

CONTINUOUS LABORATORY REARING OF *CULISETA INORNATA* (Will.) (DIPTERA : CULICIDAE)<sup>1</sup>J. McLINTOCK<sup>2</sup>

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There is a growing need for knowledge of species of North American culicine mosquitoes that can be reared continuously in the laboratory. Potter (1951) has pointed out that there is no such thing as a standard representative test insect for studies of insecticides in the laboratory. The same applies to repellents. *Aedes aegypti* (L.), *Culex quinquefasciatus* Say, and *Anopheles quadrimaculatus* Say are the mosquitoes most commonly used in the laboratory for insecticide and repellent tests in North America. These species represent mainly the southern mosquito fauna of the continent; there has been no laboratory colony to represent the hordes of pest mosquitoes that infest the Canadian plains and sub-Arctic. Studies in outdoor insectaries in Western Canada have had to be made with specimens reared from larvae taken in the field or from eggs laid by captive wild females, these procedures usually being hindered by high mortalities in the resulting mosquito populations. A lack of specimens, caused by autumn frosts, has invariably interrupted studies of the biology of these important insects. Techniques have been developed for continuous rearing of the following species under laboratory conditions: *Anopheles quadrimaculatus*, *Anopheles punctipennis* (Say), *Aedes aegypti*, *Culex pipiens* L., *Culex quinquefasciatus*, *Aedes atropalpus* (Coq.) (Trembley, 1944, 1945); *Anopheles albimanus* Wied. (Rozeboom, 1936; Burgess, 1950); of these, only *A. punctipennis* and *C. pipiens* occur in Western Canada, and they are comparatively rare. Of some 20 species occurring in southern Manitoba,

where the present work was started, only *Culiseta inornata* (Will.)—because of its stenogamous habit—showed any promise of becoming a laboratory animal.

Dupree was probably the first to observe that *C. inornata* (= *C. consobrinus* Desv.) would mate in very confined quarters (Mitchell, 1907). This fact was apparently overlooked until Owen (1937) made the same observation and suggested that this species (*Theobaldia inornata* (Will.)) would make a useful experimental animal; in a footnote he recorded his maintaining a colony of this species through five successive generations. Later (1942) he reported rearing this species continuously in the state of Wyoming, using a "semi-balanced" aquarium for the larvae. The principal ingredients of this aquarium were obtained every three months from a "vernal-autumnal" pool.

Encouraged by Owen's success, I began attempts to rear *C. inornata* in Canada in the autumn of 1942 at the Virus Laboratory of the Manitoba Department of Health and Public Welfare and the Children's Hospital of Winnipeg, Winnipeg, Manitoba, and have continued intermittently until the present. It soon became apparent that establishing a colony would be no simple problem. A balanced aquarium maintained by additions of material from a natural pool was out of the question in Manitoba. It was necessary to find an artificial medium in which the larvae would grow. During the winter of 1942-43 a small colony was maintained with difficulty for five generations, a hay infusion inoculated with paramecia being used as the larval diet. This larval rearing medium was difficult to duplicate. Numerous larval rearing media were tested during the summer of 1943, when an abundant supply of wild females was available. During the winter of 1944-45 a small colony was kept alive through

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four generations at Macdonald College, Quebec (from Manitoba stock); here the larval diet was yeast alone in water for one generation, and dried cereal grass in water until the colony died out. From August, 1946, to February, 1948, another colony was maintained at the Virus Laboratory of the Manitoba Department of Health and Public Welfare and the Children's Hospital of Winnipeg, Winnipeg, through 16 generations on a larval diet consisting of a mixture of flour and yeast in water. Here the larval mortality was variable and on more than one occasion the colony was reduced to a few individuals. During the past three years another colony was established at the Livestock Insect Laboratory, Lethbridge, Alberta, and was maintained for eight generations without signs of reduced vigor. The colony was finally allowed to die out. A routine method of rearing was developed which, I believe, will be applicable wherever the simple necessary materials and equipment are available.

*C. inornata* has much to recommend it as a laboratory insect. Besides its stenogamous habit, it is a large species, larger than the other North American mosquitoes that have been reared continuously in the laboratory. The male pupae are distinctly smaller than those of the females and the two sexes can readily be separated in this stage. The eggs are normally laid in the evening; but if in the evening gravid females are deprived of water they will deposit their eggs at any time during the day when placed over water, particularly against a dark background. The eggs are laid in "rafts" on the surface of the water, which facilitates harvesting. The female requires from one-half to one hour to deposit her raft. The eggs are pearly white when laid and turn dark brown in about two hours at 20° C., which simplifies the gathering of eggs of known age. The larvae can be reared in complete darkness or in light. The adults, both males and females, feed readily on almost any fluid diet presented to them; no preliminary warming of the diet is necessary to induce them to feed. The

females will imbibe from a free fluid surface or through a membrane. The species is readily available in North America. It is found throughout the United States and the Mexican table land (Owen, 1942). In Canada it is found as far north as the 51st parallel in Manitoba and the 55th parallel in Alberta. Finally, *C. inornata* is a known laboratory vector of western equine encephalitis (Hammon and Reeves, 1943), St. Louis encephalitis (Hammon and Reeves, 1943a), and Japanese B encephalitis (Reeves and Hammon, 1946).

Rearing the Larvae. The greatest obstacle to the continuous rearing of *C. inornata* in the laboratory has been the lack of a suitable technique for rearing the larvae. The successful rearing of mosquito larvae in general depends on obtaining a balance between the concentration of food substances, volume and depth of medium, concentration of the larvae, and temperature. In rearing *C. inornata* it is also essential to keep the surface of the medium free of a pellicle, which if allowed to form will quickly smother the larvae.

The dietary requirements of the larvae of *Aedes aegypti* were studied by Atkin and Bacot (1917), MacGregor (1929), Trager (1935, 1935a, 1937, 1948), Trager and Subbarow (1938), Golberg and DeMeillon (1947, 1948, 1948a), and Golberg, DeMeillon, and Lavoipierre (1945) and of *Culiseta* (= *Theobaldia, incidens* (Thom.) by Frost, Herms, and Hoskins (1936). The work on these two species indicates that mosquito larvae require proteins, lipoids, vitamins of the B group, and various salts in the diet. During the present study no attempt was made to determine the specific dietary requirements of *C. inornata*, but the above information was kept in mind in seeking suitable culture media for the larvae. Nor was any attempt made to keep the cultures sterile, although all apparatus used was kept as clean as it was convenient to do.

The object of this investigation was to find a routine method for *C. inornata* that would be as simple as possible and that could be duplicated anywhere. All the

necessary dietary requirements for mosquito larvae are found in yeast, and the majority of the media recommended in the literature for mosquito larvae include yeast or yeast extracts in one form or another. Early in this investigation the methods employed by Trager (1935) (without sterilization), Woke (1937), Granett and Powers (1937), and Granett and Haynes (1944) for *Aedes aegypti*, by Phillips and Swingle (1940) for *Culex quinquefasciatus*, and by Frost, Herms, and Hoskins (1936) for *Culiseta incidens* were tried with *C. inornata*. Various modifications of their techniques were also tried, as well as media consisting of hay infusions alone or containing wheat grains or cultures of paramecia, and others containing various proprietary preparations of powdered milk and baby foods. The most helpful publications encountered were those of Trembley (1944) and Bates (1941), and the medium eventually found most satisfactory was a modification of Bates' "standard" for anopheline larvae.

*C. inornata* should be reared at a temperature of 20–21° C. It can be reared at 25° C. and development is more rapid at this temperature, but larval mortality is slightly higher and the adults are not as large and robust as those reared at the lower temperatures.

On being gathered, the egg-rafts were placed in enamel pudding pans eight inches in diameter and 2½ inches deep. Five to six rafts were placed in each pan, which contained a medium consisting of 100 mg. of Difco Brain Heart Infusion, 50 mg. of baker's yeast (Fleischmann's Royal), and 0.5 gm. of ground whole-wheat bread crumbs in 1250 ml. of Bates' (1941) "medium S." The bread crumbs were prepared by slicing whole-wheat bread, drying the slices in the sun or in an oven at 45° C., and then grinding them as fine as possible in a mortar. Bates' medium S consists of 0.5 gm. of calcium sulphate ( $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ ), 0.5 gm. of sodium chloride ( $\text{NaCl}$ ), and 1 gm. of magnesium sulphate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ) in 1 litre of distilled water. A hatching pan prepared in this way accommodated about

1000 first-instar larvae. The pan was covered with a piece of window glass and a slow stream of air was bubbled continuously through the medium.

At 20–21° C. the eggs hatch in about 57 hours. The larvae were left in the hatching pans and allowed to complete their first moult there. The first moult occurs in 2–2½ days after the eggs hatch and the larvae were not transferred to the larval trays until this moult was completed and the integument had darkened. Each larval rearing tray received 150 second-instar larvae, picked up, counted, and transferred by means of a medicine dropper pipette.

The larval rearing trays were enamel instrument or photographic trays measuring 15 x 10 x 2 inches. Each tray contained 2½ litres of Bates' medium S, to which had been added 100 mg. of Difco Brain Heart Infusion, 50 mg. of baker's yeast, and 240 mg. of ground whole-wheat bread crumbs. The trays, containing these ingredients, were made up on the same day as the hatching pans, i.e., on the day the eggs were laid. These trays also were covered with window glass and air was bubbled continuously through the medium. Each second day after the trays were made up until pupation began, 50 mg. of the baker's yeast and 240 mg. of the bread crumbs were added to each tray. Since pupation began on about the tenth day after hatching, each tray received seven increments of the diet, making a total of 350 mg. of yeast and 1.7 gm. of bread crumbs.

An average of 90 per cent of the larvae pupated in five to twelve days, the majority pupating in seven days. The males began to pupate first, followed about two days later by the first female. The pupae were removed from the trays each day by means of a dipping tube and transferred to an enamel pudding pan containing distilled water through which air was bubbled continuously. The pupal pan was kept in the emergence cage.

An air stream of 1–1½ litres per hour delivered through a medicine dropper pipette to each hatching pan and larval

rearing tray was sufficient to prevent the formation of a pellicle. Too fast an air flow caused the medium to splash on the glass cover and trap some of the younger larvae. A satisfactory source of air pressure was a standard laboratory compressed-air system with a regulator to reduce the pressure to a steady five pounds per square inch or lower, depending upon the number of trays to be aerated. Also satisfactory was a laboratory blower or a Cornelius air compressor (The Cornelius Co., Minneapolis, Minn.), the compressed air being stored in a three- to five-gallon storage tank and brought up to 15-18 pounds' pressure twice a day. The outward flow of air from the storage tank was controlled by a needle valve. The beneficial effect of the air stream was probably purely mechanical, for on many occasions I have taken the larvae of *C. inornata* from pools in which there was no dissolved oxygen detectable by the Winkler method.

Bates' medium S was superior to distilled, tap, well, or pond water. In Winnipeg, tap water that had been allowed to stand exposed to the air for two days before using was satisfactory. But at Lethbridge mortality of the larvae was almost 100 per cent in media prepared with tap water. Well water and pond water gave erratic results in different localities. Difco Yeast Extract may be substituted for baker's yeast but larval mortality with the former was slightly higher. After several days the larval rearing media contained populations of bacteria and protozoa that probably originated in the bread. For some time domestic flour (Canada Approved, vitamin B) was used instead of bread, but the larval mortality varied considerably from generation to generation and this was believed due to greater variation in the numbers of microorganisms in the flour.

Feeding and Handling the Adults. Although Owen (1942) obtained a few eggs on three occasions from females fed only on sugar solutions or raisins, *C. inornata* usually requires a blood meal for the production of eggs. When mated and hungry, the females will feed readily on the

human arm, either when the arm is inserted in a cage or when the females have to feed through gauze or screen as when a cage is applied to the arm. I have not been able to induce unmated females to pierce the skin for a blood meal but an occasional female will do so while in the act of mating. However, as such a method of providing a blood meal is too slow and tedious even for a colony of moderate size, a quicker and more reliable method was sought.

In studies of virus transmission by mosquitoes it is a common practice to infect the mosquitoes by allowing them to feed on a suspension of the virus soaked up in a cotton pledget. This method was adapted to feeding blood to *C. inornata*. For some unknown reason, the adults are unable to imbibe whole defibrinated, heparinized, citrated, or oxalated blood but both males and females readily take up such blood when it is diluted with a ten per cent solution of sucrose. Dilutions containing one volume of the sugar solution to six volumes or less of blood can be imbibed. The most satisfactory mixture was one volume of the sugar solution to three volumes of defibrinated blood, mortality being lowest and egg production highest on this mixture. No comparative tests were made on the blood of different species, but for practical purposes beef, pig, or sheep blood was satisfactory. A solution of the sugar in distilled water was kept on hand and added to the blood as soon as possible (one to two hours) after it was collected and defibrinated. The mixture will keep in the refrigerator at 1° C. to 5° C. for two to three weeks. For cage feeding, the blood and sugar mixture, well shaken, was soaked up by absorbent cotton held in gauze bags. Much of the blood and sugar mixture passes through the insects undigested but this also occurs when the females feed on the arm. Unmated females that have fed on the blood will produce apparently normal rafts, but the eggs in these rafts never hatch.

The emergence cage, which was also used as a feeding and egg-laying cage,

had a wooden frame 12 inches square and 22 inches high. The top and back were of three-ply wood and the sides were plates of window glass that slid in grooves in the wooden frame. The front was of unbleached cotton with a sleeve of the same material. The bottom three inches of the frame were made of 1- x 3-inch boards, which were grooved on their upper edges to receive the window glass. The bottom of the cage was an inverted galvanized iron tray with sides one inch high and perforated with nail holes one inch apart. On the inside, the bottom of the cage was covered with fine sand about one inch deep and a layer of blotting paper was placed on the sand. The cage stood in a galvanized iron pan 15 inches square and 4½ inches deep. The pan was kept filled with water to just above the level of the bottom of the cage; this served to keep the sand and blotting paper moist and maintained a satisfactory humidity in the cage. Food for the adults was provided by two gauze bags about one inch in diameter filled with absorbent cotton and soaked in the blood and sugar mixture described above. Two small gauze bags each containing four or five boiled raisins were also supplied. The four gauze bags were hung by strings about six inches long from the ceiling of the cage. The blotting paper became soiled with the faeces of the insects and had to be replaced about once a month. The growth of moulds on the blotting paper was retarded by sprinkling it with table salt. A cage of this type accommodated 1500-2000 adult mosquitoes.

At 20°-21° C. the adults emerged in about two days after pupation. The males began to emerge first, and mating took place as soon as the females emerged, sometimes before the latter were dry. Consequently it was almost certain that single females observed resting on the walls of the cage had mated.

The adults may be handled in different ways, depending on the use to be made of the colony. For maintaining the colony, the adults were left in the emergence cage and a water surface for egg-laying was provided by placing in the bottom of

the cage two pans measuring 10 x 4½ x 1½ inches half-filled with water. The surface area of water provided for oviposition should be at least half that of the bottom of the cage. Under the artificial conditions in the cage the females apparently find the water surface by chance. If the free water area is too small, too many rafts are deposited on the moist blotting paper on the bottom of the cage. No air was bubbled through this water since the movement of the water surface disturbed the females resting there; instead, the water was renewed every second day. The egg-rafts were removed from the emergence cage each day. At temperatures fluctuating between 60° and 70° F., Owen (1942) obtained his first egg-rafts five to seven days after the first blood meal. At temperatures of 20°-21° C., I did not obtain the first egg-raft earlier than seven days after the first meal of the blood and sugar mixture. If a number of egg-rafts of approximately the same age were required, the females were deprived of a water surface until the tenth or twelfth day.

When confined in lantern chimney cages for experimental purposes, the adults were supplied the blood and sugar mixture soaked up in thin gauze pledgets one inch square. The pledgets were placed on top of the gauze or marquisette covering the chimney and covered with a watch glass. Each day these were renewed or alternated with boiled raisins.

The eggs of *C. inornata* can be stored for a limited time at 4°-5° C. if first allowed to darken. At these temperatures development proceeds very slowly so that when placed in the refrigerator, after aging for two to three hours at 20°-21° C., they will not hatch for about 21 days. Any time before this they can be removed from the refrigerator and will hatch normally. Temperatures of 4°-5° C. are fatal for eggs that have not yet darkened.

By this method the size of the colony that can be reared and maintained depends solely on the space and equipment available. With two constant-temperature cabinets large enough to hold three hatching pans, 14 larval rearing trays, and one

emergence cage, a colony of 1500-2000 adults can be maintained throughout the year.

Summary. A method is described for the continuous rearing of *Culiseta inornata* (Will.) in the laboratory. The larval rearing medium is a modification of one already recommended by Bates for *Anopheles* spp. Its successful application to *C. inornata* depends (1) on preventing a scum, or pellicle, from forming on the surface of the medium, (2) on rearing the first-instar larvae in a medium of high concentration, (3) on transferring the larvae only after the first moult is complete to a medium in which the concentration of food is gradually increased as the larvae grow, and (4) on reducing the concentration of second- and later-instar larvae to 150 in 2½ litres of the medium.

The adults are stenogamous. They feed readily and produce fertile eggs on a diet consisting of one volume of a ten per cent solution of sucrose to three volumes of defibrinated pig, beef, or sheep blood. This diet was presented to the adults soaked up in absorbent cotton or in thin gauze pledgets and required no other treatment to induce the females to feed. The blood and sugar diet was supplemented by boiled raisins.

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## ABSORPTION OF RADIOACTIVE DDT BY RESISTANT AND NONRESISTANT MOSQUITOES

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The use of radioactive insecticides appears to be a promising approach to studies of absorption and distribution of the toxicant in insects. Comparing the amount of insecticide absorbed by resistant and nonresistant strains may be of value in explaining causes of resistance. Sternburg, Kearns, and Bruce (1950) and Sternburg and Kearns (1950) determined by chemical analysis the amount of DDT absorbed and some of the metabolites formed. Lindquist, Roth, Yates, Hoffman, and Butts (1951), using radioactive DDT, found that the Orlando strain of resistant house flies absorbed considerably larger amounts of DDT than did susceptible flies.

The objects of the experiments reported

in this paper were to determine the amounts of DDT absorbed by resistant and nonresistant *Aedes nigromaculis* (Lud.) larvae and whether any of the DDT absorbed by the resistant larvae was degraded into other less toxic products.

### TESTS WITH *Aedes vexans* AND *A. sticticus*

Tests were first made with nonresistant *Aedes vexans* (Meig.) and *A. sticticus* (Meig.) larvae and pupae to develop satisfactory technique for preparation of the material. Information on the mortality and the amounts of DDT absorbed by larvae of these species at high and low temperatures was also obtained.

Methods. Tests were made in the laboratory at 70° F. unless otherwise stated, with a mixed culture consisting of approximately 55 per cent of *vexans* and 45 per cent of *sticticus* obtained from eggs

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