

LABORATORY BEHAVIOR OF *CULEX PIPiens* *QUINQUEFASCIATUS* AND THE EFFECTS OF TEPA, MÉTÉPA, AND APHOLATE UPON ITS REPRODUCTION¹

ELMO M. MCCRAY, JR. AND HERBERT F. SCHOOF

One of the major problems facing any investigator in the laboratory study of arthropod vector biology is integrating the experimental requirements of the investigation and the environmental requirements of the test animal. The experimental design and conditions can usually be adjusted to the requirements of the test organism, but occasionally, to obtain specific data, the laboratory specimens must be induced to perform under conditions dictated by experimental necessity.

These conditions prevailed when studies were initiated on the effect of several mutagenic compounds upon *Culex pipiens quinquefasciatus*. Initial attempts to cage single pairs or small numbers of the test strain were unsuccessful in two respects: mating did not occur consistently, and the percentage of individually caged *Culex* females that would readily take a blood meal from a live host was too low to validate the test results.

This paper describes the approaches used to modify the laboratory behavior of *C. p. quinquefasciatus* so as to achieve the objectives of the experimentation and reports on the influence of several chemosterilants on larval and adult *C. p. quinquefasciatus*.

A. STUDIES ON LABORATORY BIOLOGY

In experiments in the use of sterile *Aedes aegypti* males (McCray *et al.*, 1961), known numbers of sterile and normal males were combined with known numbers of normal females, and the numbers

of eggs deposited and hatched were determined. The number of *Ae. aegypti* females per replicate was held to a minimum (25) to reduce the hours necessary to count the total eggs and larvae produced. With *C. p. quinquefasciatus*, however, small numbers of individuals would not mate, and the females would not take a blood meal. Because working with large numbers of individuals in a large cage posed a number of technical problems, initial efforts were directed toward developing techniques for getting consistent mating, blood feeding, and oviposition from small colonies and single individuals.

I. FEEDING TESTS. Since *C. p. quinquefasciatus* is primarily an avian feeder, the insectary strain had been given no blood source other than chicken for hundreds of generations. It produced approximately 200 eggs/raft, and more than 95 percent of the eggs routinely hatched within 24 hours after oviposition. The females would feed on mammals only rarely.

To obtain a strain that would accept mammalian blood, groups of approximately 4,000 each were offered no blood hosts other than rabbit. In one group of 4,000 adults, 12 took a blood meal and oviposited. The mean number of eggs/raft was 30. These eggs hatched normally; the progeny were reared with no difficulty, and these F₁ individuals were used to establish a new strain. Of the approximately 350 adults produced, 49 females fed on rabbits and oviposited. These were used to establish an F₂ colony, and this procedure was followed through the F₅ generation. By this time the strain was feeding readily on rabbits, and the number of eggs/raft had increased to 100-120, with 95 percent or more

¹ From the Biology Section, Technical Development Laboratories, Laboratory Division, National Communicable Disease Center, Health Services and Mental Health Administration, Public Health Service, U.S. Department of Health, Education, and Welfare, Savannah, Georgia 31402

hatching the day after oviposition. The F_5 was the first generation to feed readily on humans, but its larval development, pupation, and adult activity were not different from those of the parent colony. Now, after several years of being maintained with a rabbit blood-host, this strain continues to produce approximately 100 eggs/raft (half the number they produced when a chicken was the sole blood-host). However, when individuals from this strain are offered chickens, they feed avidly and produce about 200 eggs/raft.

Although a mammalian feeding strain was now available, the adult females still would not consistently take a blood meal when caged individually or in small numbers. Since individual females that would take blood any and every time it was offered were required for the experimental design, "forced feeding" of citrated whole blood was attempted. Initial efforts with heparinized human blood and beef blood were unsuccessful, though many different methods were tried. All of these early methods, however, were based on the idea of stimulating the females "to seek a blood meal." All such attempts met with erratic results. Only when the concept of "seeking a blood meal" was abandoned was success achieved. The new approach was based on the mosquito's readiness to feed on sweetened solutions. Since normally the mosquitoes are fed 10 percent sucrose or honey water, sucrose was incorporated in citrated beef blood at that level. Both males and females fed readily on this formulation.

The whole beef blood contained 100 g. of sucrose and 3.25 g. of sodium citrate per liter. It was placed in 50 ml. plastic vials, frozen at 0° F. for hemolysis, and stored at this temperature. For feeding, 5-10 ml. of the thawed blood was placed in a plastic feeding vial suspended in the upper cell of a 1-gallon, 2-compartment mating-oviposition cage (Figure 1). The vial was constructed so that approximately 100 holes, made with a hot needle, extended around the vial in a 1" wide band about $\frac{3}{4}$ " from the bottom. The inside of the vial was lined with a single layer

of paper toweling to act as a wick. A similar strip of celluloid or plastic was placed inside the paper wick to force the paper firmly against the sides of the vial, to aid in the capillary movement of the sucrose-blood solution up the wick and to retard drying. The adults, male and female, came to the feeder, inserted their proboscis through the perforations in the vial and ingested the sucrose-blood solution.

The feeder was replaced daily, and after about 4 days the females produced egg rafts of approximately 100 eggs/raft. About 95 percent hatched within 24 hours, and no difference could be detected in the progeny produced. The adult life span of the females fed only sucrose-blood was not markedly reduced, but the males fed only this solution from time of emergence began to die rapidly after 1 week. However, by feeding males 10 percent sucrose-water the first 4 days after emergence and then switching to the 10 percent sucrose-blood, healthy, vigorous and sexually active specimens were produced. Both sexes apparently were feeding upon the sucrose, and the females were not coming to the feeder to take a blood meal. This fact was established by several tests in which the females refused to feed upon plain citrated blood and, when a choice was offered between feeders with sucrose-water and sucrose-blood, chose the sucrose-water. By the simple expedient of dissolving the sucrose in blood instead of water, they were forced to ingest the blood as well. The substitution of honey for sucrose was not satisfactory. This strain has now been maintained for 7 years upon sucrose-blood from artificial feeders. A new supply of blood is obtained quarterly from a local slaughter house and formulated and stored in the plastic vials at 0° F. until needed.

2. MATING TESTS. Adults refused to mate consistently in small containers or when caged in small numbers, regardless of the type of feeding involved. However, by using large numbers (2,000) in small screen cages (12" x 12" x 12"), regulating light intensity and by introducing swarm-

MOSQUITO MATING AND OVIPOSITION CAGE WITH BLOOD-SUCROSE FEEDING DEVICE.

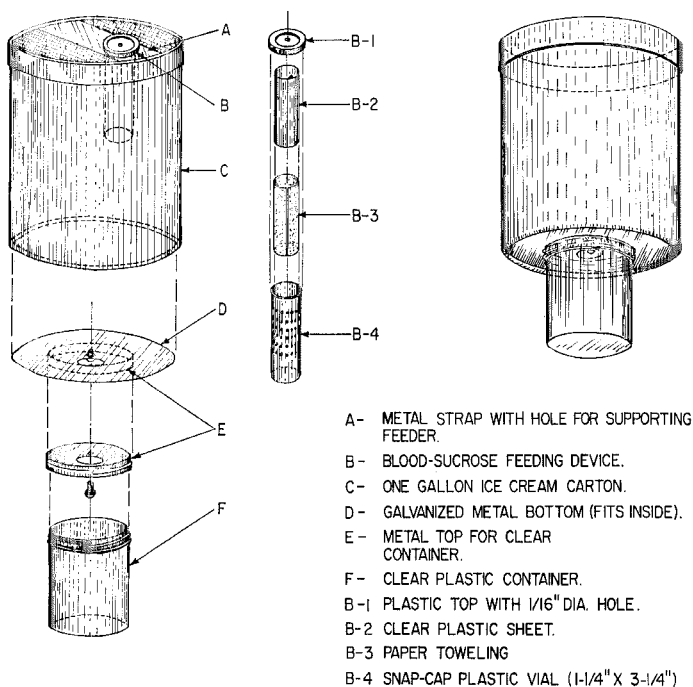


Figure 1.

ing stands, some mating was obtained. The progeny were used to establish an F_1 colony and successive, separate generations eventually produced a strain from which consistent mating of 10 pairs in gallon containers and reasonably consistent mating of single pairs in pint containers could be obtained. In several tests to determine the percentage of females successfully mated under these conditions, mixed adults were caged together for 7 days and individual females were removed, placed in pint containers similar to the large mating-oviposition cages (Fig. 1), and supplied the sucrose-blood in small plastic feeders. Ninety percent of these individually caged females were fertile, and when fed nothing but 10 percent sucrose-

blood, they lived approximately 50 days and oviposited an average of three times during their life span. The egg rafts produced after the females were 30 days of age were small (10-20), but the mean viability of the eggs was better than 95 percent.

B. STUDIES ON MUTAGENIC AGENTS

1. MATERIALS AND METHODS. Three mutagenic compounds, metepa,² tepa and apholate,³ were used in tests with the adults. Metepa was used with the larvae.

² Furnished through the courtesy of Interchemical Corporation, New York, New York.

³ Furnished through the courtesy of American Cyanamid Company, Princeton, New Jersey.

To determine the effects of different exposure periods and concentrations of metepa upon larval development and subsequent adult sterility, enamel rearing trays 9" x 15½" x 2½" containing 200 third instar larvae each in 1 liter of water were treated with technical metepa in 95 percent ethanol at 10, 20, and 30 ppm. At each concentration, the larvae were exposed for 24, 48, or 72 hours. After exposure the larvae were removed from the metepa, rinsed thoroughly, and placed in untreated larval rearing media to complete their development. Twenty-five adult males from each of the larval exposures were then caged with 25 normal females and offered sucrose-blood continuously. The egg rafts produced were then collected and held individually to determine hatch and subsequent larval development.

To determine the effect of the continuous exposure of larvae to metepa from the third instar until pupation, technical metepa in 95 percent ethanol was added to trays containing 1,500 third instar larvae in 1 liter of larval rearing media. Metepa concentrations of 10 and 20 ppm were used. After pupation in the metepa solution, the adult males produced were handled as described in the previous paragraph.

To measure the effect of adult tarsal uptake from residues of tepa, metepa, and apholate, a collapsible plywood box having inside dimensions of 12" x 12" x 12" was used. A clear plexiglass top was placed on the box to facilitate observations and subsequent removal of the exposed adults which did not rest upon the plexiglass top, but upon the plywood sides and bottom. The plywood surfaces were treated with 500 mg./sq. ft. of technical material in 95 percent ethanol and air dried from 12 hours to 12 days. In the initial tests, adult virgin males (4 days old) placed in the exposure chamber for 4 hours with 12- to 36-hour-old residues of metepa died during the exposure period. To determine whether the mortality was caused by contact with the residue or by vapors, a con-

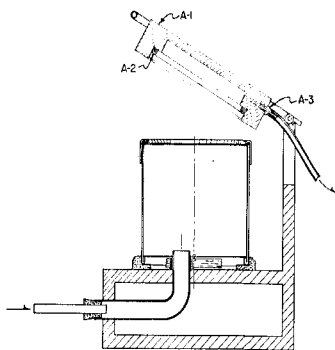
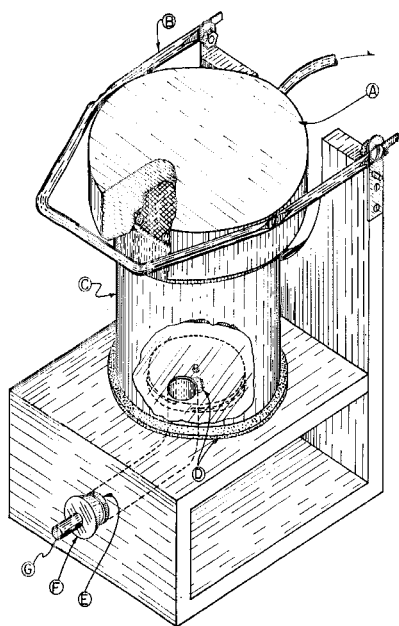
stant, gentle flow of air was passed through the chamber during the exposure period, which did not disturb the adults as they rested normally upon the residues. No mortality occurred. This technique with a 4-hour exposure time was then used in all further tests. Because of the possible toxicological hazard of removing the males from treated panels by mouth aspirator, a vacuum device (Fig. 2) was constructed that would gently transfer the treated males directly to the mating cages. The specimens were then fed 10 percent sucrose until mated 24 hours later. When the virgin females were placed with the treated males, only sucrose-blood was supplied continuously. Egg rafts were collected daily. Eggs were counted, held individually, and permitted to hatch.

2. RESULTS. In larval tests where third instar larvae were exposed for 24, 48, and 72 hours to 10, 20, and 30 ppm of metepa, some pupation did occur prior to completion of the 72-hour exposure. Total pupation in all concentrations and exposure periods ranged from 80-98 percent; the 80 percent pupation occurred with the larvae exposed to 30 ppm for 72 hours. The adults emerged normally and with a normal sex ratio. Normal virgin females, when mated with males from these tests, produced normal numbers of eggs with 90 percent hatch. Of the 96 rafts evaluated in these tests, only eight had a hatch rate less than 76 percent, and none of the rafts were sterile.

When third instar larvae were placed in 10 and 20 ppm metepa and left until 24 hours after pupation, eggs from normal virgin females mated with males exposed to 10 ppm had 83 percent hatch while those from females mated with males exposed to 20 ppm had 70 percent hatch.

In those residue tests in which 4-day-old adult males were exposed to tepa residues 12 hours old for 4 hours and then caged with normal virgin females (25/25), little or no sterility was produced (Table 1). Identical tests with apholate residues 7 days old resulted in 98 percent hatch of the 3,887 eggs collected.

ADULT MOSQUITO VACUUM ASPIRATOR



SECTIONAL SIDE VIEW

- A- HINGED AIRTIGHT TOP
 B- SELF-ALIGNING HINGE
 C- MATING AND OVIPOSITION CAGE.
 D- CAGE SEALS
 E INLET TUBE -1" INSIDE DIAMETER
 F- RUBBER STOPPER $\frac{1}{4}$ " HOLE TO FIT ASPIRATOR
 G- ASPIRATOR INLET

- A-1 SOLID PLEXIGLASS TOP
 A-2 RUBBER "O" RING
 A-3 VACUUM OUTLET NIPPLE

SAVANNAH, GA. - AUG. 66, B-100

Figure 2.

Tests with metepa residues 14 hours old produced marked sterility (Table 2). Initially, it was thought that a male, after being treated with metepa, would either be totally sterile or completely normal. Thus, any given egg raft produced by a female mated to a treated male, would consist of all or no viable eggs. This was not the case. It was found that 18/68 rafts (26 percent) were completely sterile (zero hatch); 41/68 (60 percent) had 5 percent hatch or less; 64/68 (94 percent) had 25 percent hatch or less; and only

one raft had more than 30 percent hatch (37 percent). Identical tests using metepa residues 7 days old resulted in zero hatch from 4,568 eggs; but when residues of metepa 12 days old were used, of the 6,245 eggs produced, 84 percent hatched.

Since, of the three compounds tested, metepa was the only one showing any marked effectiveness as a residue on plywood, metepa residues 7 days old were used for treating males which were then combined with normal males and females in a ratio of 10:1:1. Of the 1,325 eggs

TABLE 1.—Egg hatch from females mated with males exposed to tepa residues 12 hours old.

Test	Number Eggs	Number Larvae	Percent Hatch
A	1792	1553	87
B	1204	1089	90
C	1513	1076	71
D	803	618	77
Total	5312	4336	Mean 82
Control	1660	1528	92

used for evaluation in these tests, 27 percent hatched. Although most of the rafts were of approximately 100 eggs each, the remainder were exceedingly small. Fifty percent of the rafts produced were totally sterile, whereas 31 percent had better than 90 percent hatch; there was no correlation between size of raft (number of eggs/raft) and percentage that hatched. For example, one raft of 120 eggs had zero hatch, but a raft of only 27 eggs had 100 percent hatch.

DISCUSSION. The initial efforts in this study were directed toward obtaining laboratory specimens that would behave in such a manner that consistent mating and blood feeding could be obtained from small numbers and individually caged specimens. By selecting for several generations those specimens that would mate in a confined space, one phase of the problem was solved.

To be certain that every test female obtained blood, "forced feeding" appeared to be a logical approach. Mammalian blood was used for this purpose simply because avian blood was not as readily available, and it was not anticipated that *Culex* would show a marked reluctance to accept mammalian blood. Once this characteristic was manifested, and the problem was solved, the reduction in number of eggs/raft was a point considered worthy of reporting. Several possible explanations for this reduction in egg raft size have been postulated. The most apparent is the obvious difference in mammalian and avian red blood cells; avian red blood cells are nucleated and mammalian

TABLE 2.—Egg hatch from females mated with males exposed for 4 hours to metepa residues 14 hours old.

Test	Number Eggs	Number Larvae	Percent Hatch
A	2197	270	12
B	1391	62	4
C	1893	114	6
D	1676	58	3
Total	7157	504	Mean 7

ones, in general, are not. While this difference would suggest that the absence of some or all of the nucleic materials from the mammalian red blood cells is responsible for the reduction in number of eggs/raft, no biochemical studies were made to clarify this point.

Although the previously mentioned alterations of the behavior of the mosquitoes used in this study probably resulted in specimens that were not like the ones in the field, those specific qualities being examined were not so altered that the data obtained lost any of their validity.

The observations made during the tests with larval exposure to metepa indicated that as the concentration was increased until it produced appreciable sterilization of adult males, the material became toxic to the larvae and many died; many of those that became pupae failed to become adults or died shortly after emergence. Similar results with apholate and with metepa have been noted in other studies (Mulla, 1964). Dame *et al.* (1964) found that larval exposure of *Ae. aegypti* to tepa at 10 ppm reduced adult emergence slightly, but was highly toxic to larvae of *A. quadrimaculatus*.

All data available at the present time indicate that the practical field sterilization of adults by larval treatment under natural conditions presents many problems in addition to those mentioned above. The three compounds, tepa, metepa and apholate, are relatively toxic to mammals and have an affinity for the reproductive glands; when tested on rats they produced testicular atrophy and reduced numbers of

offspring (Gaines and Kimbrough, 1964). Also these compounds polymerize rapidly and combine readily with materials in the larval environment, thereby requiring a much higher initial application rate to obtain dosage levels that would produce any significant sterility, and the effective life of the material would be comparatively short. These characteristics are particularly important since *C. p. quinquefasciatus* breeds in polluted water.

Although in tests with these three compounds applied as mists to adults, apholate was the most effective (McCray and Schoof, 1967), the data from those tests with residues on plywood were quite different. Metepa, the least effective when used as a mist, was the only one that produced any obvious sterility, but its period of activity was brief when used as a residue. Thus, under natural conditions, repeated applications would be required within short periods of time.

In general, the use of these compounds would appear to be impractical for use in a routine manner for a mosquito control program. However, the search should be continued for other chemosterilants, and new approaches and unique application methods should be devised to explore thoroughly the adaptation of chemosterilization to field practices.

SUMMARY. To obtain specified experimental data about the effects of tepa, metepa, and apholate on *Culex pipiens quinquefasciatus*, the laboratory strain which received blood meals only from chickens and mated only when caged in reasonably large numbers and in large cages, were artificially fed a 10 percent sucrose-citrated beef blood-solution. While

the average number of eggs/raft was reduced from 200 to 100 by the shift to mammalian blood, viability remained above 95 percent. Successive selection through several generations resulted in specimens that would consistently mate in small containers and in small numbers. Some mating was obtained with single pairs. Observations about life span, survival, egg production, and behavior were recorded. Larval treatment with metepa produced little or no sterility in adult males. Residues of 500 mg./sq. ft. of tepa and apholate on plywood surfaces produced little or no sterility in adult males 4 days old. Metepa residues up to 7 days old did produce sterility ranging from 93 to 100 percent, and when treated males were combined with normal males and females in a 10:1:1 ratio, 50 percent of the egg rafts produced were totally sterile and only 27 percent of all eggs that were produced hatched.

References

- DAME, D. A., WOODWARD, D. R., and FORD, H. R. 1964. Chemosterilization of *Aedes aegypti* (L.) by larval treatments. Mosq. News 24(2):1-6.
- GAINES, T. B., and KIMBROUGH, R. D. 1964. Toxicity of metepa to rats with notes on two other chemosterilants. WHO/Vector Control 9:1-19.
- MCCRAY, E. M., JR., JENSEN, J. A., and SCHOOF, H. F. 1961. Cobalt-60 sterilization studies with *Aedes aegypti* (L.). Proc. 48th Ann. Mtg. N.J. Mosq. Exterm. Assoc., March 28-31, 1961. pp. 110-115.
- MCCRAY, E. M., JR., and SCHOOF, H. F. 1967. Mist application of chemosterilants to adult male *Culex pipiens quinquefasciatus*. J. of Econ. Entomol. 60(1):60-63.
- MULLA, M. S. 1964. Chemosterilization of the mosquito *Culex pipiens quinquefasciatus*. Mosq. News 24(2):212-217.