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### Spermiogenesis of Eupyrene Sperm in Prepupae, Pupae, and Adults of *Heliothis virescens* (Lepidoptera: Noctuidae): An Ultrastructural Study

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**ABSTRACT** We have examined the ultrastructure of postmeiotic eupyrene sperm maturation in testes of prepupae, pupae, and adult *Heliothis virescens* (F.) males. Emphasis was placed on the structures present in the elongating sperm tail. In each young spermatid, each cell has a nucleus, acrosome derivative, and numerous mitochondria in a large volume of cytoplasm. These mitochondria coalesce to form a body, the nebenkern. As the sperm cell begins to elongate, the nebenkern divides into two mitochondrial derivatives (MDs) of unequal size. The MDs have an outer and inner membrane, and as they elongate and coil in the sperm tail, cristae develop on the inner membrane. Concurrent with cell elongation is the disappearance of most of the cytoplasm present in the cell. The axial filament has a typical insect system of 9 + 9 + 2 tubules and is present along most of the length of the sperm tail as are the MDs. A prominent system of tubules, the manchette, forms around the MDs in a later stage of development. Two extracellular structures, the satellite body and the radial mantle, also undergo a complex series of changes during the maturation of the sperm cell.

KEY WORDS Insecta, membranes, axial filament, mitochondrial derivative

SPERM FORMATION in insects is an extremely complex process. Postmeiotic maturation of sperm in insects involves a highly ordered sequence of developmental changes. In *Heliothis*, it begins with a cyst of 256 round spermatids and terminates in the production of a bundle of 256 highly elongated sperm. In Lepidoptera, the situation is even more complicated because both apyrene and eupyrene sperm are produced (Gatenby 1917, Bowen 1922, Chen & Graves 1970). Thus, the testis from a single male contains two vastly different types of sperm cells in various stages of maturation.

Our primary interest in the ultrastructural analysis of eupyrene sperm formation in Heliothis virescens (F.) was to gather sufficient information to permit a step-by-step comparison with the process in sterile backcross (BC) Heliothis males (La-Chance 1984). Here we describe the ultrastructural changes that take place in normal prepupae, pupae, and adult Heliothis males. A complete and detailed description of all structures present and changes that occur during the process of eupyrene spermiogenesis would be extremely lengthy. Therefore, we stress the structures and processes that are affected in BC males and provide less information on structures and processes that are similar in normal males and sterile BC males. The process of apyrene sperm formation is very similar in normal and BC males and is not considered in detail here.

#### **Materials and Methods**

Insects were from a laboratory colony reared on standard wheat germ diet (Berger 1963). Rearing

rooms were maintained at  $27 \pm 1^{\circ}$ C and 12:12 (LD) photoperiod. "Prepupae" indicates a late fifth-stage larvae that had ceased feeding, buried into the media, and would pupate within 24 h. To estimate the relative number of various types of sperm cysts and bundles present in prepupae, pupae, and young adult males, the single fused testis was removed in saline. The organ was then transferred to a microscope slide in saline, and the testicular envelope was torn open to release the contents. The testicular envelope was discarded, and cysts were dispersed by gently stirring with a fine dissecting needle. A drop of 2% glutaraldehyde was then added and mixed through the slide contents. A coverslip was then gently dropped onto the preparation, and the contents were examined in dark field at 125×. Round sperm cysts and elongated sperm bundles were counted. The sperm cysts eventually became sperm bundles.

For transmission electron microscopy, the testis was dissected into saline, fixed for 3 h in 3% glutaraldehyde in 0.1 *M* phosphate buffer (pH 7.2), and then transferred through four 30-min washes of phosphate buffer. The tissue was then treated for 1 h in 1%  $O_3O_4$  and rinsed in distilled water for 1 h. After dehydration in a graded series of EtOH (30–100%), three 15-min washes in propylene oxide, 4 h in 1:1 propylene oxide and Spurr's resin, the testis was imbedded in Spurr's resin mixture and cured at 70°C for at least 8 h. The testis was either stained *en bloc* in 2% uranyl acetate in water at 60°C overnight prior to dehydration, or the sections (600–1,000 Å) were stained with saturated 4% aqueous uranyl acetate and counter-stained with

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Stage	Round cysts	Early elongating bundles	Later elongating bundles	Fully elongated coiled bundles	Total	Total apyrene
Prepupae	$800.0 \pm 28.3$	$112.8 \pm 10.6$	$73.4 \pm 8.6$		986.2	
Pupae						
1 d	$807.0 \pm 28.4$	$106.4 \pm 10.3$	$263.6 \pm 16.2$	_	1,177	_
3 d	$606.0 \pm 24.6$	$150.0 \pm 12.2$	$208.6 \pm 14.4$	_	964.6	а
5 d	$213.6 \pm 14.6$	$149.2 \pm 12.2$	$750.0 \pm 27.4$	_	1,112.8	$183.6 \pm 13.6$
$7 \mathrm{d}$	$99.0 \pm 9.9$	$172.0 \pm 13.1$	$541.4 \pm 23.3$	$206.8 \pm 14.4$	1,019.2	$756.6 \pm 27.5$
9 d		$4.4 \pm 2.1$	$45.2~\pm6.7$	$709.2 \pm 26.6$	758.8	$922.6 \pm 30.4$
Adult						
$<\!24$ h		—	_	$1,072 \pm 32.7$	1,072	$1,\!900.4~\pm~43.6$

Table 1. Types of eupyrene sperm cysts present in the testis of H. virescens males at various stages of development. Each mean is the average of five males  $\pm$  SD

<sup>a</sup> Immature apyrene cysts are present but difficult to count.

lead citrate. Sections were examined and photographed with a Phillips-300 electron microscope.

#### Results

Certain aspects of spermatogenesis in H. virescens males were described by Chen & Graves (1970), Chase & Gilliland (1972), and Loeb & Birnbaum (1981). Larvae contain a pair of testes that fuse in the prepupal stage. Testes of the fourthstage larvae contain cysts of spermatogonia and early spermatocytes. A few spermatocytes undergo meiosis during the fourth instar, but most spermatocytes in the first meiotic division are found in the later fifth-stage larvae and prepupae. Types of cysts and sperm bundles found in the prepupal, pupal, and adult stages are shown in Table 1. In prepupae, the testis contains mostly rounded cysts of spermatocytes in meiosis. Loeb & Birnbaum (1981) found that in prepupae each cyst contains 256 cells, but the number varies depending on rearing temperature and day length. Some of these spermatid cysts have begun to elongate slightly. The pupal testis contains the same types of cysts and bundles, although many of the cysts of spermatids present in the prepupae have elongated somewhat by this stadium. As pupal age increases, the number of elongating eupyrene bundles increases, and the number of less mature cysts decreases. Interestingly, each testes (prepupal, pupal, or adult) contains roughly 1,000 eupyrene cysts or bundles in various stages of maturation. Bundles of apyrene sperm begin to appear in the 3-d-old pupae and are easily differentiated and counted in 5-d-old pupae. In late pupal stages and in the young adults, most of the sperm bundles are either distinctly elongated coiled eupyrene sperm bundles or typical apyrene bundles. Cell divisions cease about 3 d before the adults emerge (Chen & Graves 1970)

Because of the continuous production and maturation of eupyrene sperm cysts in *Heliothis*, it is difficult to correlate a particular maturation stage with a developmental stadium. Widely varied maturation stages are present in virtually all pupal stages (Table 1). We attempted to arrange the figures to present the sequence of major changes that take place from the youngest to oldest eupyrene cells in the male testis, but this was not always possible. Because we discuss many structures in the cell, it is not always possible to refer to the figures in numbered order.

In the young spermatid cyst, each cell has a large nucleus with condensed chromatin material. The nuclear envelope is composed of two membranes and contains many nuclear pores (Fig. 1 and 2). The developing acrosome is located next to the nucleus, and cells are interconnected by cytoplasmic bridges (Fig. 3). The 256 eupyrene sperm cells in the cyst are embedded in cyst cells that surround the bundles. Cytoplasm of the cyst cells contains mitochondria, Golgi bodies, and endoplasmic reticulum (Fig. 3 and 9). Cytoplasm of the eupyrene cells also contains large amounts of smooth endoplasmic reticulum (Fig. 8). Multivesicular bodies are commonly present in the cytoplasm of spermatids (Fig. 7, 11, and 12). These bodies represent a type of lysosome and are reportedly involved in the uptake and digestion of protein and in cell lysis (Dingle 1972).

Numerous mitochondria are present in the cytoplasm of spermatids (Fig. 1 and 2). These mitochondria aggregate and later coalesce to form large bodies. The mitochondrial aggregates develop long fingerlike extensions that interdigitate (Fig.  $5 \text{ and } \overline{6}$ ). This process has been described in detail by Pratt (1970) for Murgantia histrionica and by André (1959) for Pieris brassicae. The process of mitochondrial anastomosis forms the nebenkern, which has been described for several insect species (Favard & André 1970, Phillips 1970). When the spermatid cells begin to elongate, the nebenkern divides into two separate bodies-the early mitochondrial derivatives (MDs) (Fig. 6 and 7). During this period of elongation, longitudinal sections show that the length is many times the width of the cell and that MDs are also elongating, extending from the nuclear to the tail portion of the cell. The two MDs are of different size from the moment of separation and continue to maintain this size dimorphism throughout spermiogenesis (Fig. 6-9). In elongating eupyrene cysts, one of the MDs appears



Fig. 1-6. Cross sections through eupyrene sperm cells in various stages of maturation in normal male testes. Bar = 1  $\mu$ m. (1 and 2) Prepupal round spermatid stage showing numerous mitochondria (M), nucleus (N), and nuclear membrane pores (NP). (3) Prepupal spermatid cell. Developing acrosome (AD) located next to N, cytoplasmic bridge (CB) connecting two cells in the cyst and multiple Golgi complexes (G). (4) 5-d-old pupae; section through spermatid cell at level where only one MD is present near axial filament (AF). (5 and 6) Prepupal early spermatid stages with nebenkern (NB), AF, N, and newly formed MDs.



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Fig. 7-12. Cross sections through eupyrene sperm cells in various stages of maturation in normal male testes. (7) Cell from 5-d-old pupae contains two MDs, axial filament (AF), two multivesicular bodies (MV), and Golgi body (G). Bar = 1  $\mu$ m. (8) Prepupal cells with two MDs, AF, and smooth ER. Bar = 0.25  $\mu$ m. (9) Section from 5-d-old pupae includes two eupyrene cysts in different stages of maturation. Cyst at bottom in more advanced stage with each cell containing two MDs and AF, upper cyst less advanced. Bar = 1  $\mu$ m. (10) Eupyrene cells from 1-d-old pupae. MDs show varying shapes, some doughnut shaped. Cells rich in G and ER inclusions. AF is normal. Bar = 1  $\mu$ m. (11 and 12) Cells from 5-d-old pupae. Satellite body just forming on outer cell membrane, MDs have "pork chop" appearance. (11) MVs evident and AF with accessory fibers. Bar = 0.50  $\mu$ m. (12) Bar = 0.25  $\mu$ m.



Fig. 13-17. Cross sections through eupyrene sperm cells in various stages of maturation in normal male testes. (13 and 14) Eupyrene sperm cells from 5-d-old pupae. Satellite bodies (SB) with septa connecting body to cell membrane, manchette tubules (MA) around MDs (see Fig. 23), and axial filament (AF) with accessory fibers. (13) Bar = 0.25  $\mu$ m. (14) Bar = 0.50  $\mu$ m. (15-17) Stages of AF. Bar = 0.25  $\mu$ m. (15 and 16) From 5-d-old pupae. (15) In younger cells AF has nine doublets in outer circle and central doublet. (16) Accessory fibers in center. Bar = 0.25  $\mu$ m.

to be either C-shaped or donut-shaped (Fig. 10). At later stages, MDs can exhibit a typical "pork chop" appearance (Fig. 11) or present a variety of other shapes (Fig. 12–14).

In short, the appearance of MDs varies greatly in various sections mainly because the shape of MDs varies considerably from the anterior to posterior part of the tail and because MDs undergo continuous structural changes as the sperm bundle matures. Comparisons of many micrographs indicate that MDs are of different size and that one is somewhat longer than the other. In many sections, one MD is much larger than the other, in some sections they are of nearly equal size, and in other sections only one MD is seen (Fig. 7-12).

As the eupyrene cells continue maturation, distinct cristae form along the inner membrane of each MD (Fig. 21). They are clearly seen in some cross sections and not in others. Cristae greatly increase the surface area of the inner membrane of the MDs. In the later elongating bundles, a number of microtubules form around and between each MD (Fig. 12, 13, and 23) and create a manchette similar to that described by Pratt (1970). In the young spermatid stage, an axial filament is present (Fig. 4, 5, 7, and 8). The formation of the axial filament in insect sperm has been described in detail by André & Thiery (1963), Baccetti & Bairati (1964), Phillips (1966, 1970b), and Pratt (1970). In *Heliothis*, the axial filament in young eupyrene cysts consists of a circular arrangement of nine double fibers and two fibers in the central core (Fig. 15). Later in maturation, nine much darker, more dense fibers are beginning to form, one associated with each doublet (Fig. 16). These eventually develop into the accessory tubules of the flagellum (Fig. 17).

Two extracellular structures form on the outer membrane of the cell. In the elongating bundles. a tiny, dark structure forms on the cell membrane (Fig. 11 and 12). This structure eventually develops into the satellite body and becomes a prominent feature of the sperm tail in more developed eupyrene cells. It can be observed in younger stages as an indistinct spot located more or less randomly in each spermatid tail (Fig. 11 and 12). In more mature cells, the structure is much larger and is located much nearer to the axial filament. The satellite body is situated between the outside of the cell membrane and the inside of a continuous cyst cell membrane that envelops all of the spermatids. Small septa radiate from the satellite body toward the cell membrane (Fig. 13, 14, and 22). The other extracellular structure is the radial mantle, which is characteristic of Lepidopteran sperm and forms only in the most mature eupyrene sperm bundles (Fig. 18-20). In the most mature testicular bundles, the radial mantle is well developed, and the "spokes' of this structure radiate from the outer membrane of the cell into the intercellular matrix.

One of the major features of maturation in sperm cells is the progressive loss of cytoplasm. Younger cells contain a large amount of cytoplasm, whereas more mature cells have less cytoplasm. In addition, multivesicular bodies are no longer seen and the tail portion of the cell consists mainly of the MDs, axial filament, satellite body (Fig. 18–20), and radial mantle.

#### Discussion

Previous studies on the testicular sperm of Lepidoptera were reported by Zylberberg (1963), Smith (1968), Phillips (1970a,b), Riemann (1970), Friedlander & Wahrman (1971), and Friedlander & Hauschtech-Jungen (1982) and by several studies reported in Baccetti (1970). Throughout the process of sperm maturation, the MDs are a prominent feature. In the youngest spermatid cysts, the cytoplasm contains numerous mitochondria. These mitochondria form fingerlike extensions that fuse or coalesce with one another to form a large structure, the nebenkern (Retzius 1904, André 1959, Lai-Fook 1982a,b). André (1959) described the formation of the nebenkern from individual mitochondria that he called chondriosomes. He divided nebenkern formation into two phases: the "agglomeration" of mitochondria into a very large body and the unwinding of the nebenkern, "se déroule," into two long filaments that coil around the flagellum in a helix. Growth and fusion of numerous mitochondria into a nebenkern is common in insect sperm and also in crustacea (Nicotra & Mura 1985). The nebenkern divides to form MDs. These structures are of different size and always next to each other. They elongate with the sperm tail and have varying morphology depending on the location of the section along the tail and the stage of eupyrene bundle maturation. Phillips (1970a) has shown that MDs in 18 species of insects are of unequal size. MDs have an inner and outer membrane. A complex network of cristae eventually develops in the inner membrane, and the cristae persist until the bundle reaches the duplex region of the male reproductive tract (LaChance 1984).

Maturation of the sperm bundle involves the disappearance of a large volume of cytoplasm. In the round spermatid and elongating stages, each sperm has considerable cytoplasm in the tail portion. Multivesicular bodies are also commonly present. As maturation progresses, less and less cytoplasm is seen in the sperm tails, and the multivesicular bodies also disappear. This does not imply that the two events are causally related. In the mature sperm bundles in the testis (Fig. 18–20), the cytoplasm is distributed over a much larger area and appears diminished as the spermatid elongates.

The complex system of microtubules that surrounds the elongating MDs was studied by Phillips (1970b). The sheath of tubules is often called the manchette and is found in the spermatids of mammals, birds, and reptiles as well as insects. Their role is not exactly known but they probably function in the sperm elongation process and disappear completely before the sperm cell is fully mature.

The axial filament or flagellum (André 1961, Phillips 1970b), also called the axoneme by some authors (Baccetti 1972), is another major feature in the sperm tail. It is first observed near the nucleus and nebenkern in young eupyrene spermatids. As maturation progresses, the axial filament becomes more distinct. At first, the axial filament contains nine doublets of tubules in the outer circle (periphery) and two tubules in the central core. Later, a third darker-staining accessory tubule (Baccetti 1972) appears near each doublet and produces the typical pattern in insects of 9 + 9 + 2. The accessory tubules contain glycogen-like bodies, which accounts for their darker staining (Baccetti 1972). Phillips (1970b) in his extensive study of the flagellum in insect sperm found that the "typical insect sperm flagellum has 9 singlet accessory tubules peripheral to the 9 doublets of the flagellum. Most insect sperm flagella have a central pair of singlet tubules which are similar in morphology to the 9 peripheral accessory tubules.

Two extracellular structures are also found in association with the outer membrane of the sperm



Fig. 18-23. Cross sections through eupyrene sperm cells in various stages of maturation in normal male testes. Bar =  $0.25 \ \mu m$ . (18-20) Sections through eupyrene sperm bundles in adult males showing variation in morphology of MDs and satellite bodies (SB). Radial mantle (RM) surrounds each cell. (21) Cell from adult male. Note deep, cristae (C) in one of the MDs. (22) From 5-d-old pupae. Note SB and septa (S) connecting it to outer cell membrane. (23) From 5-d-old pupae. Manchette fibers surround the MDs.

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tail. The satellite body first appears as a tiny black spot on the plasma membrane of immature eupyrene cells. It is located some distance from the axial filament and the MDs (Fig. 11 and 12). As sperm cell maturation occurs and the cell disposes of its cytoplasm, the satellite body becomes much larger and more distinct. In later, more mature sperm cells it is located near the axial filament and the MDs. The satellite body has also been called the reticular appendage (Phillips 1971, Lai-Fook 1982a,b) and the clear band (Yazumi and Oura 1964). The satellite body is attached to the cell membrane by septa, which measure about 50 Å in diameter in Anagasta (Riemann & Thorson 1971). Once formed, the satellite body is a permanent feature of the sperm tail as the eupyrene bundle descends to the duplex region of the male reproductive tract, but it disappears when the sperm bundles dissociate in the spermatophore.

The radial mantle (Fig. 18-20) is the last major structure to appear in the sperm, while the bundle is still located in the testis. This structure was studied in detail by Phillips (1971) and called lacinate appendages (André 1961). As described by Phillips (1971), "elaborate laminated appendages project radially from the plasma membrane like the blades on a paddle wheel and extend from the anterior tip posteriorly along most of the length of the Lepidopteran spermatozoa." In Heliothis, as in other Lepidopteran insects, the spokes of the radial mantle form a circle around the sperm tail. They radiate from the outer membrane into the matrix that surrounds the sperm. This structure is most conspicuous in the eupyrene sperm of adults. However, the radial mantle disappears as the eupyrene bundles leave the testis and descend into the seminal vesicles and the duplex region. Riemann & Thorson (1971) discuss the relation of the radial mantle to the formation of the sperm sheath. The radial mantle or lacinate appendage is found only in Lepidoptera in eupyrene sperm (Baccetti 1972).

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