STUDIES ON SPERMATHECAL FILLING IN AEDES AEGYPTI (LINNAEUS). I. DESCRIPTION[1]

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In a study on the female reproductive system of *Culex pipiens,* Kulagin (1901) illustrated for the first time a flask-shaped sac opening into the vagina, but he mistook it for the accessory gland. Not having seen Kulagin's study, Christophers (1923) clearly recognized that the accessory gland of mosquitoes was a separate structure from the dorsal diverticulum of the vagina and he termed this sac the *caecus.* Brelje (1924), apparently unaware of Christophers' work, pointed out Kulagin's error and described and illustrated the connections between the accessory gland, bursa copulatrix, spermathecae, and vagina in *Mochlonyx, Culex, Culiseta,* and *Aedes.* He termed the dorsal diverticulum of the vagina the bursa copulatrix. Brelje also discovered that the male mosquito deposited seminal material into this sac. Christophers (1960, p. 679) stated in his monumental work on *Aedes aegypti* that "the *caecus* . . . is a relatively small structure with the characters of a mucus gland," thus repeating Kulagin's confusion of two very different organs. In 1957 Burcham rediscovered both the structure and function of the bursa in *Aedes* and subsequently located Brelje's paper. Unfortunately Burcham's thesis was not published. Not having seen the earlier work, Hodapp *et al.* (1960) again rediscovered the structure and function of the bursa of *Aedes.* Some of this curious history was brought to light by Curtin and Jones (1961).

It has been known for a long time that female mosquitoes store sperm within from one to three spherical organs called *spermathecae* (Dufour, 1851), but it was not until 1957 that Burcham first explored some of the problems of how the sperm reach the thecae. He stated that a few sperm reach the storage organs within one minute after coitus, and he noted that few to numerous sperm were present within them in two minutes. He further remarked (p. 80) that the number ". . . steadily increased up to about five minutes after coitus" and that "the bursa copulatrix was essentially emptied within five minutes after mating." Schwartz (1961) found that sperm reached the thecae of *A. aegypti* between the 40th and 156th seconds after coitus. Spielman (1964), working with the same species, reported that sperm do not reach the thecae during the first 30 to 35 seconds after coitus but fill the organs in a period between 40 and 300 seconds.

It is the purpose of this paper to give a more detailed description of spermathecal filling in *A. aegypti* (Bangkok strain) than is currently available. A subsequent paper will deal with some of the physiological aspects of filling.

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Materials and Methods

The mosquitoes were reared in an insectary at 26° to 29° C. with a relative humidity ranging from 70% to 80%. The eggs were hatched in freshly boiled tap water that had reached room temperature and approximately 100 larvae were pipetted into a stender dish containing 250 ml. of water and a small pellet of Purina dog chow. When the larvae pupated, the pupae were sexed by placing them laterally on an ice cube and examining the pronounced differences in their external genitalia. The pupae were pooled in a beaker of water according to sex and placed in a one-cubic-foot screened cage so that the sexes would be completely separated at emergence. Adults had continuous access to 5% sugar water and the majority of the females were not offered a blood meal. Most of the studies were made on 3- to 7-day-old virgins. In the great majority of the experiments, the mosquitoes were forced to copulate by the technique of McDaniel and Horsfall (1957). Wheeler's modification (1962) of this technique proved essential to many of the observations required. Adults were generally anesthetized with nitrogen just before use. A fast-drying, non-toxic adhesive (Dekadhese) was applied to the head of Ward's #1 or #2 insect pin and gently but firmly pressed to the dorsal surface of the thorax. A series of males and females were thus arranged. The female-bearing pin was inserted into a cork glued to a microscope slide and the preparation placed under a dissecting stereo-microscope. The male-bearing pin was inserted into an adjustable holder allowing for gross and fine vertical movements. The male was moved up and down until his terminalium came into contact with that of the female at an angle of about 90°.

Dissections were made by grasping the thorax of the mosquito with sharpened Dumont #5 microforceps and the terminalium was placed into a small drop of buffered *Drosophila* saline (Ephrussi and Beadle, 1936). One finely sharpened needle was used initially to extract the organs to be studied. Further dissections, if needed, were made using two needles. Some extractions were made by using a second pair of microforceps to pull out the desired organs. Further experimental details are given in the text.

Results

1. *Efficiency of spermathecal filling after forced-copulation*

Virgins of the Bangkok strain force-copulated from 4 to 221 seconds and averaged 31.3 seconds with a standard error of 1.6 seconds (132 observations). In 18 cases which were allowed only one to five seconds of coital contact, only one was inseminated. In 8 cases which were allowed 9 to 10 seconds of coital contact, five (62%) were inseminated. In 23 cases which were allowed 15 seconds coital contact, 21 (91.3%) were inseminated.

Out of 737 cases of forced-copulation, the bursae in 8 females were observed to have what appeared to be only male accessory gland material and no visible spermatozoa. Three of these cases could be accounted for because the male used was found to be sterile and had no sperm cells available. Nevertheless, this agametic male copulated readily with 6 females in rapid succession. In two other cases, a male's freshly isolated terminalium had been used to copulate intact females. But three of the cases could not be accounted for, which presumably

indicates that on rare occasions a normal male can ejaculate only accessory gland material.

Both sperm and male accessory gland material were present in all bursae of 41 (82%) of 50 females which were force-copulated and in all but one, sperm reached the spermathecae. Of these, 41.5% had sperm in all three thecae, 48.8% had sperm in two thecae, and 9.7% had sperm solely in the large median theca.

During the first 10 minutes after forced-mating, sperm moved about within the thecae of all females examined (28 cases; 44 thecae with sperm). In those females which were dissected 30 minutes to 6 hours after forced-mating, sperm moved in

TABLE I

The extent and degree of spermathecal filling and motility of stored sperm in Aedes aegypti after different periods of unrestrained mating. Ten females used for each period

Time free coitus	Mean degree thecal filling	Large spermatheca			Lateral spermatheca			Lateral spermatheca			Bursa	
		No.	Degree	No. with motile sperm	No.	Degree	No. with motile sperm	No.	Degree	No. with motile sperm	No.	Condition
3 hrs.	2.70+	10	4+	3	10	3+	3	10	1+	3	10	Distended
6 hrs.	2.63+	10	4+	1	8 2	3+ 2+	2 2	9 1	1+ 2+	2 1	10	Distended
24 hrs.	2.70+	10	4+	8	10	3+	8	10	1+	10	10	Partially distended*
48 hrs.	2.57+	9 1	4+ 3+	10	10	3+	10	8	1+	8	3	Partillly distended**
72 hrs.	2.70+	10	4+	10	9 1	3+ 4+	10	6 2	1+ 2+	8	10	Empty

* Some sperm present in 50% of bursae; undulations of sperm seen in only one bursa.
** A few sperm seen in one bursa.

30% to 62% of the thecae containing them (20 females; 56 thecae with sperm). Twenty-four hours after forced-mating, sperm were active in 80% of the thecae containing them (5 females; 10 thecae with sperm).

2. *Efficiency of spermathecal filling with cage-copulated mosquitoes*

Using Spielman's technique (1964), individual virgin females of the Bangkok strain were introduced into a 4 × 6 in. lantern chimney containing a number of unmated males, in order to obtain information on the time required for normal mating to begin, and to determine the time of coital contact under free mating conditions. The chimney was shaken to stimulate flight and copulation. In 5 cases, 13 to 32 seconds elapsed before copulation occurred (mean of 19.4 seconds). The mating time under these conditions ranged from 9.5 to 16 seconds, with a mean of 13.2 seconds. Ten seconds of coital contact under free mating conditions usually resulted in insemination. The mean coital time of 13.2 seconds is in close agree-

ment with Spielman's value of 13.7 seconds for the Johns Hopkins strain of *A. aegypti*. Using larger cages, Roth (1948) and Burcham (1957) obtained a mean coital time value of about 16 seconds.

Three-day-old unmated males and females, 10 of each, were placed in each of five one-cubic-foot cages, and allowed to copulate freely for 3, 6, 24, 48, and 72 hours. The females were dissected and the condition of the bursae, the extent and degree of filling of the three spermathecae, and the motility of the stored sperm were noted. The degree of filling was qualitatively judged as follows: 4 +, numerous sperm; 3 +, many sperm; 2 +, few to many sperm; 1 +, very few sperm; and 0, no sperm detectable. The data for the five groups are summarized in Table I. Of the 50 females examined, 92% had sperm in all three thecae and 8%

TABLE II

Copulatory behavior and potency of a single six-day-old male Aedes aegypti when offered 12 virgin fenales in a 20-minute period

Virgin female number in order of presentation to the male	Seconds of coitus	Notes
1	10.2	Female escaped after coitus
2	13.6	Bursa full; 2 thecae with sperm
3	14.2	Bursa partially filled; 2 thecae with sperm
4	22.4	Bursa partially filled; 1 theca with sperm
5	10.8	Female dislodged the male; female not inseminated
6	68.0	Uninseminated
7	13.8	Female dislodged male; female not inseminated
8	25.8	Uninseminated
9	—	Male repeatedly attempted to clasp female's terminalium but aedeagus did not erect
10	22.8	Uninseminated
11	—	Male repeatedly erected aedeagus but did not establish good genital contact; uninseminated
12	—	Male erected aedeagus but only feebly clasped female's cerci and did not establish good genital contact

had sperm only in two thecae. Numerous sperm were in the large median theca in 98% of the cases, 94% had many sperm in one lateral theca, and 84% had a few sperm in the other lateral theca. One to 6 hours after free mating, sperm were actively moving in 26.7% to 33.3% of the thecae containing them (30 females; 72 thecae with sperm). After 24 hours, 86.7% of the thecae contained active sperm (10 females; 30 thecae with sperm). After 48 and 72 hours, sperm were active in all of the thecae containing them (20 females; 56 thecae with sperm).

It is evident that data from forced-mating of virgins are significantly different from data from unrestrained or free mating between virgins. Thus, coitus lasts about twice as long with forced-mating, and the extent of insemination and thecal filling is more variable with forced than with unrestrained matings. Furthermore, sperm within the thecae become active more rapidly following forced-mating. The reasons and significance of such differences are not clear.

3. *Potency of individual males and spermathecal filling*

Each of five previously unmated males which were 6 days old were offered 4 to 12 virgin females in succession within a 10- to 20-minute period, using the forced-copulation technique. These males successfully copulated with 4 to 9 females and inseminated 3 to 5 of these. Data on one male which attempted to copulate with 12 females in a 20-minute period are shown in Table II. This individual was able to establish good genital contact for 10 to 68 seconds with 9 females, at least three of which he inseminated. When this male was subsequently dissected, his accessory glands appeared essentially like those of a once-mated male, but his seminal vesicles were shrunken and possessed only a few spermatozoa. As illustrated by the data in Table II, copulation can occur without insemination and even prolonged coitus (as with female #6) does not necessarily result in insemination. Erection of the male's aedeagus was not necessarily followed by copulation (as with females 11 and 12, Table II). In an earlier study (Jones, 1961), it was shown that when virgin males are allowed to copulate freely with a great excess of females in a cage for one hour, they inseminate about five females and their seminal vesicles are usually completely devoid of sperm and their accessory glands are greatly reduced in diameter and have little secretory material. This is in striking contrast to the repetitively force-mated males which only rarely get rid of all the seminal vesicle sperm and apparently ejaculate much less accessory gland material into the females. Conceivably, this difference in the amount of accessory gland material in the ejaculate could account for some of the differences already noted in the last section.

Two unmated males were presented to 6 virgins each and allowed to force-copulate with each one for 15 seconds, and the females were then dissected after 23 to 69 seconds. The first male inseminated 4 of the 6 females. The single, large, median spermatheca in all 4 of the 6 females had few to numerous spermatozoa; three of the females additionally had one of the lateral thecae with few to numerous spermatozoa. The second male inseminated 5 of the 6 females. Very few to numerous spermatozoa were found in the median theca in all 5 cases. Four of the females additionally had a few sperm in one lateral theca. In all of the above cases, the sperm in the thecae were inactive.

4. *Number of spermatozoa in the reproductive system*

Some preliminary estimates on numbers of spermatozoa were made on different portions of the male and female reproductive systems after forced-mating, using squashed whole mounts stained with aceto-lacto-orcein after Carnoy fixation. Sperm heads took the stain strongly and these were counted at a magnification of 970 × with the aid of an ocular grid. The inherent counting errors are considered large because the sperm often failed to spread evenly and this failure was especially evident with spermathecal squashes.

As shown in Table III, the terminal testicular chamber of one unmated male had approximately 700 spermatozoa. The terminal testicular chamber of three repetitively force-mated males had from 333 to 1209 spermatozoa (mean of 741.3). The sperm duct (vas deferens plus vas efferens) of one unmated male had 370 spermatozoa. The seminal vesicles of three unmated males had from 3700 to 6309

sperm (mean of 5132.3), while the seminal vesicles of two repetitively force-mated males had from 485 to 1374 sperm (mean of 929.5).

The bursae of 6 females which were freshly inseminated by a single highly potent male (he force-copulated in rapid succession with 7 females) were dissected within one to two minutes after each ejaculation. This one male ejaculated 254 to 2655 spermatozoa, and progressively fewer sperm were released with each succeeding forced-copulation (Table III). In sum, this male delivered 6126 sperm to 6 females and he did not inseminate the seventh female.

TABLE III

Numbers of spermatozoa in aceto-lacto-orcein squashes of different tissues of Aedes aegypti

Tissue	No. cases	Estimated numbers of spermatozoa
Terminal chamber of testis of unmated male	1	700
Terminal chamber of testes of repetitively, force-mated males	3	333; 682; 1209 (mean, 741.3)
Sperm duct of unmated male	1	370
Seminal vesicles of unmated males	3	3700; 5388; 6309 (mean, 5132.2)
Seminal vesicles of repetitively force-mated males	2	485; 1374
Bursa 1 to 2 minutes after:		
First ejaculation	3	1142; 1744; 2655 (mean, 1847)
Second ejaculation	1	554 (?)
Third ejaculation	1	1248
Fourth ejaculation	1	937
Fifth ejaculation	1	478
Sixth ejaculation	1	254
Bursa 1 to 1½ hours after first ejaculation	3	377; 584; 1142 (mean 701.0)
Bursa 1 to 1½ hours after second ejaculation	1	119
Spermathecae		
Large	4	141; 319; 325; 600 (mean, 346.3)
Small	4	68; 200; 250; 500 (mean, 254.5)
Thecae from 6 females after first ejaculation and 1–1½ hrs. after copulation	10	73-400 (mean 225.5)

The spermathecae of four females were dissected about one hour after forced-copulation with previously unmated males. Counts of spermatozoa in the large median theca varied from 141 to 600 (mean of 346.3), and counts of spermatozoa in one of the lateral thecae varied from 68 to 500 (mean of 254.5). Other counts made on spermathecae of unspecified size ranged from 73 to 400, with a mean of around 226.

The bursae of three females were dissected one to 1½ hours after forced-copulation with fresh unmated males. The number of spermatozoa that remained in the bursa after thecal filling had occurred was estimated to vary from 377 to 1142 (mean of 701) (Table III). These counts are considered to be much more reliable than direct spermathecal counts and suggest that of 1847 sperm (Table III) deposited in the bursa, about 1146 (62%) can reach the thecae. This, in turn, suggests that about 660 sperm reach the large theca and about 486 reach one of the lateral thecae. If these suggestions are correct, then the direct thecal counts of sperm are in error by a factor of about 2.

From these various estimations the following suggestions can be made. (1) The unmated male has about 5000 sperm available within his seminal vesicles. After rapid repetitive forced-mating, the male can ejaculate about 82% of the sperm within his vesicles. (2) The male ejaculates progressively fewer spermatozoa into each successive female. (3) Sixty-two per cent of the sperm initially deposited in the bursa reach the thecae, and 38% remain in this sac immediately after thecal filling.

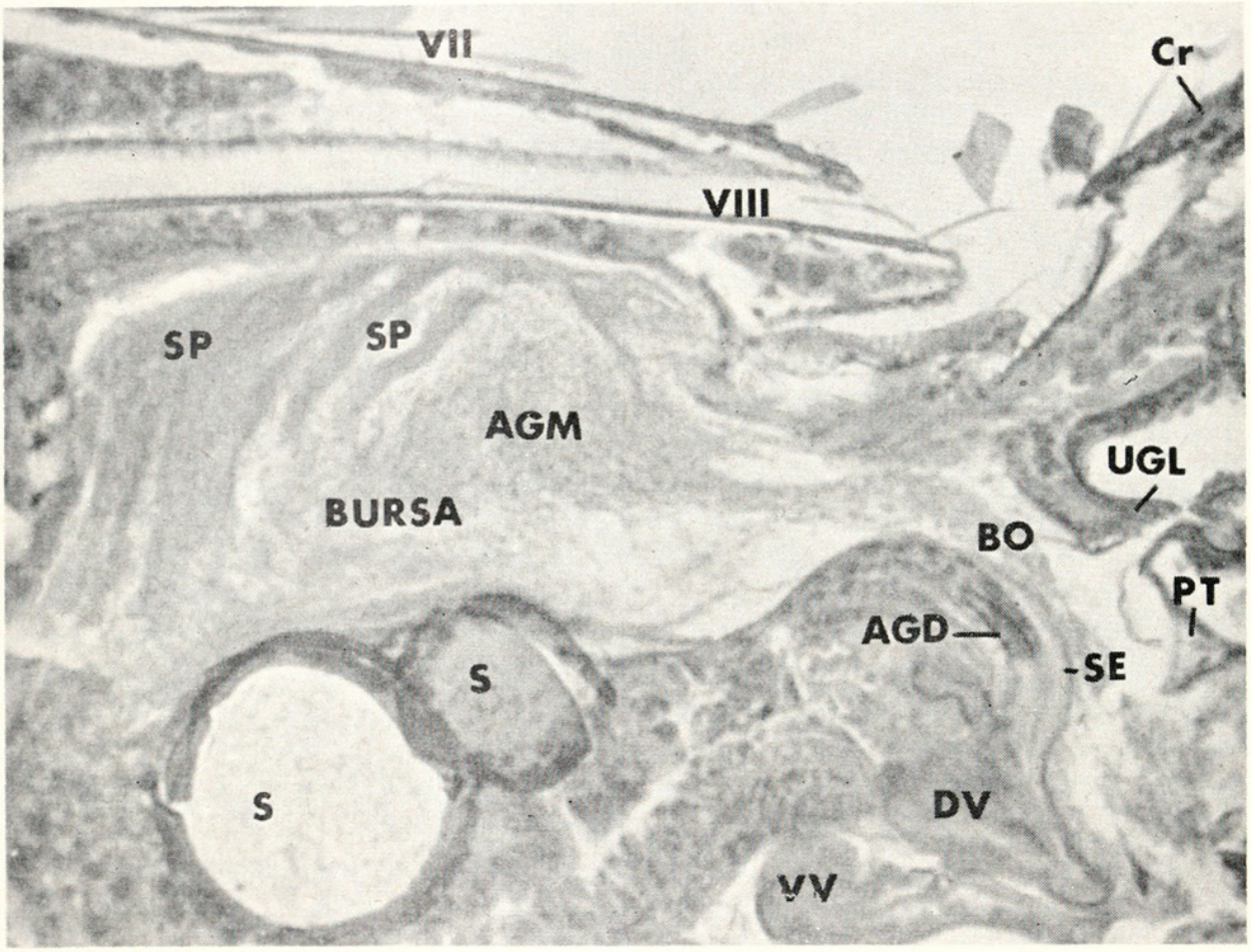

FIGURE 1. Sagittal section of *Aedes aegypti* female immediately after ejaculation of the male, showing the seventh (VII) and eighth (VIII) abdominal segments, cerci (CR), upper genital lip (UGL), bursal orifice (BO), accessory gland duct (AGD), spermathecal eminence (SE), dorsal valve (DV), ventral vaginal valve (VV), and spermathecae (S) of the female. Note the spermatozoa (SP) in packets in the dorso-anterior portion of the bursa and the large amount of finely granular male accessory gland secretion. The phallotreme of the male is shown at PT.

5. *The composition of the ejaculate*

The normal ejaculate consists of a relatively small amount of spermatozoa and a much larger amount of an acidophilic holocrine secretion of the male's accessory glands. Presumably, the spermatozoa are contained in a small volume of fluid within the seminal vesicles, and it is assumed that some of this seminal fluid is added to the ejaculate. When the ejaculate is seen under the dissecting microscope, it appears whitish; when viewed with a compound microscope, it has a greyish yellow cast.

The male accessory gland secretion includes a clear to finely granular material, round to ovoid granules of at least three different sizes, a few free nuclei, and even some intact, round to ovoid accessory gland cells of various sizes with large granular inclusions. If the accessory glands are ruptured in an open drop of saline, the exuding material does not usually vacuolate and the cells, free nuclei, and granular inclusions do not ordinarily lyse, although the cells may become swollen. The accessory gland secretion in an open drop of saline forms a dense, viscous, sticky mass which will rapidly clog a micropipette. However, if the saline mount is quickly covered with a layer of immersion oil before the glands are ruptured, then the exudate can be generally drawn into a micropipette without clogging.

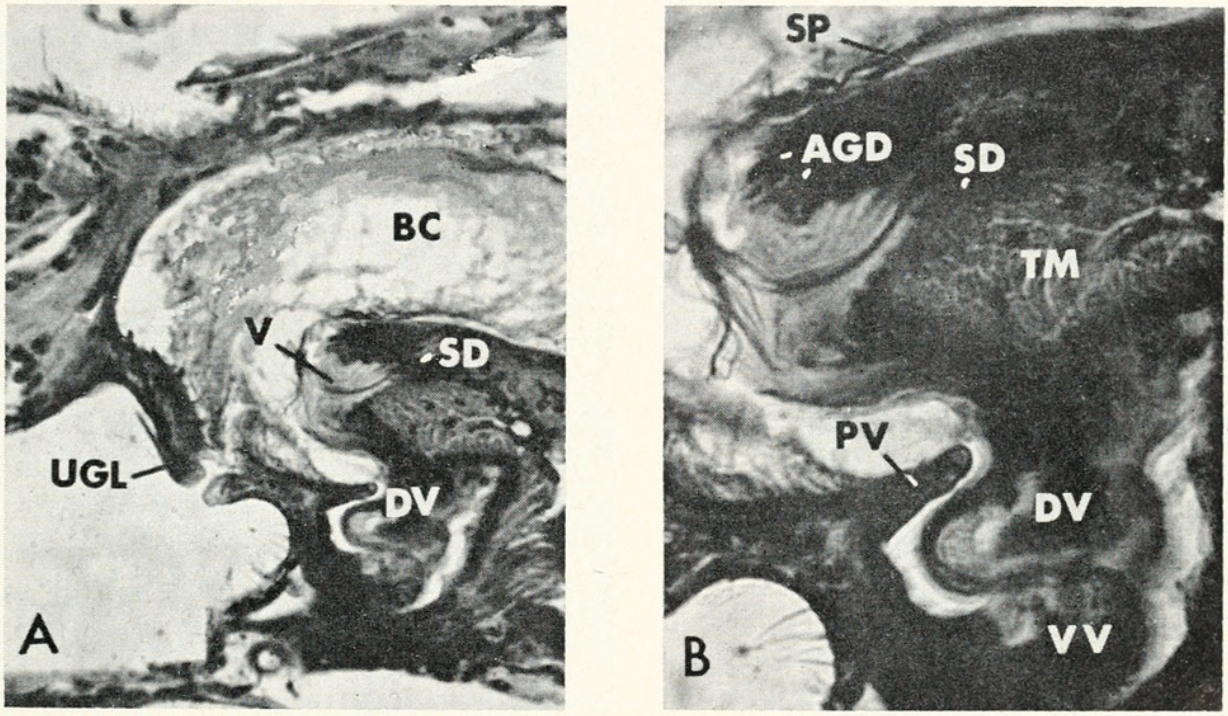

FIGURE 2. Sagittal section of *Aedes aegypti* female during spermathecal filling. In A is shown the upper genital lip (UGL), the swollen bursa (BC), the vestibule (V), the spermathecal duct (SD), and the dorsal vaginal valve (DV). B is the same section, at greater magnification, showing the sperm (SP) assembled in packets on the ventral floor of the bursa, and sperm making the U-turn into the vestibule. Note the position of the accessory gland duct (AGD), one spermathecal duct (SD), the posterior vaginal valve (PV), dorsal vaginal valve (DV) and ventral valve (VV). The transverse muscles of the spermathecal eminence are shown at TM.

6. *Some changes in the ejaculate within the bursa*

At the very moment of deposition, the locomoting packets of sperm are ejected dorsally above the accessory gland secretion (Fig. 1). In sagittal sections, the freshly inseminated bursa measures about 300 to 350 μ in length and varies from 78 to 100 μ in depth. The bursal orifice measures from about 18 to 25 μ. The spermatozoa measure about 250 μ (Christophers, 1960). While most of the sperm rapidly spread to the edges of the bursa, some of them become trapped in the granular portion of the accessory gland secretion. As Spielman (1964) has pointed out, many sperm rapidly assemble on the ventral wall of the bursa facing

the orifice (Fig. 2B, SP). Those sperm at the blind anterior end of the bursa tend to remain quite active for about 17 minutes in oil-covered drops of saline, after which they tend to become noticeably less visible and less active. Those sperm at or near the bursal orifice tend to be especially active.

Thirty seconds after ejaculation, the bursal wall in sagittal sections measured 2.2 to 3.3 μ. Two to three minutes after insemination, the bursal wall was greatly swollen (7.5 to 12 μ thick), hyaline, and the cells had large colorless vacuoles. The bursal wall was swollen and vacuolated for at least one hour after insemination. In fresh unstained whole-mounts of freshly inseminated bursae, we have seen what appeared to be a very delicate membrane surrounding the seminal mass, but no such membrane was visible in any of the histological sections.

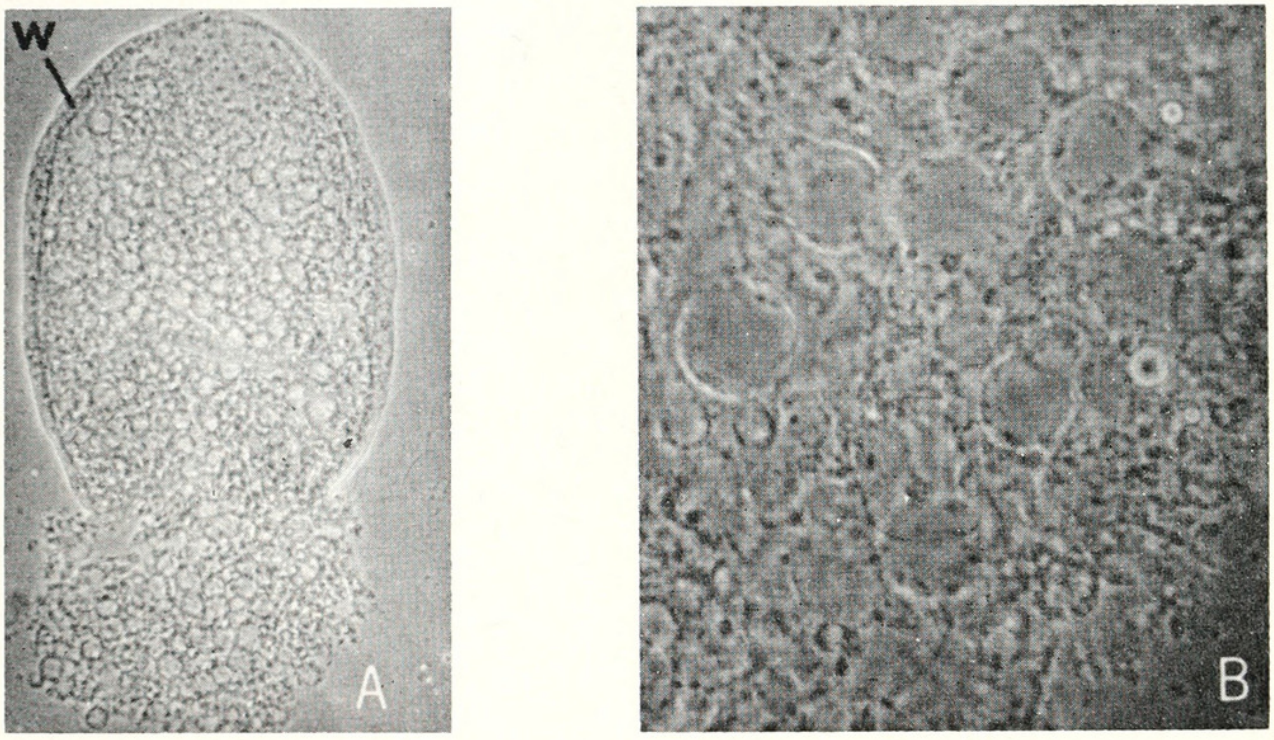

FIGURE 3. Unstained saline whole-mount dissection of the bursa of *Aedes aegypti* about 10 minutes after insemination, showing the fully vacuolated ejaculate. Note the swollen wall (W) in A and, at greater magnification (B), the large vacuoles and granules within the ejaculate. The largest vacuole in B is about 20 microns in diameter.

Three to five minutes after insemination, large vacuoles first appear within the granular portion of the ejaculate and they steadily increase in number until the bursal contents become filled with vacuoles within a granular matrix (Fig. 3). The large vacuoles measure about 20 μ in diameter. These vacuoles are very clear in whole mounts but may be indistinct in sectioned material. The ejaculate may be fully vacuolated within about 10 minutes at 27° C. At 36° to 37° C., the bursal contents fully vacuolate in about one minute. The completely isolated, freshly inseminated bursa will fully vacuolate in a drop of saline covered with a layer of immersion oil. When a male's accessory glands were crushed in the vicinity of the freshly dissected virgin bursa, the glandular exudate did not vacuolate. If the female is hosed with carbon dioxide for 5 minutes three seconds after coitus, the seminal material within the bursa does not vacuolate.

In many preparations in which the bursal contents were vacuolating or had already fully vacuolated, the sperm were not visible through the intact wall of the bursa, but when the bursa was opened, sperm were found. Bursal sperm retain a highly variable amount of undulatory activity for about 6 hours and sometimes for as long as 24 hours after insemination. In a number of cases, however, the sperm within the bursa were mostly inactive within about 75 minutes after ejaculation.

Bursae filled with vacuolated seminal material were observed up to 6 hours after insemination. Twenty-four hours after insemination, the bursae were partially distended; the wall was no longer thickened and vacuolar, and the contents of the sac were finely granular, had a yellowish brown cast, and were devoid of any vacuoles.

The bursa was never observed to contract in saline or oil-covered saline preparations of either uninseminated or inseminated *A. aegypti*. Histological sections showed the bursa to be completely devoid of muscles.

7. Structure of the spermathecae and their ducts

The median spermatheca of *A. aegypti* measures about 100 μ in diameter, and each lateral theca measures approximately 75 μ in diameter. The thecae are completely devoid of muscle and were never observed to contract in any type of preparation examined. When the spermathecae of virgins are opened under a deep layer of oil, no bubble escapes. When the thecae are crushed in a drop of xylene containing sudan black, a colorless halo of fluid appears immediately around them. This colorless watery fluid within the thecae did not react with phenol red, neutral red, sudan III, British Drug House Indicator, or with Hydrion papers.

Although the spermathecal ducts are covered by a single layer of evenly spaced circular muscles (Curtin and Jones, 1961), we have never seen these ducts contract in saline or oil-covered saline whole-mounts. The spermathecal ducts, when stretched out in saline, measure about 265 μ in length, and the clear lumen of these varies from about 2 to 3.5 μ.

8. *Speed of sperm transfer*

Twenty-two females were used to study how rapidly bursal sperm could reach the thecae after forced-mating. The females copulated with males for 9 to 54 seconds and were immersed in liquid chloroform 2 to 45 seconds thereafter. Three of the females did not become inseminated, although the males had copulated with them for 9 to 25 seconds. Eight of the females were killed 2 to 24 seconds after coitus and in three of them (37.5%), sperm had reached the thecae. In the first of these three cases, the female (which had copulated for 53.8 seconds and was killed in 18 seconds) had only a very few sperm in two thecae. In the second case, the female (which had copulated for 28 seconds and was killed in 20 seconds) had many sperm in the large theca and a few sperm in one lateral theca. In the third case, the female (which had copulated for 15 seconds and was killed in 24 seconds) had only a few sperm in the large theca. In the five other cases, however, sperm were found only in the bursa and none reached the thecae. Eight out of the 22 females were immersed in chloroform 30 seconds after coitus and were

then dissected. In only one of these females had sperm reached the large median theca. The last three females were killed 40 to 45 seconds after forced-mating, and in all three cases many sperm had ascended to the thecae, and in two of the females sperm were in two thecae. Thus, following uninterrupted forced-coitus, while a few sperm apparently are capable of reaching the thecae of a few females as early as 18 seconds after copulation, in most cases the sperm begin to fill the thecae between 30 and 45 seconds.

That highly variable results can be obtained is shown by the following data. Coitus of 7 pairs was permitted for exactly 15 seconds, after which the females were killed at 10-second intervals from 30 to 90 seconds after forced-mating, by exposing them to very strong ether fumes. Only a few sperm reached two thecae after 30, 40 and 50 seconds. Many to numerous sperm were found in two thecae after 60, 70, 80 and 90 seconds.

In spite of the variability of the data, it is evident that spermathecal filling in *A. aegypti* is a rapid event, and we are much inclined to agree with Burcham (1957) and the data presented by Spielman (1964) that no transfer occurs after the first 5 minutes following coitus.

9. *Histological observations on the reproductive tract of inseminated females before, during and following spermathecal filling*

Spielman (1964) stated (p. 341) that ". . . 5 minutes after coitus, sperm were scarce in the anterior portion of the . . . bursa'" and that one hour after coitus "the bursa was filled with coarse material and the remaining sperm were compressed into the posterior end and into the upper atrium." Our observations are not in agreement with these statements. The bursae of 22 mosquitoes were dissected 23 minutes to 6 hours after forced-copulation and in all cases many to numerous sperm were still present in the distended bursae. Dr. Spielman has kindly permitted us to examine the sections which he prepared for his studies on spermathecal filling after free mating. Our observations on his material follow.

Sections made 30 seconds following coitus showed that the bursa was distended with ejaculate, the majority of the sperm being antero-dorsally located, but at least one dense packet of sperm was seen on the ventral wall of the bursa at its orifice. No sperm were observed in either the atrium, vestibule, thecal ducts, spermathecae, or common oviduct. The ventral tuft just inside the female's ventral genital lip slanted dorsally into the bursal orifice but did not block this opening. In some sections, the posterior valve of the vagina was pressed against the surface of the dorsal valve, but in other sections a gap of variable dimensions was seen between these two vaginal valves. The ventral valve in some sections was pressed against the dorsal valve.

Thirty-five seconds after coitus, sections showed some male accessory gland material within the lumen of the upper vagina, but no sperm were detected. Numerous sperm were still in the dorsal portion of the bursa and at its blind anterior end. Many sperm in the bursa were seen in ventrally-directed arcs. Dense packets of sperm were seen making a sharp U-turn from the bursal orifice into the spermathecal vestibule (see Fig. 2). The heads of these sperm were in contact with the intima of the spermathecal ducts. No sperm were visible in the upper vagina, thecae or common oviduct. The ventral tuft slanted upward into the

bursal orifice, blocking the center of it about half way. The spermathecal eminence appeared to be elevated and the vestibular opening seemed shifted into a position dorsal to the ventral tuft. The posterior vaginal valve in some sections was pressed against the dorsal valve.

Sections made 43 to 60 seconds after insemination of the bursa showed sperm, and, in one case, male accessory gland material, free in the lumen of the upper vagina (Fig. 2). Many sperm were flattened against the surface of the dorsal vaginal valve. No sperm were visible in the lower vagina or in the common oviduct. The bursa was distended, and many sperm were ventrally aligned in dense packets at the bursal orifice. The median ventral tuft slanted halfway across the bursal orifice. The spermathecal eminence still appeared elevated. Many sperm were seen making the U-turn into the vestibular opening (Fig. 2). Sperm were observed inside two spermathecal ducts, and a few sperm were seen inside the spermathecae. The posterior vaginal valve in some sections touched the dorsal valve.

Ten minutes after insemination, sections showed sperm still present in the upper vagina, mostly flattened against the dorsal valve. No sperm were visible in the lower vagina or the common oviduct. Numerous sperm were still present in the bursa and many of them were aligned on the ventral wall at the orifice. The ventral tuft appeared to completely block the vestibule in one series of sections made at 10 minutes.

In sections made one hour after coitus, sperm were still within the upper vagina, free in its lumen, and against the dorsal valve; and for the first time, sperm were seen in the lower vagina and in the lumen of the common oviduct. Numerous sperm were still observable throughout the distended bursa. The vestibule appeared fully open to the bursal orifice. The ventral tuft did not block the vestibule. Sperm were still detectable within the spermathecal ducts and within two thecae. Sperm were especially dense at the entrance to each of two thecae.

According to Spielman (1964, p. 341), the common oviduct contains ". . . masses of agglutinated immobilized sperm" one hour after coitus. Dissections made on the Bangkok strain after forced-copulation showed a few undulating sperm within the common oviduct at 57 to 69 minutes in three females, but no sperm were visible in the oviducts of two other females. Two hours after forced-copulation, one female had undulating sperm in her common oviduct as far as the ampullae, but no sperm were seen in the oviduct of another female dissected at the same time. No sperm were visible in the common oviduct of one female dissected 6 hours after forced-copulation.

Our observations indicate that in both freely-mated and force-mated *A. aegypti* many sperm remain within the bursa following spermathecal filling, and that most of them are not concentrated at the bursal orifice. It is of considerable interest that the female has no anatomical device that could account for the fact that those sperm which are aligned at the open bursal orifice no longer attempt to reach the spermathecal vestibule. Even sperm which are free within the lumen of the upper vagina are no longer oriented towards the vestibule after spermathecal filling.

Numerous dissections were made on force-mated and freely-mated specimens

before, during, and following spermathecal filling, and in not one case were sperm ever detected within the spermathecal ducts. It is difficult to reconcile this with the presence of a few sperm in these ducts in sectioned material made one hour after free-mating. It is possible that during dissection the sperm within the ducts quickly entered the spermathecae.

10. *The behavior of spermatoza*

Observations were made on the activity of spermatozoa within various portions of the unmated male's intact reproductive system in oil-covered saline whole-mounts. The spermatozoa often exhibited intense whirling or spiralling activity within the posterior testicular chamber. They were either inactive or showed a highly variable degree of activity within the sperm ducts, and sometimes undulated within the seminal vesicles. Sperm retained activity within the testes for 54

TABLE IV

Changes in motility of sperm with time in thecae isolated from force- and freely-mated Aedes aegypti one hour after insemination. Ten females used for each of the two groups

Time after isolation of thecae	% Thecae with moving spermatozoa	
	Force-copulated*	Freely mated**
less than 10 mins.	46.4	33.3
1 hour	85.7	58.3
2 hours	64.3	8.3
3 hours	42.9	0
4 hours	10.7	0
5 hours	3.6	0

* Twenty-eight thecae contained sperm.
** Twelve thecae contained sperm.

to 270 minutes and undulated in some seminal vesicles for 60 to 348 minutes. Sperm within the sperm ducts, if active at all, were generally active for less than 38 minutes and for not more than 196 minutes.

The spermathecae of force-mated and freely-mated mosquitoes were isolated one hour after copulation in a drop of saline and the preparation covered with a layer of immersion oil so that the activity of the sperm within the thecae could be noted at hourly intervals. As shown in Table IV, sperm were generally more active and remained active for a longer period in the force-mated than in the cage-mated group. While no activity was observed three hours after isolation of the thecae from the cage-mated mosquitoes, sperm were actively moving in 42.9% of the thecae of the force-mated females at this time.

The following types of activity were observed in spermatozoa released from seminal vesicles into a drop of saline covered with a layer of immersion oil: (1) very rapid locomotion with the short, sharp, thin, stiff, needle-like head piece tilting up and down as the long thin tail made rapid, large wave undulations. These explosive progressive locomotory movements occurred either in a generally straight line in any direction, or the sperm would circle about briefly. In saline drops

which were not covered with a layer of oil, locomotions were only rarely observed. (2) Very rapid and intense coiling or lashing motions of the tail were observed, especially when the sperm occurred in clusters and when the head was at an interface. Many times the tails of clustered sperm whirled or lashed synchronously. (3) Slow, smooth, regular, large wave undulations of the tail were observed when sperm were congregated at the saline/oil interface. (4) Irregular undulations or oscillations of highly variable amplitudes were observed in sperm which had ceased locomoting or which had stopped the smooth regular undulations. Different portions of the tail were capable of undulating at very different rates and with different amplitudes. The waves moved away from the head piece.

Observations were made on the activity and survival time of sperm released from the seminal vesicles. Highly variable results were obtained, depending upon the technique and the location of the sperm within the preparation. When the seminal vesicles were ruptured into an open drop of saline, many sperm which reached the edge of the drop quickly lost their motility in one to three minutes. Those sperm which did not reach the edge of the drop undulated irregularly and lost all activity fairly rapidly. However, those sperm still inside the torn vesicles tended to remain active for about four minutes in open saline drops. When the vesicles were ruptured in a very small amount of saline that had been covered first with a drop of immersion oil, the sperm were often intensely active for two to 15 minutes, especially around the surface of the vesicles. Generally, the sperm in such preparations lost all activity in three to 87 minutes after release. Those sperm which moved out into the layer of oil very quickly ceased moving. When the vesicles were opened in a moderate-size drop of saline that had been covered with a layer of immersion oil, those sperm which were strongly oriented at the saline/oil interface remained active for 16 to 60 minutes, but those which did not reach the edge of the drop tended to lose their activity in about one minute. The sperm inside the seminal vesicles remained quite active for two to 119 minutes (in most cases for about 6 minutes); highly variable numbers undulated feebly for 182 to 328 minutes. Sperm on the outside of the vesicles tended to have their heads oriented to the vesicles' surface and were often very intensely active for about four minutes.

In coverslipped saline mounts in which air bubbles had been trapped, the sperm head was frequently strongly oriented to the saline/air interface.

To study whether chemotaxis might be involved in sperm migration, seminal vesicles and bursal sperm were released in the vicinity of intact male accessory glands, male accessory gland exudate, and onto freshly excised vaginal tissues. In many cases, the tip of the sperm head was strongly oriented to all of these tissues, but sperm did not specifically congregate around them. The head of seminal vesicle and bursal sperm did not become specifically oriented to fat body, testes, somatic muscles, bursa, female accessory gland, or its duct, spermathecal ducts, intact or crushed spermathecae, common oviduct, or even to a freshly laid egg.

To determine whether the sperm head would be oriented in or against the direction of a moving stream, the intact seminal vesicles were dissected into a drop of saline on a glass slide next to a long rectangular coverslip supported on two sides by capillary glass rods. The thin space between the coverslip and slide was filled with saline. The seminal vesicles were cut open so that the sperm

poured out into the saline just under the edge of the coverslip and a strong current was produced by withdrawing saline at the other end. In all such preparations, the sperm heads were precisely aligned in the direction of the flowing stream of saline. Dead sperm were not precisely aligned. This type of rheotaxis of live *Aedes* sperm is exactly the opposite of that of bull or human sperm which are known to orient the head piece against the direction of a moving stream (see, *e.g.*, Rothschild, 1962).

Thus, the head of the spermatozoön of *A. aegypti* becomes oriented toward certain tissues and becomes aligned in the direction of a flowing stream, and the tail piece is capable of propelling the cell rapidly.

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Summary

1. With the forced-copulation technique, the Bangkok strain of *Aedes aegypti* can ejaculate within the first five seconds of coitus but usually does so within 10 to 15 seconds. The male force-copulates for 31.3 seconds, and 82% of the females become inseminated. In 90% of them, spermatozoa reach two of the three thecae. With naturally-mated mosquitoes copulation is significantly shorter in duration, all of the females become inseminated and in 92% of them spermatozoa reach all three thecae.

2. The terminal chamber of the testis of an unmated and repetitively force-copulated male has about 700 spermatozoa. Each sperm duct has about 370 sperm. The seminal vesicles of unmated males have about 5000 spermatozoa, while the vesicles of repetitively force-mated males have about 930.

3. With rapid repetitive force-copulation, the male ejaculates progressively fewer spermatozoa into the bursa of each successive female. Sperm counts made on 6 ejaculates from one male varied from 254 to 2655.

4. Counts on spermatozoa remaining in the bursa after spermathecal filling indicate that 62% of them leave the bursa, and suggest that about 660 sperm reach the large theca and 486 fill one of the lateral thecae.

5. Most of the sperm deposited in the bursa quickly spread to the edges of the sac, and many become aligned on its ventral wall. The wall of the bursa greatly swells two to three minutes after insemination. Shortly thereafter the accessory gland secretion within the ejaculate begins to vacuolate and may be fully vacuolated within 10 minutes and remains vacuolated for at least 6 hours.

6. With forced-copulation, a few sperm may be capable of reaching the thecae within 18 seconds but in most cases sperm begin to reach the thecae between 30 and 45 seconds after coitus. Complete thecal filling can occur in 90 seconds and probably is terminated within the first five minutes or less after coitus. Fol-

lowing spermathecal filling many active sperm remain in the bursa for some time. Following spermathecal filling those sperm at the bursal orifice no longer make the U-turn towards the open spermathecal vestibule.

7. Spermatozoa within the isolated but intact male reproductive system may remain active for five to six hours in oil-covered saline whole-mounts. Spermatozoa released from seminal vesicles in oil-covered saline drops exhibit four types of movement: (a) brief, rapid, explosive, progressive locomotion, (b) rapid synchronous coiling when the cells are in dense clusters and the head is at certain interfaces, (c) smooth undulations *in situ,* and (d) irregular undulations or oscillations. The heads of sperm of *Aedes* become oriented in the direction of a moving stream.

8. Sperm released from the seminal vesicles may become strongly oriented toward the male accessory glands and its exudate, and to freshly excised vaginal tissues, but they do not specifically congregate about these tissues in oil-covered saline whole-mounts. Seminal vesicle sperm do not become oriented to freshly excised fat body, testes, somatic muscles, bursa, female accessory gland or its duct, spermathecae or their ducts, ovary, common oviduct, or a freshly laid egg.

Conclusions

Many sperm deposited in the bursa of female *Aedes aegypti* (Linnaeus) rapidly locomote around the male accessory gland secretion of the ejaculate and assemble on the ventral floor of the sac at its orifice where they undergo rapid and violent synchronous coiling movements. The strong orientation of the sperm head to certain interfaces presumably guides the long, thread-like contracting cells over a U-shaped route directly into the vestibule where they first contact the opening of the spermathecal ducts. Bundles of sperm swiftly ascend the ducts, presumably only in female fluids, and simultaneously reach two or three thecae. Shortly after sperm begin to fill the already fluid-filled thecae, the bursal wall swells and presumably secretes material into the ejaculate. After this the accessory gland secretion of the ejaculate begins to vacuolate, and a short time after this, the still active sperm at the open bursal orifice stop moving into the vestibule.

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