# STUDIES ON THE ARTIFICIAL INSEMINATION OF THE MOSQUITO *AEDES AEGYPTI* (LINNAEUS)[1]

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A successful method by which female mosquitoes could be artificially inseminated could conceivably provide the means for the establishment of laboratory colonies which do not normally mate under laboratory conditions. To date, the inability to colonize many species of mosquitoes has been the limiting factor in the study of various aspects of their biology, of their importance and potentiality as disease vectors, and of their hereditary characteristics.

This study was made to determine the feasibility of artificially inseminating *Aedes aegypti* (Linnaeus) mosquitoes. The ramifications of the problem included the study of their reproductive organs, the characteristics of the spermatozoa, the design and trial of insemination equipment, and the development of insemination techniques.

The establishment and maintenance of a mosquito colony was a primary requisite for the study. Dormant viable mosquito eggs adhering to paper toweling were placed in a pan of distilled water. Upon hatching the larvae were fed a mixture of finely ground dog food and brewer's yeast. To prevent the accumulation of larval food only small quantities were given at a daily feeding. Excess food, which eventually decomposed, was found to be detrimental to larval growth and development. The larvae were allowed to pupate and emerge in a rearing cage which was approximately 22 x 22 x 22 inches large. The adults were provided honey as a source of food. A rabbit was placed in the cage approximately every third day as the source of the blood meal necessary for mosquito egg development. The female mosquitoes oviposited upon moist paper toweling placed within the cage for that purpose. The toweling with the adhering eggs was periodically removed and stored in a humidor for future use.

The majority of the pupae were allowed to emerge in the rearing cage. However, a small percent of the female pupae were transferred to small plastic boxes, 1 x 1 x 1 inch large, and placed in holding cages. The holding cages were assembled by merely placing a lamp chimney within the cover or bottom of a Petri dish. A piece of bobbinet cloth was used to cover the other end of the chimney. The female pupae were easily separated from the male pupae, for they are distinctly larger than the males. In this manner it was possible to establish and maintain a suitable virgin female population for the study.

## INTERNAL MORPHOLOGY OF REPRODUCTIVE ORGANS

The nature of the study required a thorough working knowledge of the morphology of the mosquito's reproductive organs. The description of these organs, which follows, is concerned principally with the internal structure.

It should be noted that the differences found in the adult external terminalia, especially that of the male, have been widely used in classification of mosquitoes. Discussions of the morphology of the external male and female genitalia can be found in the literature.

MALE. Figure 1 is a ventral view showing the internal and external male reproductive organs. It will be seen that the male generative organs consist of a pair of testes which are dorsally located in the 8th abdominal segment, from which the sperm is carried by the lateral sperm

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ducts to the common sperm duct. Ducts from the accessory glands join the common sperm duct shortly before it terminates in the phallus. The phallus is surrounded by an elaborate arrangement of claspers and various chitinous structures which are collectively termed the genitalia.

FEMALE. The internal female reproductive organs are shown in a dorsal view in figure 2. The two ovaries, which lie dorsolaterally, are found in the 6th and 7th abdominal segments. From each ovary arises a short lateral oviduct. The lateral oviducts unite to form the common oviduct which extends posteriorly to its external opening, the bursal pouch, in the

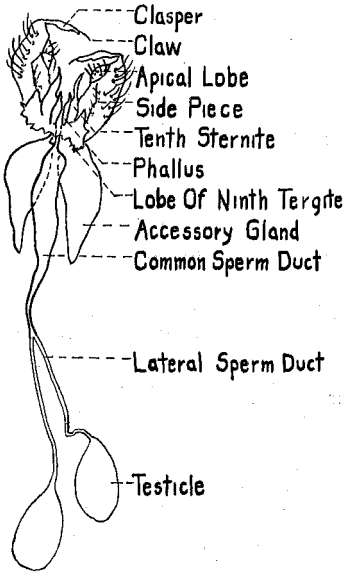


Figure 1

240 μ

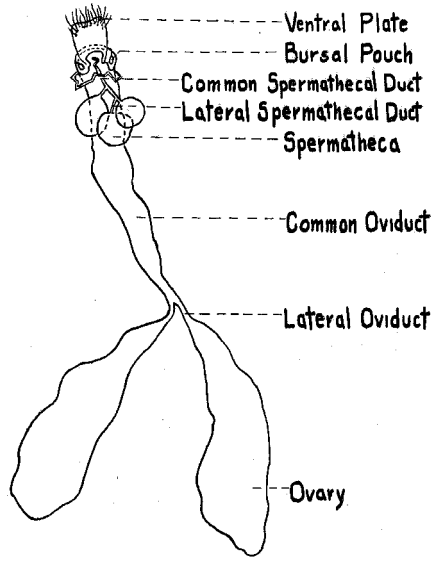


Figure 2

320 μ

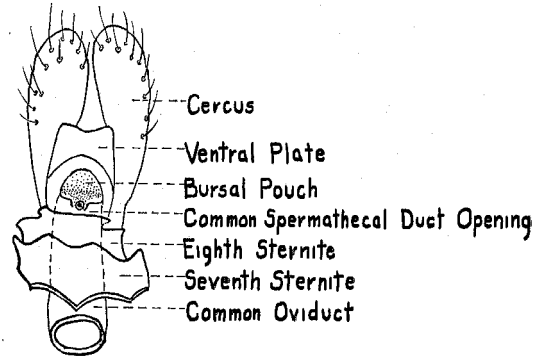


Figure 3

160 μ

8th segment. The three spermathecae which are readily observed as perforated chitinous sub-spherical bodies are attached by long narrow ducts which open jointly into the bursal pouch.

Figure 3 shows the opening of the common oviduct and the spermathecal duct opening from an external ventral view. The bursal pouch is a purse-like folding membrane normally drawn within a protective sheath formed by the 8th abdominal segment. The spermathecal duct opening, which is only 8 microns in diameter, is surrounded by a rigid sclerotic disc located medianly on the anterior dorsal surface of the bursal pouch. The ventral plate and the adjacent bursal pouch were pulled out for the purpose of the illustration.

## EVALUATION OF SPERMATOZOA

An evaluation of the sperm characteristics involved consideration of their appearance, abundance, and physiological requirements.

MORPHOLOGY. The spermatozoa consist of a long filamentous tail attached to an equally slender needle-like body. The sperm cells were found to average 0.7 microns in diameter and 132 microns in length. Of their over-all length, the body piece occupied 22 microns. No distinct division of the body piece into a head and a middle piece was observed in living or stained sperm when using 97X oil immersion lenses and 15X eyepieces on either a standard compound microscope or a phase microscope.

The filamentous tail of each sperm cell contained four or five large undulations which occupied its entire length. Small secondary waves within these undulations gave the tail a sinuous appearance.

Spermatozoa were stained only in their body region by basic stains. Giemsa's spirochete stain and Heidenhain's iron haematoxylin stain were the dyes used. The stain reaction indicated the presence of chromatin material in this region.

The definite body and tail regions could also be readily observed as the unstained living spermatozoa moved actively about in their characteristic rotary motion.

ENUMERATION. The number of sperm cells per cubic centimeter was determined with the aid of a double Improved Neubauer hemacytometer. Knowing the original fluid volume, the number of sperm available was then readily calculated.

A series of five counts showed the average number of sperm available to be 15,000. Since it was impossible to be sure that all the sperm had been removed from the reproductive organs, this number was not considered to represent the total number of spermatozoa. It was, however, believed to be an estimate of the average number of sperm present upon dissection and available for transfer.

PHYSIOLOGY. Some of the more important physical factors which are known to effect physiological activities were investigated. Temperatures above 40 degrees Centigrade were found to be lethal to the spermatozoa. The minimum lethal temperature was not determined, but reduced sperm activity was noted at 20 degrees Centigrade. The use of a heat absorbing thick frosted-glass filter on the microscope lamp was found to be essential for the reduction of heat produced by the lamp. The type of cool light described by Patton (1943) was also satisfactorily used to prevent heat fixing the sperm.

Sperm in uncovered drops generally remained active 15 minutes. Sperm in covered drops usually remained active 30 minutes. This was thought to reflect some deleterious effect due to oxidation. However, similar results were observed when covered and uncovered drops containing sperm were placed in an atmosphere of carbon dioxide.

A modified Belar (1929) saline solution having a pH of 6.9 was found to be the most satisfactory perfusion medium for sperm transfer. The determination of the osmotic pressure, the proper salt balance, and the hydrogen ion concentration was necessarily based upon that of the body fluid, and a paper concerning this problem is in preparation for publication at a later date.

### INSEMINATION STUDIES

Attempts were made to devise equipment which could be used to satisfactorily carry and deposit viable sperm in the female mosquito. A uniform system was established for the handling of the mosquitoes preparatory to and following attempted artificial inseminations, and several seemingly logical insemination procedures were investigated.

EQUIPMENT. Five syringes were investigated to determine a means of transferring the sperm to the female.

Syringes utilizing a machinist's type of micrometer caliper, a tuberculin syringe, and a variable transformer as their source of force to produce motion were investigated. However, a syringe assembled by merely fitting a glass micropipette within a short, approximately ten inches long, piece of transparent Polyethylene medical tubing was found to be the easiest to operate and the most satisfactory of all the syringes investigated. Its low cost of production, simplicity of construction, and lack of movable parts and connections requiring gaskets were also definitely advantageous.

The tubing had an 0.07 inch inside diameter and an outside diameter of 0.11 inch. De Khotinsky cement, medium grade, was used to secure the conduit to the micropipette. The micropipette tip was drawn from 0.07 inch soft glass tubing. The technique used was essentially that outlined by Barber (1914). A common laboratory microburner and one made from the tip of a number 27 Becton, Dickinson Yale syringe needle were used in drawing the glass. A finished micropipette tip had an outside diameter no greater than 50 microns and a bore diameter of less than 12 microns.

In operation the free end of the tubing was held in the operator's mouth, and movement in the system resulted from his application of positive or negative air pressure. The force providing the movement within the system could therefore be applied without the use of the operator's hands. Thus, the left hand was used for focusing the microscope or moving the object being worked upon, and the right hand could be used to direct the micropipette. It was found that with practice it was possible to discontinue the use of a micromanipulator and to hold and direct the micropipette with the right hand. The resulting spontaneous freedom of motion in all planes was an advantage of paramount importance.

A balsa wood holding block and staples fashioned from minuten insect pins were designed and found to be satisfactory for holding the females securely, without injury, during the insemination operation. Merely securing the mosquito to prevent flight was found to be unsatisfactory, since the abdominal movements of the female made complete immobilization necessary. Even though it was possible to hold the abdomen firmly enough to prevent lateral or verticle motion, it was not possible to prevent an avoiding reaction of the terminalia. This avoiding reaction consisted of a retraction of the genital region within the eighth abdominal segment to an even greater extent than to which it was normally retracted. This made the insertion of the micropipette tip into the bursal region exceedingly difficult. The retraction method of transferring sperm into the bursa of the female, to be described later, overcame the avoiding reaction difficulty to some extent.

HANDLING PROCEDURE. Untreated males which were at least 24 hours old were taken from the holding cage by means of an aspirator and were anesthetized with a mixture of carbon dioxide and air. They were then placed in a carbon dioxide atmosphere on a filter paper within a Buchner funnel. The apparatus utilized for maintaining the state of continuous anesthetization was similar to that described by Williams (1946). Each mosquito's wings and legs were removed immediately prior to its being placed upon a microscope slide and dissected.

The female mosquitoes were anesthetized in the same manner as the males. The female was then placed on her back in such a position on the pinning block that the tip of her abdomen would reach

the edge of the pinning surface. The minuten staples were then placed in position with the aid of fine-pointed jeweler's forceps. Usually three staples were found to be sufficient for securing the mosquito. The staples were placed about the body at the cervical, thoracic, and posterior abdominal regions.

Following the attempt at artificial insemination, the female was placed in a lamp chimney holding cage. Twelve hours later the female was allowed to obtain a blood meal. The twelve hour holding period served to insure feeding only those females not injured by the operation, the injured usually having died during that period. A strip of moist toweling was then made available, within the holding cage, as an oviposition site.

INSEMINATION PROCEDURE. The instrumental insemination procedure involved obtaining viable sperm from the male and transferring it to the bursal region of the female.

Several techniques were investigated in attempts to obtain the sperm. Only males at least 24 hours old were used. These were seldom devoid of sperm, but it was noticed that males dissected soon after emergence did not contain a supply of active spermatozoa. This situation may be be related to the phenomenon reported by Roth (1948), who, while working with *A. aegypti,* observed that the male genitalia must undergo a nearly 180 degree rotation before copulation can be successfully completed. He also showed that it seldom required more than 25 hours for the rotation to be completed.

The dissection of male mosquitoes was carried out in a drop of physiological salt solution. Minuten insect pins mounted in wooden applicator sticks were used to sever the abdomen between the 7th and 8th abdominal segments and to remove the posterior sternites and tergites. The male reproductive organs were then cut at the point where the common sperm duct enters the phallus and were transferred to a smaller drop of saline solution. If debris had not accumulated in the dissecting medium during the course of the operation, the testes, the sperm duct, and the accessory glands were not transferred. A dissecting microscope having wide field oculars and a magnification of 30 diameters facilitated the operation.

To release the spermatozoa, the common sperm duct, lying between the two accessory glands, was cut at the point where it entered the phallus. The sperm then flowed from the common sperm duct and commenced to actively disperse throughout the drop of saline.

Attempts to squeeze sperm from intact males, as can be done in the case of drone bees, have failed. The diminutive size of the insect made it seemingly impossible to evert the phallus and force sperm from the reproductive organs by the application of external pressure.

Although Rees and Onishi (1951) were able to cause the sclerotized plates of the phallus, in *Culiseta inornata* (Williston), to be extended by stimulation shortly following copulation, no noticeable response was observed to the rubbing of the *A. aegypti* terminalia. Stimulation in this manner failed to lead to the production of an available sperm supply.

Attempts were made to bring about an ejaculation by the mosquitoes as the sympathetic resultant of a shock reaction. Ejaculation failed to be induced, however, by the methods of shock utilized.

The transfer of viable sperm into the bursal region of the virgin female mosquitoes was accomplished by several methods. An injection method was utilized by directing the micropipette tip into the bursa of the female. The sperm were then discharged directly from the syringe into the bursal pouch. In some cases, only a small quantity of the sperm suspension was introduced, and the micropipette tip was immediately withdrawn. At other times, a relatively large amount of the spermatozoal fluid was injected to form a drop which adhered to the genital region. Still other trials were conducted in which prolonged or repeated transfers were made, both with the sparse and the voluminous types of injection.

Due to the minute size of the spermath-

ecal duct opening, 8.0 microns in diameter, and its inflexible characteristic, attempts to inject the spermatozoa directly into the spermathecal ducts were unsuccessful.

In several instances all glass contacting the sperm suspension was treated with an anti-wetting agent to prevent physical damage to the sperm due to contact with the glass.

It should be stated that the technique for exposing the bursal pouch, to be next described, was incorporated in many of the injection method transfers.

The retraction method consisted of grasping the ventral plate of the female with fine-pointed jeweler's forceps and gently pulling with the right hand so as to expose the bursal pouch. The excised testes and common sperm duct of the male were then placed directly upon the extended purse-like bursal pouch and ruptured to liberate the sperm. The ventral plate was then released, and as the ventral plate and the adjacent bursal pouch retracted to their normal position within the 8th abdominal segment, the released sperm were carried along. The introduction of spermathecae containing viable sperm removed from naturally mated females was also accomplished in this manner. The spermathecae were crushed to liberate the sperm immediately prior to releasing the ventral plate.

The debris was removed 24 hours after the operation to prevent its interference with subsequent oviposition, and to eliminate it as a source of bacterial infection.

## RESULTS

To date, the artificial insemination of 105 virgin *A. aegypti* females has been attempted. Six of the mosquitoes did not survive the 24 hours holding period; the rest, however, lived at least one day and were given an opportunity to take a blood meal. Fifty-seven of the treated females became engorged, and thirteen of these individuals were induced to feed upon more than one occasion. Two blood meals were the maximum number to be taken by any of the mosquitoes. Only two of the engorged females failed to survive the minimum four days required of oviposition following the blood meal. One female survived 57 days following the attempted instrumental insemination and the blood meal.

A total of 542 eggs were laid by 22 of the test females. As a result of repeated blood meals, five of the mosquitoes laid eggs upon two separate occasions. The maximum number of eggs laid by a female during a single oviposition period was 75 eggs. The largest number of eggs obtained from a single mosquito was 85; this was, however, the sum of the eggs produced upon two separate oviposition occasions. The minimum number of eggs laid by an individual was one. Four mosquitoes were observed to have laid a single ovum, whereas a single egg was laid by another female following a second blood meal and an earlier egg batch. One egg was laid by a female which had not partaken of a blood meal, and one egg was produced by one of twenty blood-fed virgin female mosquitoes, which were untreated.

Approximately ten percent of the test mosquitoes were sacrificed to permit an inspection of their spermathecae. Unfed and fed females which had survived the attempted instrumental insemination but which had not laid eggs, and mosquitoes which had fed and oviposited were examined. In no case was a spermatheca observed which contained sperm.

None of the eggs laid by the treated females hatched to produce larvae. Many of the submerged eggs collapsed and were readily seen to be nonviable. Upon dissection, none of the remaining eggs were found to be viable.

All females which had mated naturally, and which were subjected to the techniques of artificial insemination employed, produced normal numbers of viable eggs.

## DISCUSSION AND CONCLUSION

The studies upon the instrumental insemination of mosquitoes were necessarily attempts to duplicate the phenomenon effected by a natural mating.

Marshall (1938) reported fertilization in mosquitoes to take place at the time of oviposition. At that time, the spermatozoa travel from the spermathecae and enter the egg through the micropyle as it passes the opening of the spermathecal ducts. Thus the problem was basically one of transferring viable sperm, which would fertilize eggs during oviposition, to the female.

Giles (1902) believed the Culicid spermatic element of the male to be "transferred *en masse* from the receptacles of the male to those of the female." In this study, the transfer of active sperm into the bursa of the female was successfully accomplished, for it was possible to recover the active sperm immediately following a transfer. Clean micropipettes were used in making all such recoveries.

Attempts to deposit sperm in the spermathecal ducts were unsuccessful, due to the minute size of the duct's external orifice. Watson (1927), and workers to date, met with the same difficulty in the queen bee. He, however, observed that the bee sperm was deposited in the oviduct, and that the sperm migrated by their own activity to the spermatheca of the female. At the present time, there is no evidence to indicate that such is not the case in mosquitoes.

Had the attempts at artificial insemination by transferring sperm-filled spermathecae of naturally mated females been successful, as was reported by Gottschewski (1937) using *Drosophila melanogaster* Meigen, it would not have provided a means of maintaining colonies of mosquitoes which do not normally mate under laboratory conditions, since it would require a source of naturally mated female mosquitoes.

The males and females utilized ranged from one to six days old; adult *A. aegypti* of this age group were shown by Roth (1948) to be capable of successful matings.

Future studies upon the artificial insemination of the mosquito will be conducted to seek an explanation for the failure of the sperm to survive the transfer and migrate into the spermathecae. It is hoped that sperm transfer will be facilitated in these studies by the development of a holding device which readily exposes the bursal area and at the same time prevents the avoiding reaction of the female.

The diminutive size of the mosquito imposed strict limits upon the specifications of the equipment design and upon the techniques of sperm transfer. Due to these limitations, it was not possible to obtain and transfer undiluted sperm by a pipette method, as is done for the artificial insemination of other insects.

The production of viable eggs by treated naturally mated females and of sterile eggs by females treated either before or following a blood meal indicated the technique had no deleterious effects upon oviposition. It was, therefore, concluded that the inability of the sperm to survive the transfer and to fertilize the eggs during oviposition resulted in the production of sterile eggs.

## SUMMARY

Attempts to artificially inseminate the mosquito *Aedes aegypti* (Linnaeus) were made. The important features of the mosquito culture technique, the anatomy of the sex organs, the evaluation of the spermatozoa, and the insemination studies were described. Twenty of 105 treated females laid a total of 542 eggs, but the inability of the sperm to survive the transfer and fertilize the eggs during oviposition resulted in the production of sterile eggs.

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# THE EFFECT OF CONTAMINATION UPON MOSQUITO LARVAE IN RAIN-WATER BARRELS

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For several years, six rain-water barrels located in a rather poorly-lighted place adjacent to a dense beech-maple forest in Cuyahoga County, Ohio served as favorite collecting spots for various species of mosquito larvae.

During the months of May and June, 1952, these barrels teemed with larvae of *Aedes triseriatus, Culex pipiens,* and *C. restuans,* with *A. triseriatus* dominating. On July first, the author accidently upset the contents of a kitchen garbage container in one of the barrels. It was decided to continue making regular collections from all six barrels and note whether or not there would be any differences in the mosquito populations of the barrels. Following are the results of these observations.

July 1, 1952—All six barrels contained mosquito larvae in approximately these proportions: *A. triseriatus,* 6; *C. pipiens,* 2; *C. restuans,* 1. Added garbage to barrel number six only.

July 15, 1952—Barrels one to five still maintaining the proportion of larvae as described previously. Barrel number six very foul and odoriferous, no larvae present.

July 30, 1952—No apparent changes in the mosquito population of barrels one to five. Number six teeming with *C. pipiens* only.

September 3, 1952—Barrels one to five contained only *A. triseriatus.* Barrel number six contained only *C. restuans.*

September 14, 1952—Barrels one to five contained *A. triseriatus* and *C. pipiens* in the proportion of six to one. *C. restuans* had disappeared entirely.

Barrel number six teemed with *C. restuans* only.

September 20, 1952—Barrels one to five contained *A. triseriatus* and *C. pipiens* in the proportion of one to one.

Barrel number six teemed with *C. restuans.*

October 1, 1952—Barrels one to five contained *A. triseriatus* and *C. pipiens* in the proportion of one to one.

Barrel number six contained *C. restuans* only.

From these collections made during the summer of 1952, it looked as if the addition of the garbage to barrel number six immediately eliminated all larvae; then as it was diluted by rain *C. pipiens* first appeared only to be completely replaced by *C. restuans* which remained for the rest of the season. *A. triseriatus* never did reappear in barrel six, possibly because of the contaminating material present but it was speculative as to why *C. restuans* seemed to "prefer" the contaminated barrel only in which to breed after the first of July.