SPERMATOGENESIS IN *AEDES AEGYPTI*[1]

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INTRODUCTION. Many contributions have been made in the field of mosquito cytology during the last few years. The emphasis of this work, however, has mostly been on either salivary gland chromosomes (see Kitzmiller 1963) or on somatic chromosomes (Breland 1961, Rai 1963a).

A knowledge of the behavior of chromosomes during meiosis, which on the whole has lagged behind, is gradually being acquired for various genera of mosquitoes. Early studies which provide some data on spermatogenesis include the following species: *Culex pipiens* (Stevens 1910, Lomen 1914, Taylor 1914, Whiting 1917, Moffett 1936, Grell 1946, Callan and Montalenti 1947); *Culex tarsalis* (Stevens 1911); *Anopheles punctipennis* (Stevens 1911); *Anopheles maculipennis* (DeBuck and Swellengrebel 1935); *Culiseta incidens* (Stevens 1911); *Corethra plumicornis* (Frolowa 1929) and *Theobaldia longiareolata* (Callan and Montalenti 1947). More recent accounts of gametogenesis in male mosquitoes have dealt with *Anopheles stephensi* (Rishikesh 1959); *Culiseta inornata* (Breland et al. 1964) and a preliminary report on meiosis in *Aedes aegypti* (Akstein 1962).

It is surprising that detailed information concerning the meiotic chromosomes of as important a vector species as *Aedes aegypti* is not available. The somatic complement consists of three pairs of rather large chromosomes which in good preparations are individually recognizable (Rai 1963a). The cytogenetic effects of radiation (Rai 1963b) and of the chemosterilant, apholate (Rai 1964), on the somatic chromosomes of this species have been rather extensively studied. Because of our interest in expanding these studies to meiotic chromosomes, particularly with a view to detecting and isolating heritable chromosome aberrations, it was decided to investigate the details of normal meiosis during spermatogenesis.

It may be pointed out also, that there has been a recent resurgence of interest in insect spermatogenesis. In *Drosophila melanogaster,* for example, it has been shown that each primary spermatocyte regularly forms two functional and two non-functional sperms (Peacock and Erickson 1965). It is believed that this results from a functional inequality of the two spindle poles during meiosis I. Furthermore, "a specific orientation at metaphase I relative to this polarity" of chromosome II has been invoked to explain the unusual, non-Mendelian, segregation of *Segregation-distorter,* a second chromosome locus in *Drosophila melanogaster.* In *Aedes aegypti,* an inherited male-producing factor (MP) similarly results in very highly distorted sex-ratios (Craig et al. 1960). The explanation of this phenomenon, too, may be tied up with abnormal spermatogenesis. The need for understanding the mechanics of meiotic chromosomes during normal spermatogenesis became all the greater in view of this circumstance.

MATERIAL AND METHODS. For the most part the ROCK strain of *Aedes aegypti* was used in this study. Eggs of this strain were hatched in deoxygenated water. The growing larvae and pupae were reared in tap water in either white enamel pans or pint containers at a temperature of 82° F. (± 1° F.) and a relative humidity of 80

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percent (± 10 percent). They were fed liver powder daily. The details of rearing procedures for this species have been described by Craig and VandeHey (1962).

Fourth instar larvae, pupae, and adults were dissected at regular time intervals after these stages had begun. Testes of larvae and pupae were dissected in the tissue fluids of the animals. Those of adults were dissected in *A. aegypti* saline (Hayes 1953).

Several methods of fixation and staining were used. In some cases, testes were prestained in a solution of 1 percent orcein in 45 percent acetic acid. Fat cells around the testes were removed and the aceto-orcein was replaced by a drop of aceto-lactic-orcein (Breland 1961). After 5–10 minutes, the preparation was squashed. Material was also fixed in a solution of 6 parts methanol: 3 parts chloroform: 2 parts propionic acid (Pienaar 1955). Testes were dissected in this fluid and stained with aceto-lactic-orcein. A few testes containing mature sperm were mounted in 1 percent aqueous solution of vital red.

Temporary slides were sealed with fingernail polish and photomicrographs were taken from these using a 35 mm Zeiss Ikon camera and Panatomic-X, black and white film.

Because of extensive meiotic chromosome stickiness encountered in the ROCK strain, a number of other strains of *A. aegypti* were examined also. These included another wild type, highly inbred strain, NIH, and two mutant strains designated RED-EYE and MINIATURE-APPENDAGES respectively. The description of the results in this paper is, however, based on the ROCK strain only.

RESULTS. The testes are sausage-shaped bodies lying in the dorsolateral region of the sixth abdominal segment and are covered by a light brownish layer of fat cells. Each testis is colorless, highly refractive, and divided into partitions or cysts. The pointed end of the testis is directed anteriorly; the broad posterior end is connected with the vas deferens. The two organs are not always of the same size, nor are they necessarily in the same physiological stage of development.

Most of the cells in the testicular cysts of fourth instar larvae were either in interphase or in different stages of spermatogonial mitosis. Primary spermatocytes were observed in pupae from the beginning of pupal life onward. Considerable variation in the progress of spermatogenesis during pupal development was encountered in different pupae. However, the cells of any one testicular cyst were more or less at the same stage.

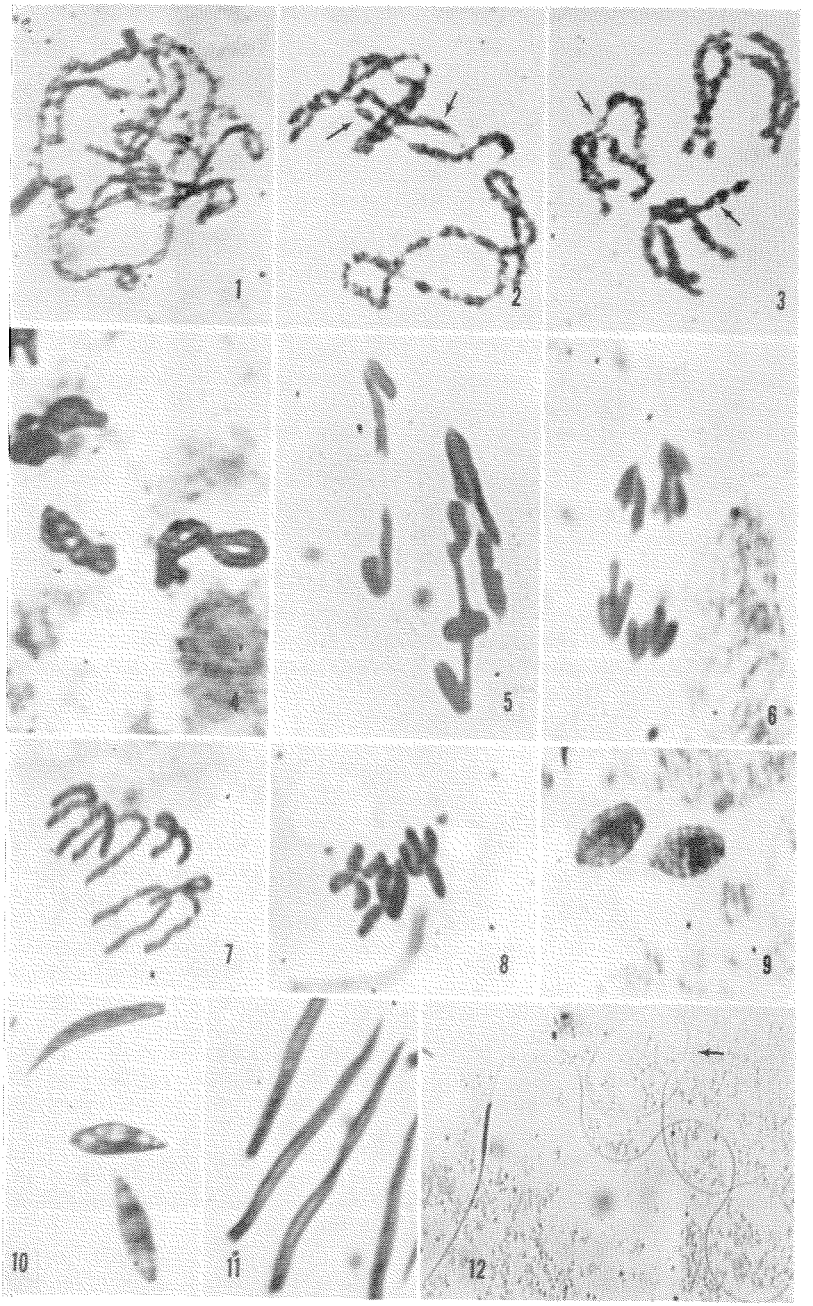
Usually in pupae 2 to 4 hours old the posterior cysts were in pachytene. This stage (fig. 1) was the earliest at which chromosomes became visibly discrete. The paired chromosomes were optically single and were completely synapsed except for a few flared regions. Pachytene was of relatively long duration. Moreover, some cells in this stage were observed in almost all preparations of pupal testes.

Diplotene was characterized by shortening and a gradual repulsion of the homol-

Explanation of the Figures on page 47

Spermatogenesis in *A. aegypti*. Figs. 1–11 ca. 1560X; Fig. 12 ca. 625X.

FIG. 1.—Pachytene
FIG. 2.—Diplotene (arrows indicate double nature of chromosomes)
FIG. 3.—Later diplotene with chromosomes more contracted
FIG. 4.—Diakinesis
FIG. 5.—Metaphase I
FIG. 6.—Anaphase I
FIG. 7.—Prophase II
FIG. 8.—Metaphase II
FIG. 9.—Formation of spermatids
FIG. 10.—Spermatids
FIG. 11.—Spermatids at a later stage
FIG. 12.—Mature sperm (arrow indicates tip of tail)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ogous chromosomes. As these contracted they appeared as three distinct pairs with rather conspicuous chromomeres (figs. 2 and 3). The double nature of each homologous chromosome was clearly visible throughout diplotene (see arrows in figs. 2 and 3). Repulsion was usually greater at the centromeric region. Chromosomes were held together presumably only at points of chiasmata or because of the remains of relational coiling. There was considerable variation in bivalent configurations. In some, the chromosome arms on one side of the centromere were entwined whereas those on the opposite side adhered at a single point or not at all. In others, the chromosomes adhered only at their tips.

Diakinesis was apparently of short duration. The chromosomes appeared very contracted with homologues held together at points of chiasmata (fig. 4).

A stage referred to as prometaphase stretch in *Culiseta inornata* (Breland *et al* 1964) and a number of other insects was observed in *A. aegypti* also. Chromosomes at this stage were characterized by a stretching much greater than that observed by Breland *et al.* (figs. 7 and 8, p. 475) for *Culiseta inornata.*

At metaphase I homologous centromeres were oriented on the equatorial plate (fig. 5). The number of chiasmata per bivalent ranged from 1–2. Chiasmata terminalized as homologous chromosomes moved toward opposite poles. This resulted in the production of either ring or rod bivalents.

The chromosomes moved individually and non-synchronously during anaphase I, the four arms of each diverging somewhat behind the centromere (fig. 6).

Telophase I was probably of very short duration, as very few preparations showing this stage were observed. The secondary spermatocyte was small and stained very densely with orcein.

During prophase II the chromosome arms became widely separated (fig. 7). In many instances the centromeres of these were observed to be oriented towards one side of the cell while the arms trailed toward the opposite side. By metaphase II this polarity had disappeared and the chromosomes were maximally contracted. They resembled small H's and X's with their short arms held togehter by prominent centromeres (fig. 8). At anaphase II the three chromosomes moved together toward opposite poles. Often their movement was so synchronous that they were not detectable as individual chromosomes. Because of the fragility of the material, four telophase II nuclei lying next to each other and resulting from the same primary spermatocyte were rarely observed.

After telophase II the chromatic material in each nucleus appeared in clumps lying along the inner edge of the nuclear membrane. Gradually the cells assumed an elliptical shape and became pointed at opposite ends (fig. 9). The fusiform spermatids exhibited what appeared to be a spirally arranged band of heteropycnotic material resulting in alternating regions of dark and light staining areas (figs. 10 and 11). Spermatids were visible in the posterior cysts of the testis of some pupae as early as 6-7 hours after pupation. Approximately half the pupae 9 to 11 hours old contained spermatids. Some spermatids were present in practically all pupae 13 to 15 hours old.

Although the details of spermiogenesis have not yet been investigated in this species, the fusiform spermatid gradually elongated into the mature sperm (fig. 12). The tail structure appeared early in developing spermatids, but was apparently destroyed by the acetic acid of the staining-fixation process (Breland and Gassner 1964). The fully formed sperm consisted of a deeply staining head about 40μ in length and a tail about 270μ long (fig. 12). The sperm appeared in pupae from 17 hours onward. At 43 hours of pupal life the posterior half of the testis was filled with sperm. These did not become motile, however, until emergence or shortly thereafter.

DISCUSSION. Although somatic pairing of chromosomes is common in the Diptera, it is not of universal occurrence throughout the group. It is suppressed in some

Nematocera like the Sciaridae (Metz 1938) and the Cecidomyiidae (White 1946), but is characteristic among the Culicidae. This more or less permanent attraction between homologous chromosomes modifies the pattern of both mitosis and meiosis. Whiting (1917) maintained that the primary spermatocytes of *Culex pipiens* initially contained optically single threads which paired during a zygotene stage. However, the existence of a leptotene stage among Diptera has been questioned by a number of workers (Bauer 1931, Grell 1946, Rishikesh 1959, and Akstein 1962). The present study has revealed that in *A. aegypti,* at the earliest stage at which the homologous chromosomes became visibly discrete, they were definitely paired for most of their length. This stage corresponds to pachytene. Thus, as in a number of other mosquitoes, visible leptotene and zygotene stages do not occur in *A. aegypti.* This obviously results from the fact that the chromosomes during the earliest stage of meiosis retained the intimate pairing of the previous mitotic anaphase and telophase. During the pachytene stage the chromosomes themselves were not visibly double. A few, rather small segments where pairing had not taken place occurred interstitially along the chromosomes. Such asynaptic regions have been reported in the salivary gland chromosomes of *Aedes aegypti* also (Mescher 1963). Whether these are comparable in origin to similar regions described in *Culex* (Grell 1946) could not be ascertained.

Shortly after the beginning of repulsion of homologous chromosomes which initiates diplotene, the previously single chromosomes were visibly double. This duplication was particularly manifest at chromomeric regions. Contrary to the observations of Akstein (1962) this duality was manifest throughout diplotene although not every preparation showed this equally well.

During anaphase I the separation of homologues was asynchronous in *A. aegypti.* The smallest pair of chromosomes usually separated first. Asynchronous behavior has also been reported for *Culiseta inornata* (Breland *et al.* 1964), *C. pipiens* (Kitzmiller 1953), *Anopheles stephensi* (Rishikesh 1959), *An. maculipennis atroparvus* and *An. claviger* (Frizzi 1947). The underlying cause for this behavior probably differs from group to group. In *Culiseta inornata* as in *A. aegypti* the smallest pair of chromosomes moves first whereas in *An. stephensi* (Rishikesh 1959) the smallest pair is the last to separate at anaphase I. Breland (1961) and Rai (1963a) described a similar asynchronous behavior in mitotic anaphase of certain species of mosquitoes. In instances where the smallest chromosomes separate first, it is tempting to correlate movement with relative size. The longer the chromosome the more time it may take for terminalization of interstitial chiasmata. However, in *An. stephensi* a different situation obtains. The small chromosomes are sex chromosomes and move last. In this species Rishikesh (1959) described the first meiotic division for these chromosomes as equational rather than reductional. This may be the reason why the sex chromosomes of *An. stephensi* separate after the autosomal bivalents.

Presumably the separation of chromosomes at anaphase II is synchronous for most mosquito species. In *A. aegypti* this synchrony in movement coupled with surface stickiness often made it difficult to see the three chromosomes individually. By contrast those of *Culiseta inornata* (Breland *et al.* 1964) are quite discrete. Those of *An. stephensi,* on the other hand, move asynchronously at anaphase II (Rishikesh 1959), the smallest ones being the first to separate.

Rai (1963a) showed that one of the larger chromosomes (chromosome II) in *A. aegypti* was submetacentric. In a number of cases an inequality in arm lengths in one pair was apparent in the present study also (fig. 7).

Whiting (1917) indicated that in *Culex pipiens* meiosis proceeds in a more or less stepwise fashion beginning in the posterior cysts of the testis and progressing forward.

However, in *A. aegypti* the testicular cysts do not necessarily develop in a sequential order from the caudal region forward. Several testes examined as whole mounts contained cysts at metaphase I in both caudal and mid-regions. Between these were cysts in the pachytene stage. The significance, if any, of this pattern is unknown. The testes as a whole were most active meiotically during the first 12 hours of pupal life. The stage of development of the germ cells was not completely correlated with the age of the pupa. Even under similar environmental conditions there was considerable variation. By the time the adult emerged, however, the posterior half of the testis was completely filled with sperm.

One of the biggest difficulties encountered in the present study presumably resulted from chromosome stickiness. This was most pronounced at metaphase I and anaphase I. Frequently, the chromosomes at these stages adhered to each other so closely as to form a single chromatic mass. Squashes in which the chromosomes did not show much stickiness as in fig. 5 were rather rare. Rearing the larvae and pupae at relatively lower temperatures (60°–70°) did not improve the quality of the squashes. It was for these reasons that a number of other strains, already mentioned, were examined for their suitability for the study of meiotic chromosomes. On the whole, the results were not much different except for the MINIATURE strain, in which the chromosome stickiness was much less. Work is under way in our laboratory to investigate if this stickiness is genetically controlled. It is of interest to note that similar factors may explain the lack of success encountered in mapping salivary-gland chromosomes in this species (Mescher 1963).

Breland *et al.* (1964) have placed considerable emphasis on the occurrence of pro-metaphase stretch and its possible bearing on dipteran ancestry. They have argued that "in view of the occurrence of a prometaphase stretch in *Boreus brumalis,* a crane fly, and *Culiseta inornata,* it is tempting to suggset that the present study adds some additional evidence" to support the view that the Diptera were derived from the Mecoptera (see Hinton 1958) rather than from Neuroptera, as advocated by White (1949). Although the finding that this phenomenon occurs in *Aedes aegypti* also appears to substantiate the views of Breland *et al.,* we are of the opinion that much taxonomic or phylogenetic reliance cannot be placed on this character. In *Periplaneta americana,* John and Lewis (1957) have indicated that pro-metaphase stretching is "facilitated by a lapse of matrix stickiness" so that the two may be correlated. Furthermore, Lewis and John (1957) have suggested that this stretching facilitates normal disjunction of chromosomes in organisms where meiosis is non-chiasmate. In the light of this, it appears reasonable to consider that matrix stickiness and the occurrence of a pro-metaphase stretch are phenomena which serve "the mechanical function normally performed by chiasmata" (John and Lewis 1957). The role of pro-metaphase stretch in *Aedes aegypti* and *Culiseta inornata* may be tied up with the fact that the chiasmata frequency in these is rather low.

SUMMARY. The mechanics of meiotic chromosomes during spermatogenesis in *A. aegypti* was studied. Fourth instar larvae, pupae, and adults of the ROCK strain were dissected at regular time intervals after these stages had begun. Meiotic chromosomes were studied principally from squash preparations stained with aceto-lactic-orcein. Visible leptotene and zygotene stages do not occur in *A. aegypti* probably because of somatic pairing of chromosomes.

Pachytene was the earliest stage at which chromosomes became visibly discrete. The double nature of each homologous chromosome was clearly visible throughout diplotene. Diakinesis was followed by a pro-metaphase stretch. Metaphase I chromosomes were often clumped and sticky. The chromosomes moved individually and non-synchronously to opposite poles during anaphase I. During prophase II the centromeres were oriented

toward one side of the cell. Chromosomes became maximally contracted at metaphase II. At anaphase II the three chromosomes moved as a single mass toward opposite poles. Shortly after telophase II the cells became elliptical and then fusiform. The developing spermatids contained alternating dark and light regions of stained material. The fully formed sperm consisted of a head 40 microns in length with a tail about 270 microns long.

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