner the ovaries were flattened, and the basal follicle number (BFN) was determined. The BFN is numerically equal to the "ovariole number" provided each ovariole contains a basal follicle. If one or more ovarioles is underdeveloped or rudimentary and lacks a basal follicle, these numbers are not equal.

RESULTS AND DISCUSSION. Based on the susceptibility of three-day-old larvae to the insecticides (Table 1), newly hatched larvae were reared in DDT or dieldrin concentrations of 0.00025, 0.0005, 0.001 and 0.002 p.p.m. Malathion concentrations included 0.0125, 0.01, 0.008, 0.004, and 0.002 p.p.m. The SL max for DDT, dieldrin, and malathion were 0.001, 0.001, and 0.0125 p.p.m. respectively, based on survival through the larval and pupal stages, complete emergence and mating. At present it is not known if these values apply to adult longevity.

Although subtle effects on growth and

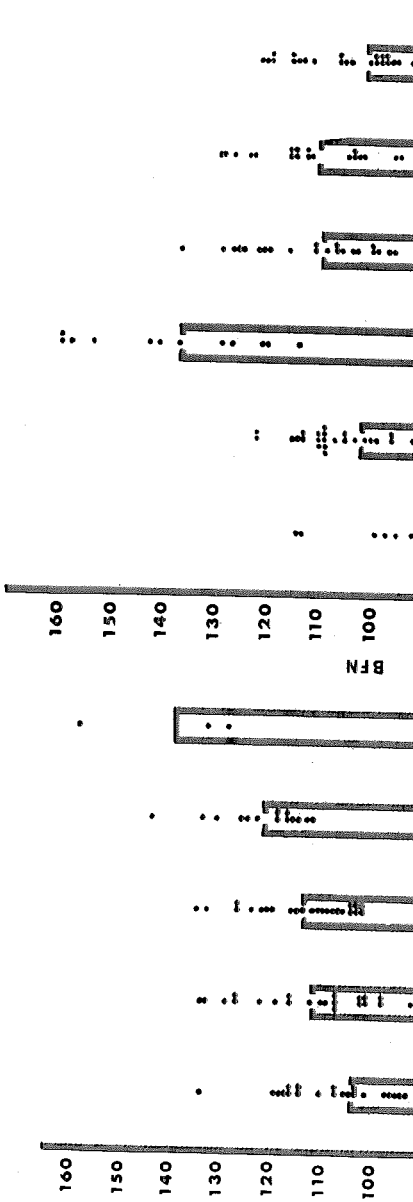

FIG. 1.—The BFN of female mosquitoes treated in the first larval instar with various concentrations of dieldrin. (C= control).

FIG. 2.—The BFN of female mosquitoes treated in the first larval instar with various concentrations of ethanol, DDT, and dieldrin.

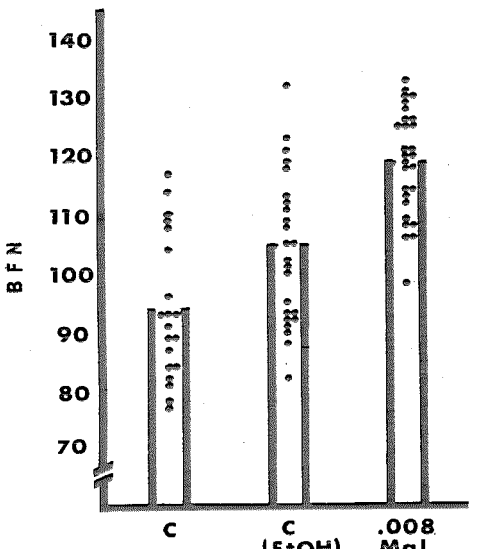

FIG. 3.—The BFN of female mosquitoes treated in the first larval instar with ethanol and malathion.

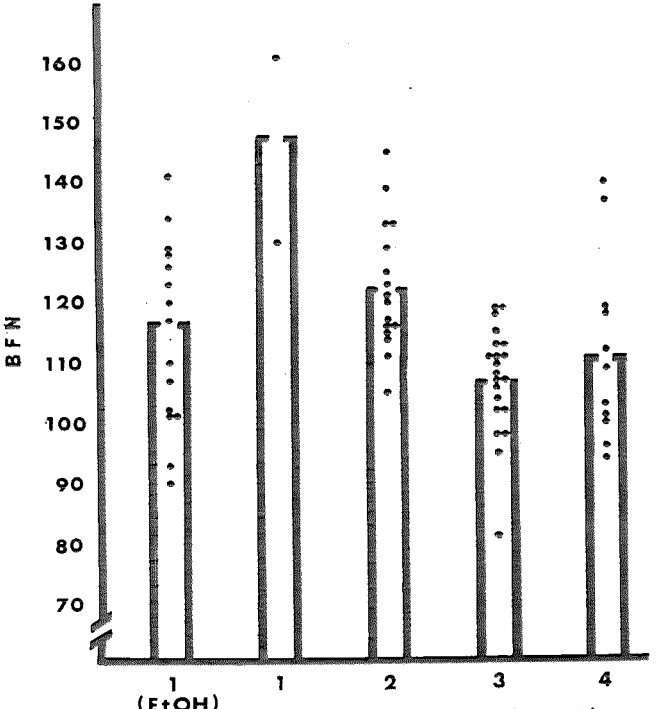

FIG. 4.—The BFN of female mosquitoes treated with DDT on various days of larval life.

Table 1.—Toxicity of insecticides to 3-day-old *Aedes aegypti* (L.) larvae, in p.p.m.

	24-hour		48-hour	
	LC50	LC90	LC50	LC90
DDT	0.0052	0.0128	0.0033	0.0090
Dieldrin	0.0042	0.0082	0.0028	0.0058
Malathion	0.05–0.1	......	......	......

rate of development were noted, the most marked effect due to sublethal dosages of insecticides involved the basal follicle number (BFN). In preliminary experiments the BFN at DDT SL max (0.001 p.p.m.) was increased 9 percent. Similar results were obtained with dieldrin (Fig. 1), the average of individual BFN being indicated by the top of each bar graph. The increase in BFN at the dieldrin SL max (0.001 p.p.m.) was approximately 14 percent, at 0.75 SL max approximately 7 percent. Because BFN was apparently not proportional to the sublethal dose and both DDT and dieldrin affected the BFN, additional experiments were conducted in which the effect of ethanol, the dispersing medium for the insecticide, was investigated (Fig. 2). Increases in BFN in groups treated with ethanol, DDT 0.001, DDT 0.0075, dieldrin 0.001, dieldrin 0.0075 p.p.m. were 19, 59, 27, 29, and 19 percent respectively above controls receiving no treatment. Ethanol, therefore, is also a contributor to the effect on BFN. In this series of experiments (Fig. 2) DDT at 0.001 p.p.m. caused 33 percent mortality by the third day and the SL max lies between this concentration and 0.0075 p.p.m. The distribution of BFN values at 0.001 p.p.m. is approximately equal to that in other groups and there is no indication that at the LC33 there has been selection of survivors having higher BFN. Although this and other experiments have confirmed that increased BFN occurs in survivors of dosages lethal to a proportion of the population, apparently the effect is greatly reduced or nonexistent at 0.5 SL max and below. In the case of dieldrin at 0.75 SL max (0.0075 p.p.m.), (Fig. 2), the BFN is approximately equal to that of control groups receiving ethanol treatment alone. This lower limit of a sublethal effect relative to SL max probably varies with each insecticide and possibly is related to the slope of the dosage mortality regression line. This may be the explanation of a 12 percent increase in BFN over ethanol treated groups at 0.64 SL max malathion (0.008 p.p.m.) as shown in Fig. 3. However, in each population of mosquitoes employed in the experiments, even though from the same strain, the BFN was variable, e.g. untreated and ethanol control groups of Fig. 2 as compared to those of Fig. 3. Therefore, any small differences between insecticides and their ability to increase BFN at the sublethal level must await further series of experiments.

Additional experiments were conducted to determine the stage in larval period when the gonads or prospective gonads directly or indirectly receive the stimulus for a greater BFN. Normally the ovaries develop slowly during the larval stages and follicles start to appear in the fourth instar (Christophers, 1960). When 0.001 p.p.m. DDT was added at various times after egg hatch (Fig. 4), addition on day 2, 3, or 4 had little or no effect on BFN. In this experiment survival of larvae receiving 0.001 p.p.m. DDT on day 1 was low and it is concluded that in this experiment this dosage was above SL max. However, limited observations at this level reflected the increase in BFN observed in previous experiments (Fig. 2) at this approximate dosage level. Although the results indicate that susceptibility to stimuli for an increased BFN exists in the first and second larval instars, the absence of the effect

TABLE 2.—Effects of lethal and/or sublethal levels of insecticide on egg production of various species.

Insect	Insecticide	Stage Treated	Dosage Level	Effect on Egg Production	Reference
Drosophila melanogaster	arsenic	adult & larva	0.001%, L?	reduced brood size	Mann, 1923
	DDT	adult & larva	SL, 5 p.p.m.	none	Kalina, 1950
	dieldrin	adult	LC 66–99	increased	Knutson, 1953
Calandra oryzae	pyrethrins	adult & larva	SL, 1.5 p.p.m.	none	Chadwick, 1962
			low L, 3 p.p.m.	reduced	
Musca domestica	DDT	adult	L	reduced; abnormal oogenesis	Lineva, 1962
		adult	SL	reduced; suppression of oviposition	Beard, 1965
		larva	SL	reduced	Anderson and Sutherland, 1966 (unpub.)
Photophormia terrae-novae	DDT	adult	SL	reduced; abnormal oogenesis	Derbeneva-Uhova and Drobozina, 1965
Glossina palpalis	DDT	adult?	SL?	reduced	Baldry, 1964 cited by Beard, 1965
Aedes aegypti	dieldrin	adult	L, 0.0075 μg	reduced	Duncan, 1963
			SL, 0.0016 μg	none	
Tribolium confusum	DDT	adult	L	reduced	Loschiavo, 1955
Leptinotarsa decemlineata	DDT	adult	SL, 6.7% mort.	increased	Kuipers, 1962

in third and fourth instar larvae subjected to 0.001 p.p.m. on day 3 or 4 does not mean that the latter stages are not susceptible but that this concentration (0.001 p.p.m.) is below their SL max. Additional experiments have shown that the SL max for third instar larvae is approximately 0.003 p.p.m. DDT, at which level there is a 20 percent increase in BFN. It is not known if the fourth instar is also susceptible at its SL max. This may be too late in larval development since follicles may appear as early as the fourth instar in this species (Christophers, 1960).

Factors other than chemicals can also induce increased BFN. According to van den Heuvel (1963), dry body weight and "ovariole number" (=BFN) per individual are generally inversely proportional to the larval habitat temperature. Since tissues other than ovaries contribute to an increase in body weight associated with decreased temperature, the significance of increased BFN can be overlooked if it is based on dry body weight. The increase in BFN per individual and also in weight of other tissues probably is due to lower temperatures reducing the rate of metamorphosis more than the rate of cellular growth. In the case of sublethal doses of pesticides, dry body weight has not been determined; however, the adults are only slightly if at all larger than the controls, and generally the rate of development is little affected. It appears that the effect of pesticides and ethanol is more specific for gonadal tissues than others. Therefore, while temperature and pesticides can induce a similar result in BFN, their methods of induction may differ. Pesticides possibly indirectly influence BFN by their effect on the larval hormonal system.

There are two possible explanations for increased BFN. The stimulus either (1) affects the gonads before or at a time when the number of ovarioles normally is determined, or (2) causes maturation of so-called rudimentary ovarioles which normally do not mature or contain a basal follicle. Experiments with larvae exposed in the first instar to SL max and subsequently fractional SL max in the following instars favor the first explanation, although the second explanation would be acceptable if the pesticide stimulus is received, recorded, and the effect is exerted at a later time. Increased BFN with treatment initiated in the third instar, at a time when some early follicular formation has occurred (Christophers, 1960), supports the second explanation. In contrast to some insect species with a fixed ovariole number (Snodgrass, 1935), the mosquito ovariole number as indicated by BFN is variable under controlled rearing conditions, and such variation at least partially may be due to physiological factors affecting maturation of ovarioles.

The apparent effect of sublethal and lethal levels of insecticide on egg production of various insects is given in Table 2. Although the effect generally seems to be one of reduced egg production, caution should be used in the interpretation of results between lethal versus sublethal and adult versus larval treatment. In some studies, e.g., *Drosophila* and arsenic (Mann, 1923), reduced brood size based on emerging adults cannot be equated with reduced egg production since the treatment may have been toxic to a portion of the larvae. In other cases where lethal levels are employed, reduced egg production is to be expected if it is based on the entire treated population, including those dead, dying, and surviving. Possible increased production can only be detected by examining the individual survivor as in the case of *Drosophila* and DDT (Knutson, 1955), although such increase may be due to selection. At levels sublethal to the population, an effect on egg production would depend on several factors. The effect would be minimal or nonexistent if (1) the dosage level is too low, (2) the adult is treated after processes toward egg production reach a stage where they are little affected, and (3) the gonads are less susceptible to toxicants or their stimuli than more vital organs. Reduced

egg production would be expected if the gonads are more sensitive to toxicants than other vital organs, e.g. chemosterilants, or if the neuromuscular system of the genitalia is paralyzed, e.g. pyrethrins (Tenhet, 1947; Parkin, 1961). Conceivably any effect may be species dependent, which might explain the differences between *Musca domestica* found by Anderson and Sutherland in 1966 (unpublished experiments) and *Aedes aegypti* reared from early larval stages with sublethal amounts of DDT. However, in this particular case *Musca* larvae were exposed continuously to approximately the same DDT level whereas in the method used for *Aedes* larvae, DDT is probably rapidly reduced in concentration by adsorption on inert surfaces or codistillation (Bowman *et al.*, 1964) onto plastic surfaces. Therefore, a short sublethal exposure may be sufficient to indirectly stimulate increased egg production but not remain to adversely affect the same tissues. It is admitted that experiments with *Aedes aegypti* presented here have dealt with potential rather than actual egg production. Investigations are continuing to determine if the potential is realized and the eggs are viable. However, it is conceivable at present that some insecticides, while controlling insect populations, likewise induce survivors to rebuild populations more rapidly than expected.

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