EFFICACY OF SYNTHETIC SUBSTANCES WITH HORMONAL ACTIVITY AGAINST IV INSTAR LARVAE OF *CULEX PIPIENS QUINQUEFASCIATUS* SAY IN AN OUTDOOR INSECTARY

C. M. WHEELER [1] AND MARIE THEBAULT [2]

This research was supported by the General Research Support Grant to the Harvard School of Public Health No. FR-5446, and by the Colonial Research Institute.

INTRODUCTION. The intriguing hypothesis that a synthesized substance exhibiting juvenile hormonal activity may be a possible "Third Generation Pesticide" was proposed by Williams (1967). In the early experimental investigations in the U.S.A., lethal effects against *Aedes aegypti* Linn. and *Culex pipiens quinquefasciatus* Say, were observed under laboratory conditions by Spielman and Williams (1966), and by Spielman and Skaff (1966). A preliminary test out of doors was reported by Spielman (1970) suggesting that the activity of the synthetic mixture was definite but of rather brief duration. More extensive trials of synthetic hormones under circumstances approximating field conditions were considered necessary in order to pursue further the hypothesis that insects may be controlled under field conditions through utilization of their own hormones.

Presented in this report are the objectives and results of such investigations. A commercially synthesized mixture of compounds having juvenile hormonal activity was used, either alone or in combination with the synergist, piperonyl butoxide, against larvae of *Culex pipiens quinquefasciatus* Say. The objectives were to determine:

a. the effect on larvae of continuous exposure at concentrations of 0.001, 0.003, 0.01 and 0.02 mg/ml.
b. the extent of action after 24, 48, and 72 hours.
c. the compatability with piperonyl butoxide.
d. whether or not the presence of an excess of larval food in the test water influences the action.
e. the effect on newly laid egg rafts.
f. possible sterilizing effect on adult mosquitoes.

MATERIAL AND METHODS. Studies were conducted in a screened out-door insectary while certain supportive experiments were conducted in the laboratory. The insectary and the laboratory are adjacent to one another in the facilities of the Colonial Research Institute, Freeport, Grand Bahama Island, The Bahamas.

The synthesized mixture of hormone-like substances was purchased from the California Biochemical Company, Los Angeles, California, hearing the designation "Synthetic Juvenile Hormone" (trade name "CALBIOCHEM") No. 420476, Lot No. 860023.[3]

Colonies of *Culex pipiens quinquefasciatus* Say were initiated with larvae taken from natural breeding sites in various locations on Grand Bahama Island. Mosquitoes were maintained in a completely screened, out-door insectary measuring 47′ x 36′ x 8′, and consisting of three similar rooms with an entrance vestibule from which separate doors gave access into each room. Each room contained four concrete troughs, 15′ x 3′ x 1½′, equipped with valve-controlled drains. The troughs were filled to

[1] Research Associate in Entomology, Department of Tropical Public Health, Harvard School of Public Health, 665 Huntington Avenue, Boston, Massachusetts 02115.

[2] Technician, Colonial Research Institute, Freeport, Grand Bahama, Bahamas.

[3] The description of this mixture on the label of the container read as follows: "light yellow mobile oil. Activity about 1 percent that of pure natural juvenile hormone and at least 100 times more active than dodecyl methyl ether."

a depth of 5 inches with water from the Institute's own deep well system. Purina Rabbit Chow pellets were scattered sparingly into the water to serve as larval food. In addition, three small sheaves of dried local grass were submerged at equal intervals along the bottom of each of the troughs. Boards approximately 20" x 48" were placed across each trough to provide shelter for the adult mosquitoes. Water was changed and troughs cleaned thoroughly at 2-month intervals or more often if the condition of the water so indicated.

Rabbits, chickens and guinea pigs were confined continuously in the insectary; sugar cubes and dried raisins provided supplemental food for mosquitoes. Four potted rubber plants and two small cherry trees were placed within the breeding room to form additional resting places for mosquitoes. Only three of the four troughs in each room were used for experiments requiring the presence of free-flying adult mosquitoes. Daily air temperature, rainfall, wind direction, and wind velocity were recorded. During experimental work, daily water temperatures were taken in selected test vessels and in breeding troughs.

The basic experimental trials were conducted in deep-sided white plastic "baby bath tubs" containing 5 liters of filtered water from the reserve trough. Tubs were numbered and assigned at random to treated or control groups. Tests were done in duplicate. In the first two series, the numbers were coded and the treatment applied at zero time by an individual who thereafter made no observations on the results; the code was revealed only after the tests were completed. The tubs were placed together in one trough; to stabilize water temperature within the plastic tubs, water was put into the large trough up to a depth of 1½–2", thus providing a large volume of water surrounding the tubs.

Cup tests utilized waxed paper pint-sized milk cartons in which a standard volume of water, 200 ml, was used. The cups were used in the laboratory for ovicidal trials and certain continuation trials of major experiments.

Stock solutions (10 mg/ml) were prepared by dissolving the crude synthetic mixture in 95 percent ethanol and were stored at +4° C in the dark.

Larvae used in the experiments were reared in the laboratory from egg rafts collected daily from the outdoor insectary. All larvae were examined individually under the microscope to establish the stage of development. Twenty larvae were used for each test except in cup tests when 10 larvae per cup were used.

For both tub and cup tests, "seasoned" water taken from one of the active breeding troughs was strained through several layers of cotton gauze and 180-inch mesh wire screen. Various amounts of the ethanol solution of the synthetic mixture were added to the appropriate tubs; dispersion was assured by stirring throughout the water of the respective tubs. Larvae were then placed in the containers. To determine ovicidal effects, egg rafts were placed on the surface of treated water and submerged for periods of 10 seconds or 60 seconds. In certain trials 200 ml of treated water were removed from each tub and placed in a clean cup at intervals of 24, 48, and 72 hours after the application of the mixture to the water. Larvae were segregated by developmental age (Spielman and Skaff, 1966) and 10 IV instar larvae of the "D" stage were then introduced into each cup.

Experiments were conducted in the majority of instances in three replicas of duplicate runs.

Observations in each larval test were continued until all larvae had either died, or had developed through to adults (fertility of these adults was not ascertained). Daily records were maintained for each cup or tub test, including original number of larvae used, and percent reaching maturity.

Daily temperature readings made at 8:00 a.m., 12 noon, and 5:00 p.m. ranged from 13° C to 34° C: average 22° C for the period covered by the trials now reported.

RESULTS

TITRATION TRIALS. Activity of the synthetic substances against the IV-D larvae varied with the concentration as noted in Table 1.

TABLE 1.—Hormonal effect on IV-D larvae of *Culex pipiens quinquefasciatus* of synthetic mixture alone and in combination with piperonyl butoxide (p.b.) (Summation of all tests)

Concentration mg/ml	No. of Tests	No. of Larvae Used	% Adults Mature
0.02	6	120	0
0.01	12	240	12
0.003	6	114	57
0.001	6	120	84
95% ethanol	12	240	92
Control	12	240	96
0.01 plus pb 0.0001%	6	120	0
pb 0.0001%	6	120	93

In a concentration of 0.02 mg/ml, no larvae reached maturity; while in a concentration of 0.01 mg/ml, 12 percent of the larvae reached the adult stage. Thus the effective concentration in the out-of-doors trials was roughly similar to that in the earlier laboratory trials reported by Spielman and Williams (1966) and Spielman and Skaff (1966).

The hormonal action extended for a period of at least 72 hours in daily decreasing activity in experiments designed to determine this point (Table 2). There was no effect on Ist, IInd and IIIrd larval instars.

SYNERGISTIC ACTION. In a series of experiments with piperonyl butoxide, in the presence of a 0.01 mg/ml concentration of the synthetic mixture, it was demonstrated that the synergist when used in a final concentration of 0.0001 percent apparently produced a response which increased the activity of the hormone to a level equal to that of 0.02 mg/ml concentration (Table 1). Piperonyl butoxide alone at the strength as stated above had little effect on IV-D larvae.

ACTIVITY IN PRESENCE OF EXCESS LARVAL FOOD. The presence of excess amounts of larval food in treated water produced no change in the efficacy of the mixture at any level of concentration. At each concentration powdered Purina Rabbit Chow pellets were used in 0.5, 1.0 and 1.5 gram amounts.

OVICIDAL ACTION. To determine possible ovicidal action, experiments were conducted in two ways: (a) placement of egg rafts less than 24 hours old on the surface of water containing 0.01 mg/ml concentration of the synthetic mixture, and (b) submersion of individual rafts for periods of 10 seconds or 60 seconds respectively. In both instances, rafts were subsequently maintained in the treated fluid in test cups. In all cases, larvae hatched normally. In one instance, an egg raft, although it had sunk into treated water, subsequently produced viable larvae. Resulting larvae remained in the test fluid through completion of their life cycle and developed into fertile adults.

STERILITY. Whether or not adult mosquitoes may be sterilized by the hormonomimetic mixture was investigated as follows: One breeding room was divided in half with a screened partition to include

TABLE 2.—Longevity of hormonal activity (Note: figures in ( ) denote number of IV-D larvae used

Hormone Concentration mg/ml	Activity after 24 hrs.		Activity after 48 hrs.		Activity after 72 hrs.	
	No. Mature	% Mature	No. Mature	% Mature	No. Mature	% Mature
0.02	4 (30)	13	3 (30)	10	6 (30)	20
0.01	9 (60)	15	21 (60)	35	25 (40)	63
0.003	25 (30)	83	27 (30)	90	10 (10)	100
0.001	28 (30)	93	30 (30)	100	9 (10)	90
95% Ethanol	55 (60)	92	54 (60)	90	37 (40)	92
Control	57 (60)	95	57 (60)	95	37 (40)	92

two of the original four troughs. Three baby chicks were quartered in one of these small rooms to furnish blood meals for adult mosquitoes when these were later introduced. Adult mosquitoes in the laboratory were housed for 7–10 days in a glass lamp chimney over water with sugar cubes inpregnated with a 10 mg/ml hormone solution as their only food. The mosquitoes then were released in the outdoor insectary, where they deposited egg rafts on the surface of water contained within one of the troughs. Several lots of adults were placed in this room over a period of 3 months. Seventy-six egg rafts were collected during this time; of this number, 67 percent hatched, while the remaining 33 percent produced no viable larvae (many of these eggs were examined and found to be embryonated). In a parallel control experiment virtually all eggs hatched that were contained in 10 rafts laid by adults fed on sugar alone. The result of this rough test suggests that some degree of sterility was produced by the feeding of hormone solution.

DISCUSSION. The studies above indicate that results similar to the earlier laboratory tests can be obtained repeatedly under out-of-doors conditions. Of the 5 sub-stages in the IVth larval instar, only 2 are sensitive to hormonal action according to Spielman and Skaff (1966), namely IV-C and IV-D, the others, IV-A, IV-B, and PP being refractory. The large majority of the deaths occurred in the Pharate Adult (PA_2) stage, and this phenomenon is attributable to hormonal action. The activity of the synthetic mixture lasted somewhat longer in these tests than in those previously reported. These tests were conducted with seasoned water but without added food; Spielman (personal communication) has evidence that added food shortens the duration of the hormonal effect of the mixture.

Bowers (1968) reported that piperonyl butoxide synergized with hormonomimetic mixtures. We confirmed that this synergist may be used to amplify the effect of synthetic hormones under field conditions against the larvae of mosquitoes in the concentrations stated.

Embryogenesis is disrupted after certain moths and bugs are exposed to synthetic juvenile hormones according to Riddiford and Williams (1967); mosquitoes were similarly affected in the trials reported by Spielman and Williams (1966). We found some indication that pre-gravid mosquitoes may be sterilized by ingestion of such a preparation. Unlike Riddiford and Williams (1967) we found no direct ovicidal effect.

Next steps in pursuing the "3rd generation pesticide" hypothesis now seem worth taking, namely, (a) to seek individual components which have much greater activity than that of the crude mixture itself; (b) to search for ways to prolong the duration of action under variable climatic conditions, and (c) to obtain data on toxicity of whichever highly active compounds are identified as candidates for further field trials.

In view of the relatively close correlation of the results of laboratory tests with these trials out of doors, repeated over several months, the search for individual compounds of high activity can be conducted under laboratory conditions with considerable confidence.

SUMMARY. Tests conducted out of doors over a period of several months showed that crude synthetic juvenile hormone prevented the maturation of IV-D larvae of *Culex pipiens quinquefasciatus* Say in a concentration of 0.02 mg/ml. Piperonyl butoxide enhanced the action of the mixture somewhat. Fertility of adult mosquitoes which fed briefly on sugar impregnated with the mixture seemed to be impaired. Next steps in testing the "3rd generation pesticide" hypothesis are suggested.

ACKNOWLEDGMENTS. The authors are indebted to Dr. John C. Snyder, Dean, Harvard School of Public Health, and Dr. Thomas H. Weller, Professor and Head of the Department of Tropical Public Health, for the opportunity to conduct this investigation; to Dr. Andrew Spielman of the Department of Tropical Public Health,

Harvard School of Public Health, for technical advice; and to members of the Colonial Research Institute, Freeport, Bahama, The Bahamas, for their valuable assistance.

References Cited

Bowers, W. S. 1968. Juvenile hormone: Activity of natural and synthetic synergists. Science. Vol. 161, pp. 895–897.

Riddiford, L. M. and Williams, C. M. 1967. The effects of juvenile hormone analogues on the embryonic development of silkworms. Proc. Nat. Acad. Sci. Vol. 57, pp. 595–601.

Spielman, A. 1970. Synthetic juvenile hormones as larvicides for mosquitoes. Industry and Tropical Health. Vol. VII, pp. 67–69.

Spielman, A. and Skaff, V. 1966. Inhibition of metamorphosis and of ecdysis in mosquitoes. Jour. Insect Physiol. Vol. 13, pp. 1087–1095.

Spielman, A. and Williams, C. M. 1966. Lethal effects of synthetic hormone on larvae of the yellow fever mosquito, *Aedes aegypti*. Science, N. Y. Vol. 154, pp. 1043–1044.

Williams, C. M. 1967. Third generation pesticides. Scientific American. Vol. 217, No. 1, pp. 13–17.

A METHOD FOR REARING THE MOSQUITO *CULEX PIPIENS MOLESTUS* FORSK[1]

M. BAR-ZEEV AND D. BEN-TAMAR

Israel Institute for Biological Research and
Tel-Aviv University Medical School, Ness-Ziona

This laboratory is engaged in the evaluation of chemicals, furnished by the U.S. D.A., as candidate repellents against a number of blood-sucking insects including the local mosquito *Culex pipiens molestus* Forsk. It is the most common mosquito in houses in this country, and a fierce biter which attacks mostly in the dark. Since relatively large numbers of this mosquito are necessary for the screening tests, a simple technique has been devised to rear large numbers of them. The method may also be adopted to rear other species of mosquitoes.

DESCRIPTION OF THE REARING CONTAINER. The rearing container (Fig. 1) is a commercially available plastic container (36 cm in diameter at the base, 43 cm in diameter at the top, with a 2 cm rim, and 43 cm high). Close to the bottom of the container there is a hard plastic tube B, (1.6 cm inside diameter) in the form of an inverted T. At the ends of this tube, which are outside the container, small rubber tubes A & D are connected. They can be closed and opened by clamps. At the other end of this tube, which is inside the container, a screened metal tube is attached C (3 cm in diameter and 28 cm long). Another hard plastic tube H (1.6 cm inside diameter) comes out from the bottom of the container. It has at its end a small rubber tube with a clamp I. To the end of this tube a screened metal tube J (5 cm in diameter and 20 cm long) can be connected, whenever necessary. The top of the container is covered with cheesecloth E, tightened to the container by a rubber band. On top of it is placed, when necessary, a cover F made of a plastic screen, except for the rim (3 cm) which is of hard plastic. There are four holes (5 cm in diameter) in this plastic screen. Into these holes are inserted, when required, four inverted jars G which contain about 90 cc of a 10 percent sugar solution. The plastic covers of the jars are each perforated

[1] This research has been financed in part by a grant made by the United States Department of Agriculture, Agriculture Research Service, authorized by Public Law 480.