Cell Tissue Res (1994) 275:467–479

Cell & Tissue Research

Ultrastructural examination of the insemination reaction in *Drosophila*

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Received: 4 May 1993 / Accepted: 1 July 1993

Abstract. The insemination reaction is a swelling of the female vagina caused by the male ejaculate. This postmating phenomenon is common among species in the genus *Drosophila*. It could act as a plug securing male paternity. It is not clear, however, what benefits it provides to the female. The structure formed in the female vagina is expelled in some species and disappears gradually in others suggesting different phenomena. Based on ultrastructural examination of the vaginal contents of five *Drosophila* species (*D. mettleri*, *D. nigrospiracula*, *D. melanogaster*, *D. mojavensis*, and *D. hexastigma*), we propose three terms to describe these vaginal structures: the sperm sac, the mating plug, and the true insemination reaction. Each term describes a distinct structure associated with a specific female postmating behavior. This study questions the concept of the insemination reaction as a single phenomenon and discusses its possible functions from an evolutionary perspective.

Key words: Vagina – Insemination reaction – Postmating behavior – Evolution – Sperm competition – Sexual selection – Paragonia – *Drosophila* (Insecta)

Introduction

Sexual selection is a major selective force acting on both sexes in the context of reproductive fitness (Thornhill 1979). The reproductive success of males depends on the sperm that accomplish fertilization of the eggs (Parker 1984). Females of many species, especially insects, store sperm and remate several times; therefore, it is likely that ejaculates from several males will compete for the fertilization of the eggs (Parker 1970). Males have evolved behavioral, morphological, and physiological adaptations to secure their paternity against other males. Male mechanisms to ensure paternity can be beneficial or costly to females (Knowlton and Greenwell 1984). Cases where there is conflict between the interests of the sexes pose interesting evolutionary questions (Trivers 1972; Parker 1984).

The insemination reaction, which occurs in many species of *Drosophila*, is an enlargement of the vagina produced by the male ejaculate (Patterson 1946). In intraspecific matings the reaction appears to prevent the female from remating temporarily (Patterson 1947). Therefore, the insemination reaction secures the male paternity of offspring. It is not clear, however, whether this postmating plug provides any selective benefit to the female in which it evolved (Maynard Smith 1956). Parker (1970), discussing the insemination reaction in the context of sperm competition, suggested that the insemination reaction does not have to confer selective advantages to the female; it could have evolved through competition among the males. The insemination reaction could be a trait evolving in females due to the advantage conferred to her male offspring (Fisher 1958).

The insemination reaction influences the postmating behavior of the female. Patterson (1947) stated that most females will expel an excess of sperm together with the insemination reaction materials around 6 to 8 h after mating. This suggests that the female plays an active role in removing sperm from her vagina. However, Lee (1950) and Asada and Watanabe (1987) reported that the insemination reaction disappears gradually from the vagina. A gradual clearance of the reaction suggests the possibility of an ongoing physiological process rather than a female-controlled behavior. Because the two ways of bringing the reaction to its end – expulsion and gradual disappearance – suggest major differences in female control of the outcome of a mating, we decided to compare the structures of the reaction masses formed in different species of *Drosophila*.

In the present study, using ultrastructural and biochemical techniques, we compared the structures formed in the female vagina after mating in five *Drosophila* species: *D. mettleri*, *D. nigrospiracula*, *D. mojavensis*, *D.*

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hexastigma, and *D. melanogaster*. The first four species belong to the repleta group, a large group of mainly cactophilic *Drosophila,* which show great variation in reproductive characters. *Drosophila melanogaster* was chosen because it has a postmating behavior similar to some of the cactophilic flies although it is phylogenetically distant from the repleta group. These five species previouly have been classified in the three different categories of the insemination reaction first proposed by Wheeler (1947). *Drosophila melanogaster* is listed in class I, as there is no observable insemination reaction, but the female expels a droplet of sperm after mating (Wheeler 1947). *Drosophila mojavensis* has a very striking enlargement, class III, of the vagina that disappears gradually as first demonstrated by Patterson (1946). *Drosophila hexastigma* is also listed in class III, since it has a major enlargement of the vagina, but Wheeler (1947) found that *D. hexastigma* females also extrude a solid structure after mating. More recently *D. mettleri* has been classified as class II, with a mild reaction, and *D. nigrospiracula* as class I, with no observable reaction, by Markow and Ankey (1988). However, Heed (1990) found that both of these species discard a large mass of sperm after mating.

Based on the ultrastructural and biochemical results reported here and our ongoing studies of the postmating behavior of females, we believe that among these five species there are three distinctive postmating phenomena: the "sperm sac" present in *D. mettleri, D. nigrospiracula,* and *D. melanogaster*; the "true insemination reaction" present in *D. mojavensis;* and the "mating plug" present in *D. hexastigma.* To understand the delineations of the sperm sac, mating plug, and true insemination reaction inside the female vagina (see Lee 1950 for definition of this term), the structure of the vaginal wall of each species was also examined. Our new classification brings into question the role of the insemination reaction as a single entity and contributes to our understanding of the diversity of reproductive traits among *Drosophila* species.

Methods and materials

Animals

The strains of cactophilic flies used in this study were: *Drosophila mettleri* A855 and *D. nigrospiracula* A855, both collected in Cerro Colorado, 24 kms northeast of Puerto de la Libertad (Mexico) in February, 1984; *D. mojavensis* A730 from Tonichi (Mexico), collected in March, 1978; and *D. hexastigma* A842 from Zapotitlan Salinas (Mexico), collected in March, 1983. These cactophilic flies were reared in the laboratory in 8-dram shell vials containing standard food (yeast-agar-banana-malt-syrup). Virgin males and females were immobilized and separated on ice within 24 h after eclosion. About 25 flies per vial were stored at room temperature until used in the experiment between 11 and 14 days posteclosion. The *D. melanogaster* used were the Oregon R-C strain. In this species the males and females were also separated on ice within 8 h after eclosion and used in the experiment between 4 and 6 days posteclosion.

On the day of the experiment approximately 30 virgin males and 20 virgin females were placed in Petri dishes with pieces of food on the sides. After 8 h the flies were removed and the expelled structures were collected. The structures expelled by *D. hexastigma* were collected after a 24-h period.

Electron microscopy

The vaginal structures expelled by the females and the reproductive organs of virgin and recently mated females were prepared for light- and electron-microscopic examination. *Drosophila mettleri, D. nigrospiracula, D. melanogaster,* and *D. hexastigma* females expel the structures formed in the vagina on the surface of the food media, where they can be found and collected. The expelled structures were collected from the Petri dishes and placed in fixative overnight. Females were dissected in saline solution and their reproductive organs were also placed in fixative overnight. The fixative solution contained 2.5% glutaraldehyde, 0.5% paraformaldehyde, 0.18 mM $CaCl_2$, 0.58 mM sucrose, and 0.1 M sodium cacodylate buffer, pH 7.4. After fixation the tissues were osmicated en bloc in 0.05%–1.0% OsO_4, and then dehydrated through a graded series of ethanols and embedded in Epon/Araldite. For light microscopy, sections were cut at 1-μm thickness, stained with toluidine blue, and mounted in Permount. For electron microscopy, thin sections were cut on a diamond knife, placed on clean grids, stained with lead citrate, and examined in a JEOL 1200EX electron microscope.

For light microscopy, we examined the vaginas of 8 *D. mettleri* females interrupted during the late stages of mating or immediately after mating and 3 discarded sperm-containing structures; 8 *D. nigrospiracula* vaginas of recently mated females and 2 discarded sperm-containing structures; 11 *D. mojavensis* vaginas 40 min after mating; 3 *D. hexastigma* vaginas between 1 to 4 h after mating and 5 extruded structures; and 2 *D. melanogaster* vaginas of recently mated females and 2 extruded sperm-containing structures.

For electron microscopy we examined 3 extruded sperm-containing structures of *D. mettleri* and 3 vaginas in the last stages of mating or right after mating; 2 *D. nigrospiracula* vaginas after mating and 2 discarded sperm-containing structures; 2 *D. mojavensis* vaginas 40 min after mating; 2 *D. hexastigma* vaginas from 1 to 4 h after mating and 2 extruded structures; and 2 *D. melanogaster* vaginas after mating and 2 expelled sperm-containing structures.

Electrophoresis

We used one-dimensional electrophoresis to compare the chemical contents of the sperm sacs in *D. mettleri, D. nigrospiracula,* and the mating plug in *D. hexastigma.* Fifteen virgin males and 15 virgin females, 11–15 days old, were placed in Petri dishes with small pieces of food in the sides. After 24 h 10 sperm sacs or matings plugs of each species were collected from the Petri dishes and placed in an ice-cold homogenizer with 200 μl of 5% trichloric acid. The homogenate was transferred to a plastic tube and the homogenizer was rinsed with 200 μl of 5% trichloric acid that was also added to the plastic tube. Then, the homogenate was centrifuged for 5 min, at 12000 *g* and the supernatant was carefully removed and either used immediately or stored at −90° C.

The proteins in the supernatant were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli 1970) on 10% acrylamide gels containing 0.1% SDS. The gels were silver-stained as described by Merril et al. (1983). Sigma standards were used.

Results

Drosophila mettleri

In previous studies *D. mettleri* has been classified as having a mild insemination reaction (Markow and Ankey

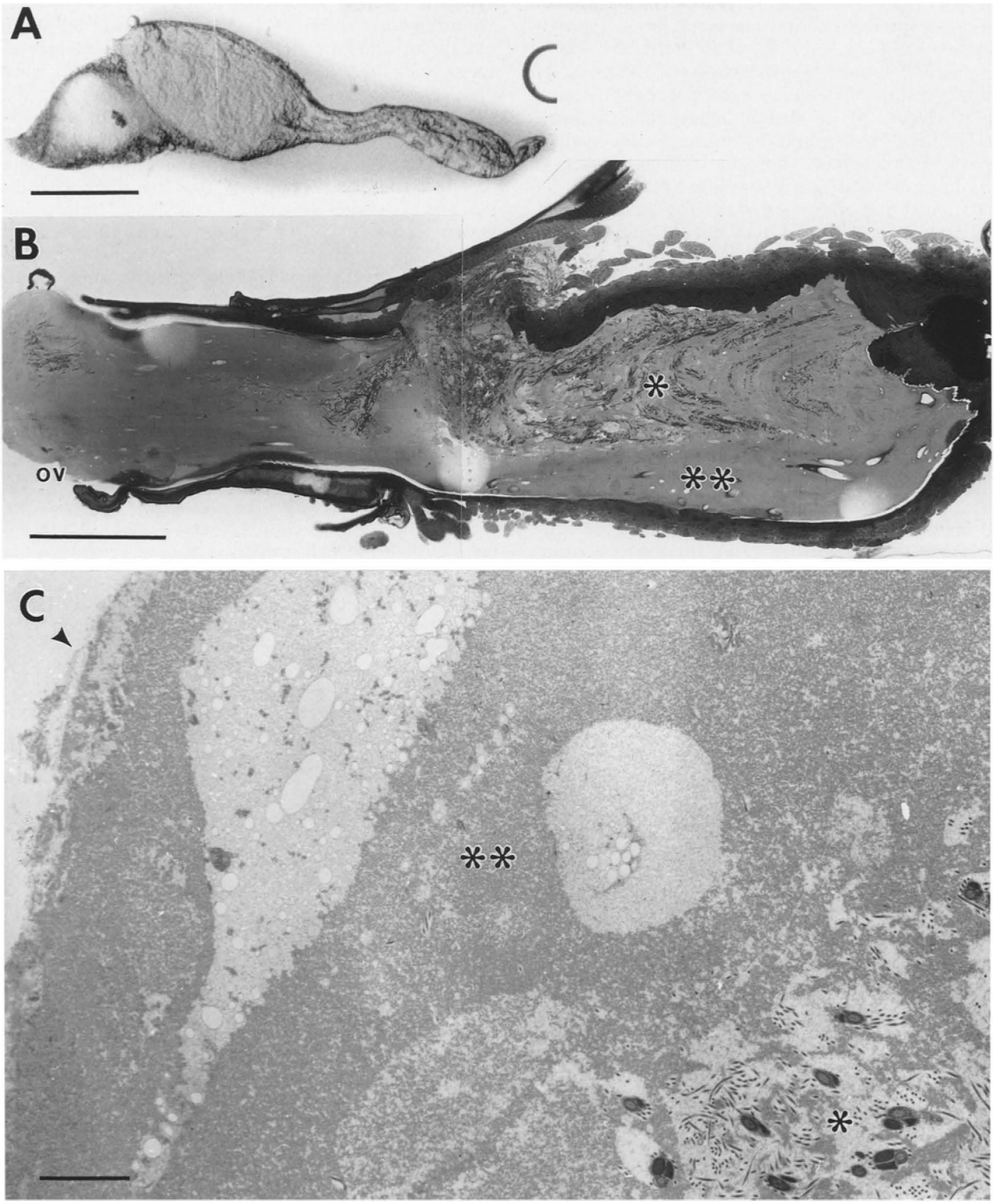


Fig. 1 A–C. *Drosophila mettleri.* **A** Light micrograph of extruded sperm sac. **B** Light micrograph of longitudinally sectioned vagina of a female interrupted in the middle stages of mating. The vagina is filled with two sperm sac substances: a lighter flocculent material densely packed with darkly stained sperm (*one asterisk*) and a denser amorphous extracellular material (*two asterisks*), which predominates in the opening of the vagina (*ov*). **C** Electron micrograph of extruded sperm sac: sperm are densely packed in light material (*one asterisk*), which is partially ringed by a denser material (*two asterisks*). No cellular membranes separate the two substances or surround the entire sperm sac (see *arrowhead*). *Scale bars:* **A** 200 μm; **B** 100 μm; **C** 2 μm

1988). After mating, *D. mettleri* females expel a whitish sperm sac of variable size and shape measuring approximately 660 × 230 μm (Fig. 1A). The extruded "sperm sacs" (Heed 1990) are compact packages composed of two clearly distinct materials: a light, flocculent material partially ringed by a denser amorphous material (Fig. 1C). Light micrographs of sections of the vaginas of recently mated females show the same two materials as seen in the extruded sperm sac (Fig. 1B).

The vaginal wall of *D. mettleri* females comprises two cellular layers: an outer layer of muscle and an inner epithelial layer with a thin chitinous lining. In the electron microscope (Fig. 2A), the muscle is seen to be one cell thick, with myofilaments oriented longitudinally, and the inner membrane of the epithelial cell layer is thrown into numerous and very compact infoldings varying little in thickness around the vagina. Both within the vagina immediately after mating and in the sperm sac after it is extruded, most of the sperm are densely packed in a light, flocculent material, which also contains numerous paragonial tubules (Bairati 1966; Perotti 1971; Fig. 2C) and presumed "fat cells." These cells contain empty membranes sacs, which probably contained lipids before dehydration (Fig. 2B). At the electron-microscope level no membranes are seen to separate the light and dark substances within the vagina (Fig. 2D) or surrounding the extruded sperm sac (Fig. 1C). An example of a transverse section of a sperm tail, containing the mature axoneme and two dense mitochondrial derivatives of similar size, is shown in the inset in Fig. 2A.

Drosophila nigrospiracula

We found that *D. nigrospiracula* also has sperm sacs, despite having been classified as having no insemination reaction at all (Markow and Ankey 1988). *Drosophila nigrospiracula* sperm sacs are also of variable size and shape but smaller on average (440 × 160 μm) than the sperm sacs of *D. mettleri*. The sperm sac in this species also comprises two materials, which have the same appearance as in *D. mettleri* (Fig. 3C). The light material, where the sperm are embedded together with the paragonial tubules, is partially encapsulated by the denser amorphous material. In contrast to *D. mettleri*, however, the sperm are not accompanied by fat cells (Fig. 2B).

The vaginal wall in *D. nigrospiracula* consists of three layers: an external longitudinal muscle layer, a middle circular muscle layer, and an inner epithelial layer lined internally by cuticle (Fig. 3A). The epithelial layer in this species also shows areas of infoldings (Fig. 3B); however, the thickness of these infolded areas varies greatly along the length of the vagina and on average is thinner than in *D. mettleri* (or *D. mojavensis*, see below).

Drosophila melanogaster

Drosophila melanogaster also has been classified as having no observable insemination reaction, but females discard a substantial portion of the ejaculate in a 'droplet' according to Wheeler (1947). These discarded structures, measuring approximately 420 × 140 μm, resemble the sperm sacs of *D. mettleri* and *D. nigrospiracula* when observed through the dissecting microscope. Light and electron microscopy of sectioned material, however, reveal that the structure discarded by *D. melanogaster* females comprises at least three materials. There is a light material where the sperm is embedded along with the paragonial tubules (Fig. 4C, E), a denser amorphous material, and a denser material packed with filamentous structures (also seen by Perotti 1971), which differ from profiles of sperm in having a regular cross-banding pattern (Fig. 4D). In this species there is a thin cellular process bounding some areas of the sperm sac (Fig. 4D).

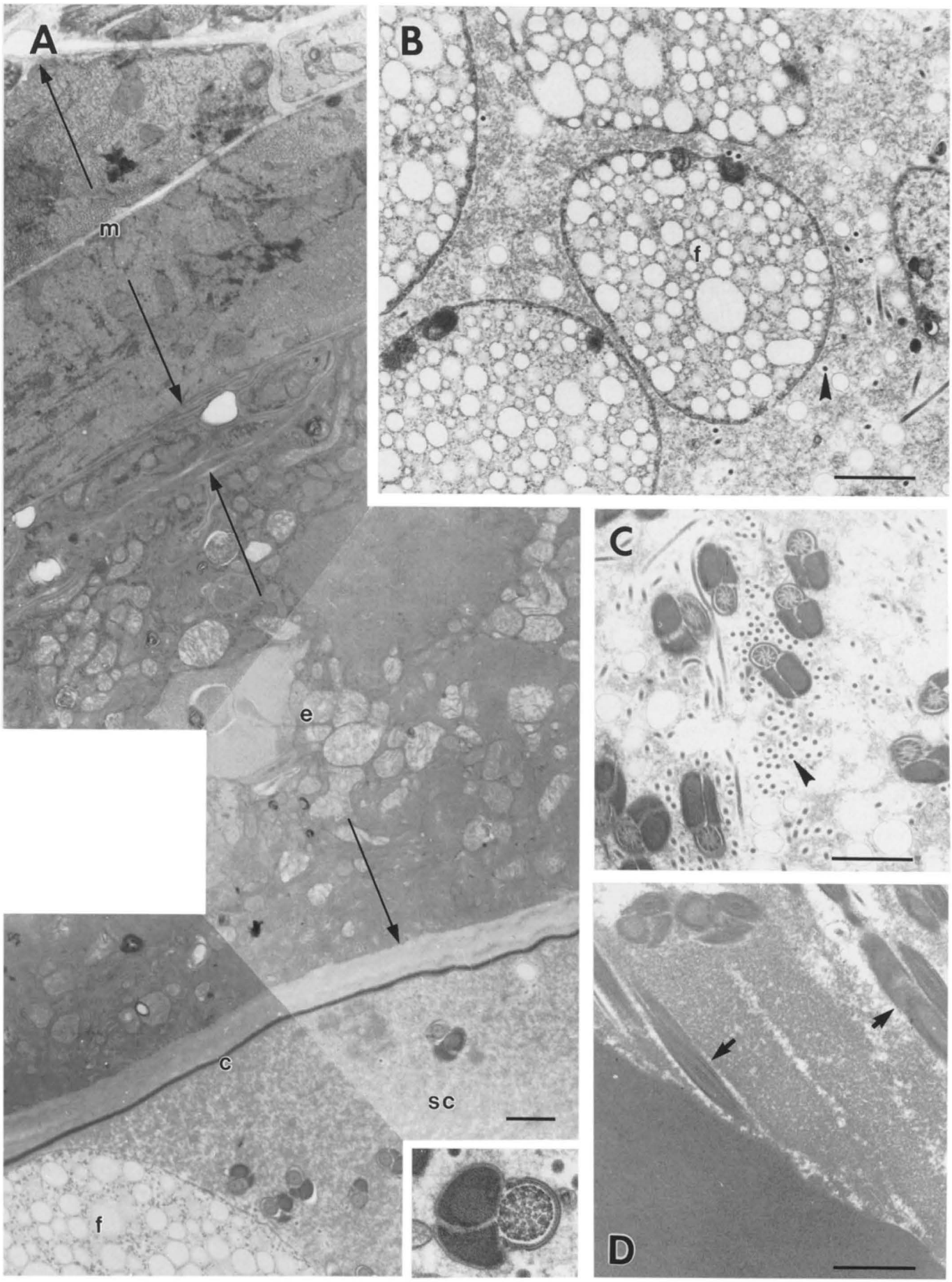
In *D. melanogster* we found significant variation in wall structure in different parts of the vagina. It varies from very thin areas, comprising only the epithelial layer and a single muscle cell layer (Fig. 4B), to areas where the muscle cells may be stacked five to six deep. The inner epithelial layer is lined internally by cuticle and also shows infoldings, which vary in thickness along the length of the vagina (Fig. 4A). Demerec (1965) described the structure, at the light microscopic level, of the vaginal wall in *D. melanogaster;* he, too, described several layers of circular muscle that do not extend over the entire perimeter of the vagina.

Drosophila mojavensis

Drosophila mojavensis has a strong "true insemination reaction" (which cannot be examined outside the female like a sperm sac because it is not discarded). The "true insemination reaction" mass, in contrast with the sperm sacs, is not formed by two distinct substances. The vagina of mated *D. mojavensis* females is filled with a uniform amorphous matrix in which sperm are embedded along with paragonial tubules. At the electron-microscopic level this matrix looks different from either of the two substances found in the sperm sacs (Fig. 5B, C). A cross section of a sperm tail with the axoneme and one mitochondrial derivative is shown in Fig. 5B.

The vaginal wall in *D. mojavensis* shows the same organization as that in *D. nigrospiracula*. There are two muscular layers, an external longitudinal and a middle circular layer, and an epithelial layer lined with chitin

Fig. 2A–D. Electron micrographs of *D. mettleri*. **A** Montage of cross section spanning entire vaginal wall in late stages of mating, demonstrating concentric layers of longitudinal muscle (two cells thick, *m*), epithelium (*e*), and cuticle (*c*). In lumen of vagina, beneath cuticle, is sperm sac (*sc*) containing sperm and fat cells (*f*). *Inset* Cross section of sperm tail at higher magnification. **B–D** Components of sperm sac inside vagina. **B** Presumed "fat cells" (*f*) and paragonial tubules (*arrowhead*) from light region. **C** Sperm and paragonial tubules (*arrowhead*) from light region. **D** Boundary between light and dense regions. Note absence of any intervening membrane. *Arrowheads* Longitudinal sections of sperm tails. *Scale bars:* **A, B, C** 1.25 μm; **D** 1 μm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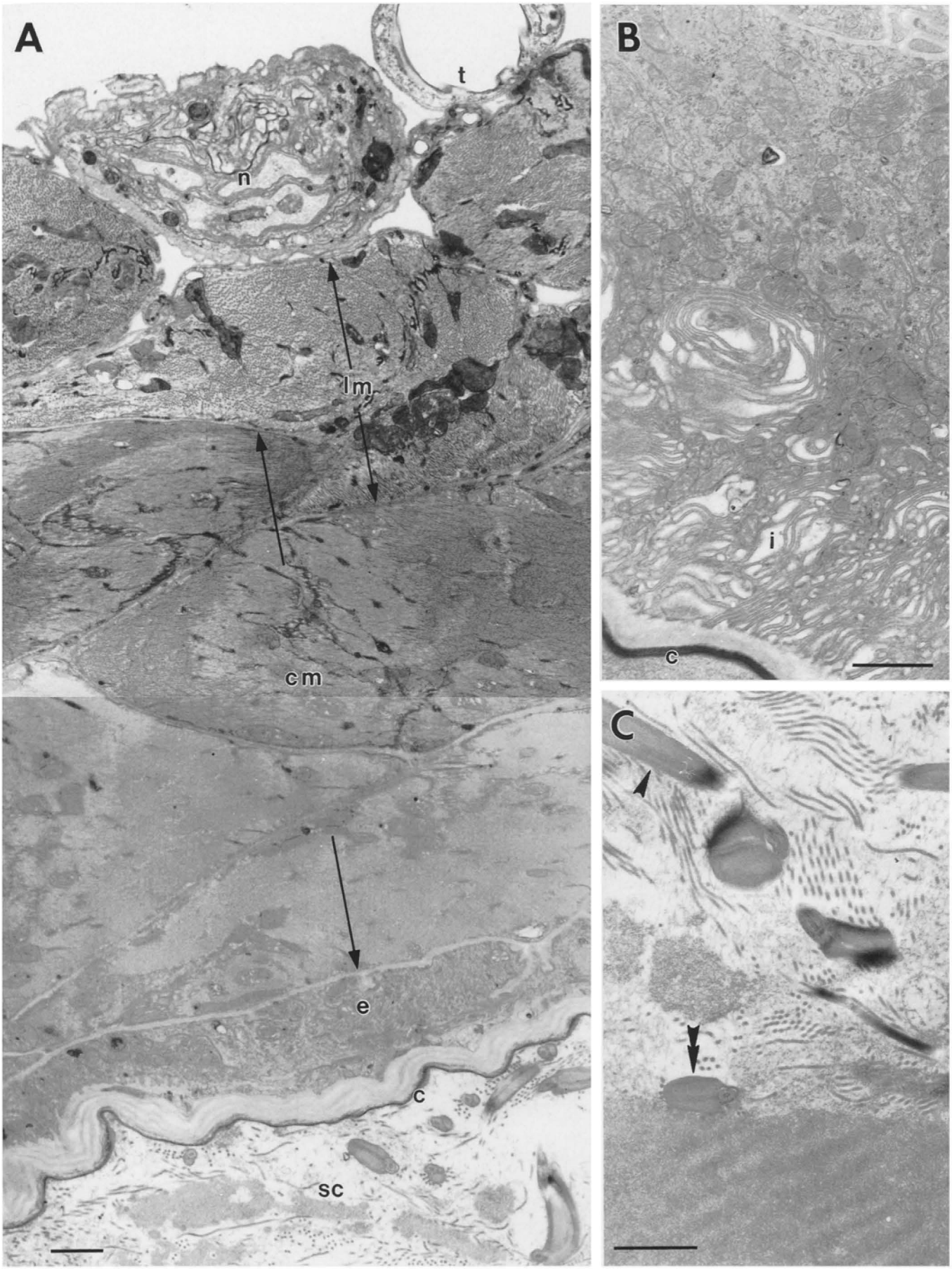


Fig. 3A–C. Electron micrographs of *D. nigrospiracula*. **A** Montage of cross section spanning entire vaginal wall 5–15 min after mating. *cm* and *lm* Circular and longitudinal muscular layers; *e* epithelial layer; *c* cuticle; *sc* sperm sac; *n* probable nerve; *t* trachea. Epithelial membranes are not infolded in this region. **B** Epithelial layer of vaginal wall in area of heavy infolding (*i*) of membranes. **C** Boundary between light and dense regions of sperm sac in vagina. All sperm (*arrowhead* longitudinal section; *double arrowhead* cross section) and paragonial tubules are in the light area. *Scale bars:* **A–C** 1 μm

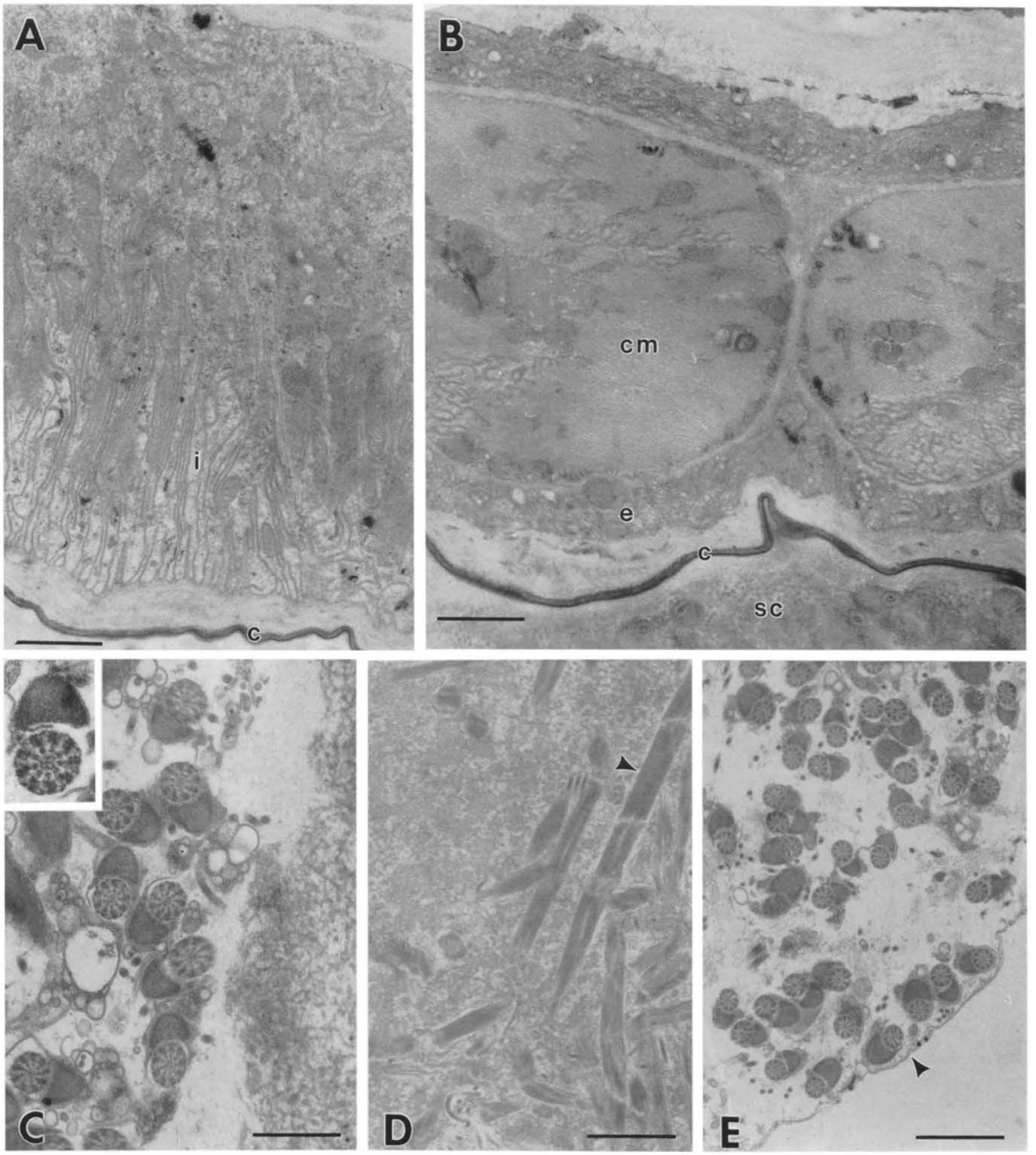
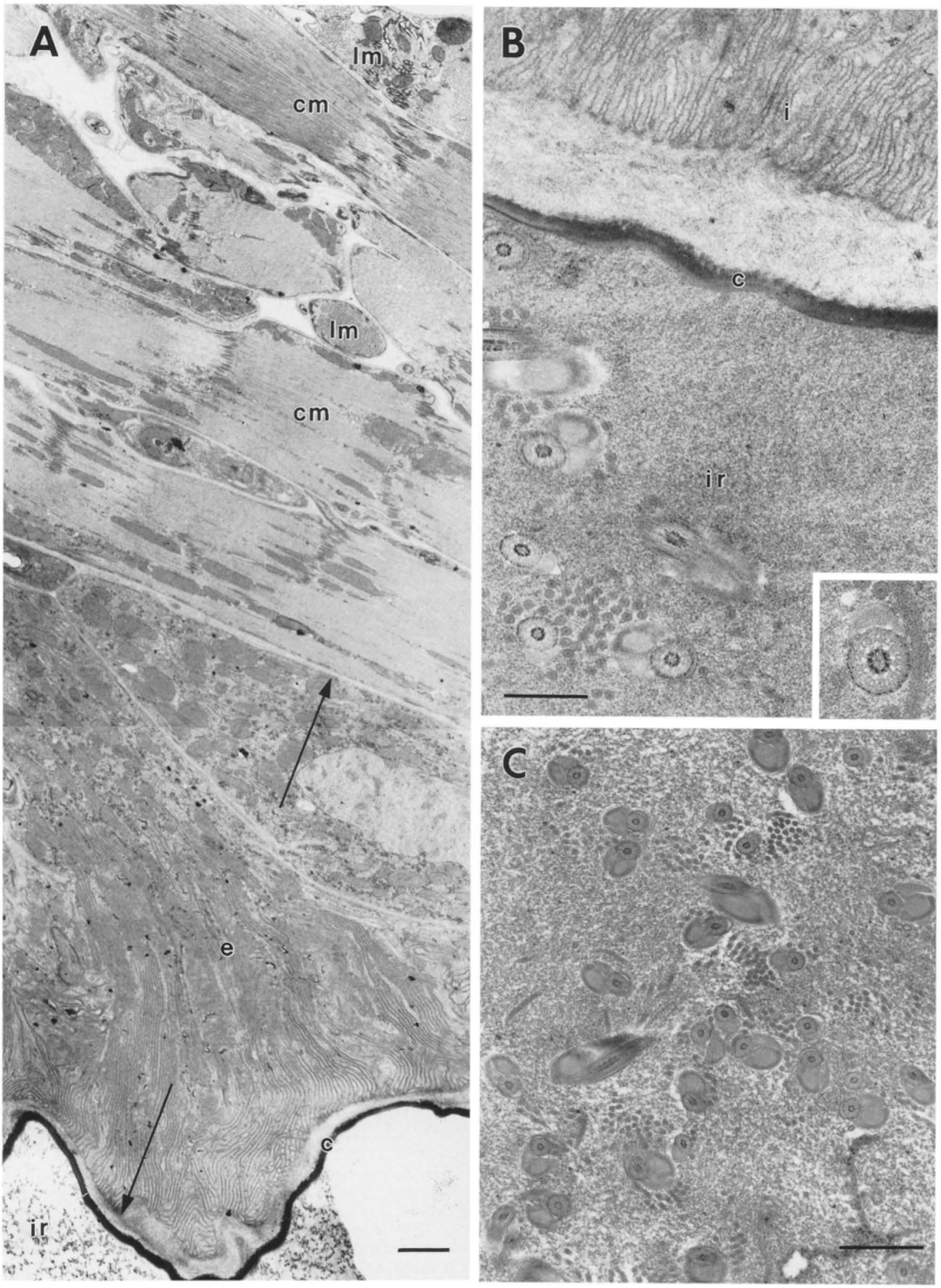


Fig. 4A–E. Electron micrographs of *D. melanogaster*. **A** Cross section of epithelial layer of vaginal wall 5–15 min after mating. *i* infoldings of epithelial cells; *c* cuticle. Inner epithelial membrane is most heavily infolded in this area. **B** Another area of same vaginal wall showing one circular muscle layer (*cm*), epithelial layer without infoldings (*e*), cuticle (*c*), and sperm sac (*sc*). **C–E** Components of extruded sperm sac. **C** Boundary between light region and moderately dense regions. Sperm and paragonial tubules in light region. *Inset* Cross section of sperm tail at higher magnification. **D** Densest material, packed with filamentous structures (*arrowhead*). **E** Outer edge of sperm sac, in area containing sperm. Note thin cellular process (*arrowhead*) bounding the sperm sac. *Scale bars:* **A, B, D, E** 1 μm; **C** 2 μm



Figs. 5A–C. Electron micrographs of *D. mojavensis*. **A** Montage of cross section through vaginal wall 45 min after mating. *cm* and *lm* Interspersed circular and longitudinal muscular cells; *e* heavily infolded epithelial layer; *c* cuticle. In lumen of vagina, beneath cuticle, is insemination reaction (*ir*). **B** Higher magnification of membranous infoldings (*i*), cuticle (*c*), and insemination reaction (*ir*). *Inset:* Cross section of sperm tail at higher magnification. **C** Sperm and paragonial tubules embedded in the insemination reaction secretion. *Scale bars:* **A**, **C** 1 μm; **B** 2 μm

(Fig. 5A). In this species the epithelial layer is very heavily infolded compared with the two species that have the sperm sacs (Fig. 5A, B).

Drosophila hexastigma

Drosophila hexastigma, like *D. mojavensis,* has been classified as having a very strong, class III, insemination reaction. However, the structure formed in the vagina after mating in this species is quite distinctive. In *D. hexastigma* the vagina always enlarges in an elongate shape. The mating plug is a solid structure, which can be extracted easily from the vagina intact; this cannot be done with the sperm sacs or the insemination reaction. Females expel this plug usually within 24 h after mating. The mating plugs are larger structures than the sperm sacs, measuring 840×220 μm, with a consistent elongate shape. Electron micrographs of sections of the vaginas of recently mated females show irregularly shaped patches of a dense homogeneous material embedded in a lighter material (Fig. 6B). The entire plug is surrounded by a thin layer of a dense amorphous substance. Most of the sperm are located under this outer layer along with paragonial tubules (Fig. 6B). Smaller numbers of sperm and paragonial tubules are also present in the light material between the patches. A cross section of a sperm tail with the axoneme and two large mitochondrial derivatives is shown in Fig. 6D.

The vaginal wall in *D. hexastigma* is very thin near the ovipositor, comprising only the epithelial layer and occasional isolated muscle cells (Fig. 6C). The wall widens abruptly deeper in the vaginal pouch, where the muscle cells may be stacked three to four deep (Fig. 6A). In no part of the wall is the epithelial membrane thrown into deep folds. The myofibrils are disorganized with no apparent preferred orientation (Fig. 6A).

Electrophoresis

Preliminary biochemical examination of the sperm sacs and mating plugs from three of the species studied here extend our ultrastructural analysis. The results of the electrophoretic polyacrylamide gel are shown in Fig. 7. The gel reveals marked variation in the chemical composition of the sperm sacs of *D. nigrospiracula* and *D. mettleri* and the mating plug of *D. hexastigma.* Within the sperm sacs, a few bands can be detected in the same position in both species. Thus, the sperm sacs, while not identical, show greater similarity with each other than with the mating plug.

Discussion

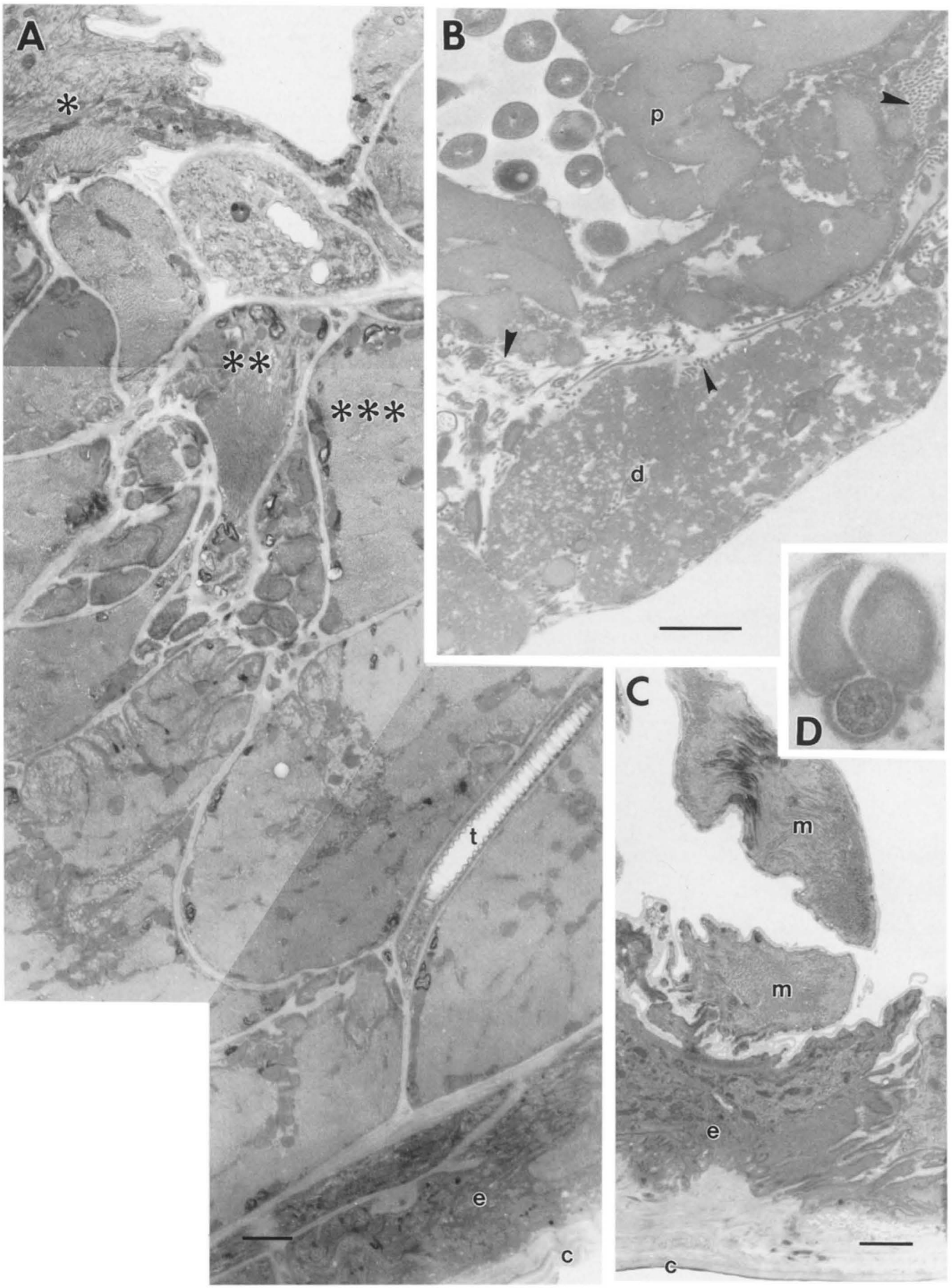
The insemination reaction as defined by Patterson (1946) appears to play a significant role in the postmating behavior of females and the fate of the ejaculate. The structures formed in the vagina after mating appear to create conflict between female and male evolutionary interests. Furthermore, there is a wide variety of postcopulatory behaviors among the species previously categorized as having an insemination reaction, suggesting diversity at the functional level. In the present study we examined the structure of postcopulatory vaginal contents and the vaginal wall as a first step in determining the role of these vaginal structures as well as to extend our understanding of the diversity of reproductive traits in *Drosophila.*

Structure of the vaginal contents and the wall

The main purpose of this study was to distinguish at the ultrastructural level structures that have been pre-

Table 1. Summary of analysis of postmating phenomena

Traits	*Drosophila mettleri*	*Drosophila nigrospiracula*	*Drosophila mojavensis*	*Drosophila hexastigma*	*Drosophila melanogaster*
Phylogenetic classification	repleta group eremophila complex	repleta group anceps complex	repleta group mulleri complex mojavensis cluster	repleta group mulleri complex longicornis cluster	melanogaster group
Wheeler's class.	Class II	Class I	Class III	Class III	Class I
Vaginal contents:					
-expulsion?	Yes	Yes	No	Yes	Yes
-structure	Two discrete materials with fat cells	Two discrete materials without fat cells	Uniform matrix	Two materials with patchy distribution	Three materials
Vaginal wall:					
-muscle layer	One muscle layer	Two muscle layers	Two muscle layers	Thin to thick; 0 to 4 layers	Highly variable (thin and thick)
-epithelial layer	Much infolding	Some infolding	Heavy infolding	No infolding	Some infolding
Present classification	Sperm sac	Sperm sac	True insemination reaction	Mating plug	Sperm sac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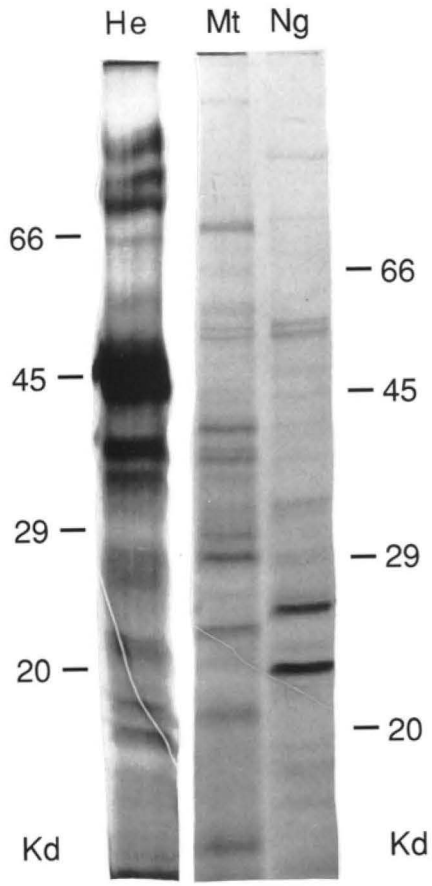

Fig. 7. Electrophoretic patterns (on 10% SDS gels) of protein samples prepared from mating plugs of *D. hexastigma* (*He*) and sperm sacs of *D. mettleri* (*Mt*) and *D. nigrospiracula* (*Ng*)

viously categorized as a single phenomenon, the insemination reaction. Our results reveal three different postcopulatory phenomena found in *Drosophila:* the true insemination reaction, the sperm sac, and the mating plug.

Our results (Table 1) can be summarized as follows. The sperm sacs in *D. mettleri* and *D. nigrospiracula* females are discrete compact structures with a large number of sperm (Heed 1990). They are formed by two substances, which are easily distinguished at the light- and electron-microscopic levels. The electron microscope revealed only one difference between the species, that the sperm sacs of *D. mettleri* have fat cells embedded in the light substance. The vaginal wall of *D. mettleri* comprises a muscle layer, an epithelial layer with heavy infolding, and a thin chitinous lining. *Drosophila nigrospiracula* has two muscle layers, an epithelial layer with little infolding, and a thin chitinous lining. *Drosophila melanogaster* has a sperm sac comprising three distinctive substances arranged irregularly in a compact unit. One of the substances is packed with a type of paragonial tubule not observed in the other species. The vaginal wall is highly variable along its perimeter, very thin in some areas and extremely thick in others.

Drosophila mojavensis exhibits a true insemination reaction, an expansion of the vagina after copulation is completed (Lee 1952). The reaction produces an amorphous matrix where the sperm is embedded. Electron microscopy shows that this matrix where the sperm is embedded looks significantly different from the matrix where the sperm is embedded in the sperm sacs or mating plug. The vaginal wall in this species is formed by three layers as in *D. nigrospiracula,* but in *D. mojavensis* the epithelial layer has very heavy infolding more comparable to that of *D. mettleri.*

Drosophila hexastigma has a mating plug, a solid structure comprising irregularly shaped dense material embedded in a lighter material. The vaginal wall in this species is very thin near the ovipositor widening abruptly in the vaginal pouch.

The true insemination reaction

Wheeler (1947) categorized *D. mojavensis* and *D. hexastigma* as having a strong insemination reaction. Our results lead us to conclude that only *D. mojavensis* has the "true" insemination reaction that we define as a mass of one amorphous material that greatly distends the vagina of recently mated females. This structure is soft inside the female, and it clears gradually from the vagina (Lee 1950; Asada and Watanabe 1987). Asada and Kitagawa (1988a) report that the insemination reaction in species in the nasuta group is soft in homogamic matings, but it hardens in heterogamic matings and the females cannot discard it. We have not examined heterogamic matings.

There are other differences between the true insemination reaction, the sperm sac, and the mating plug that support the idea of a distinct classification. In most species the insemination mass appears after copulation has finished. In such cases there is a clear chemical reaction occurring when the ejaculate from the male reacts in the female vagina (Lee 1950; Asada and Watanabe 1987, 1988; Asada and Kitagawa 1988b). This reaction causes a significant change in the volume of the vagina characterized by a great distension of the vaginal pouch. The sperm sac, on the other hand, can be observed in the female vagina soon after copulation begins. There is only a slight increase in volume of the vagina. We cannot rule out the possibility of a chemical reaction occurring during sperm sac formation in the female vagina; however, if sperm sac formation involves a chemical reaction in the female vagina, this reaction differs in timing and

Fig. 6A–D. Electron micrographs of *D. hexastigma.* **A** Montage of longitudinal section through vaginal wall about 1 or 2 h after mating. Muscle cells have myofibrils oriented in many different directions (*one, two* and *three asterisks* indicate three different orientations). *e* Epithelial layer without infoldings; *c* cuticle and *t* trachea. **B** Mating plug in same vagina, showing irregularly shaped dense patches (*p*) embedded in a lighter material, with a thin layer of dense material (*d*) surrounding the plug. Sperm are located under this outer layer (not shown here) along with paragonial tubules (*arrowheads*). **C** Thin area of the same vaginal wall near the ovipositor. *e* Epithelial layer; *c* cuticle and two loose muscle cells (*m*). **D** Cross section of sperm tail at high magnification. *Scale bars:* **A–C** 1 μm

ultrastructural appearance from the true insemination reaction.

The true insemination reaction, as found in *D. mojavensis,* is the only structure that is not expelled by the female, and it cannot be extracted intact from the vagina. There are no separate distinctive substances as in the sperm sacs or the mating plug. Wheeler (1947), reporting on *D. ananassae*, speculated that one part of the ejaculate contains the free sperm and one part is sperm-free and forms a gel-like mass, which forces retention of sperm for some time. This suggests that it is necessary to have other materials besides the sperm-containing substance to form a compact disposable structure.

The mating plug

Drosophila hexastigma, rather than having an insemination reaction, has what might better be described as a mating plug. A mating plug here is described as a structure, formed in the vagina after mating, that becomes a dense solid unit inside the female and is expelled sometime after mating. The mating plug in *D. hexastigma* always has the same elongate shape whether it is inside the female vagina or outside. In comparison with the sperm sac or the true insemination reaction, this is a dense, very compact and elastic structure and contains few sperm when extruded. Due to these characteristics, this structure inside the female vagina must act as a barrier to remating. We do not have any direct evidence, however, to prove that this structure seals the female genitalia. *Drosophila* males deliver the ejaculate into the female through a very thin-walled tube, the ejaculatory duct, which opens at the tip of the intromittent organ or aedeagus (Fowler 1973). The ejaculatory duct is too narrow to transfer fully formed the structure seen in the females after mating. This indicates that a solidification of the male products must occur inside the female. It is very difficult to observe *D. hexastigma* matings, so the time of formation, hardening, and discarding of the plug have not been observed. The distinctive appearance at the structural level is corroborated by a distinct chemical composition when compared to the sperm sacs of *D. mettleri* and *D. nigrospiracula* and suggests a difference in composition also from the true insemination reaction. Further investigation in the remating behavior of this species is required to understand the function of this structure.

Bairati and Perotti (1970) described a compact waxy plug formed in the *D. melanogaster* vagina 5–7 min after the beginning of copulation (mating lasts approximately 20 min). The function of this plug is not known. They postulated that the plug acts as a barrier to prevent loss of sperm through the vagina. At this time the relationship between the waxy plug they describe and the mating plug of *D. hexastigma* is not clear.

Sperm sac

Drosophila mettleri was classified previously as having a mild insemination reaction (Markow and Ankey 1988), while *D. nigrospiracula* and *D. melanogaster* were classified as having no observable insemination reaction (Wheeler 1947; Markow and Ankey 1988). In this study, we found these species to be similar and classify all three of them as having the sperm sac (Heed 1990). We define sperm sacs as structures with distinctive materials that stay soft inside the female vagina and do not cause inordinate distention of the vaginal pouch. These structures, containing a great quantity of sperm, are discarded by the females and become cohesive units in contact with the air.

The cohesion of the sperm sacs as compact units could be explained by the presence of a gelatinlike substance. Products from the male accessory glands contribute significantly to the male ejaculate in many insect species (Leopold 1976). These glands contain glycoproteins and mucoproteins, which might well form a compact structure in the absence of enclosing membranes. There is great variation in the chemical composition of the male secretory glands among *Drosophila* species (Chen 1984), which could explain the differences in the sperm sac structure and biochemical components among the species we studied.

In a variety of other insects the substances transferred by the male form what is called a spermatophore. Spermatophores are usually expelled by the females some time after mating. There is no precise definition for this term. The term "spermatophore" has been applied to any kind of structure that contains sperm, and therefore is so vague that we have chosen not to use it. Without providing a definition for the term, Gromko et al. (1984) reported that there are no spermatophores in the genus *Drosophila.*

Due to the small size of the male ejaculatory duct, the sperm sac, like the mating plug and the so-called spermatophores in other insect species, must be assembled in the female vagina from products of the male ejaculate. The sequence of transfer of the male ejaculate materials to the female to form the sperm sac is not known. Wheeler (1947) suggested that in *D. ananassae* the first portion of the ejaculate contains the sperm, and at the end of copulation the male transfers a sperm-free substance.

Sperm sacs are usually discarded within 24 h after mating in the three species. Behavioral work with *D. mettleri* shows that the time of exclusion of the sperm sac is correlated with the stage of maturation of the ovaries; females with immature oocytes retain the sperm sac longer (H. Alonso-Pimentel and W.B. Heed, unpublished). Correlations between the timing of discarding and physiological changes in the female are under study for the other two species, *D. nigrospiracula* and *D. melanogaster.*

The morphological variation of the vaginal wall

The five species examined exhibit significant variation in the structure of the vaginal wall. The number of muscle layers, the orientations of the layers, and the infoldings of the epithelial wall differ greatly among these spe-

cies. The vagina plays an important role in many aspects of female reproduction. The variation observed here could be due to adaptation to different female reproductive behaviors. Markow and Ankey (1988) have presented evidence that females with strong insemination reactions (class III) obtain a significant amount of material from the ejaculate. Our ultrastructural analysis shows that the infolding of the inner epithelial membrane of the vaginal wall varies among the species. The degree of infolding, high in *D. mojavensis* and low in *D. nigrospiracula,* correlates with the levels of absorption of amino acids from the male secretion found by Markow and Ankey (1988), suggesting that the infolding may provide the structural substrate for uptake of material. The variation encountered in the structure of the vaginal wall might also, however, be explained on the basis of the distant phylogenetic relationships among these species.

Future directions

In light of the variation observed in vaginal structures and postmating behavior of the female, the concept of the insemination reaction as proposed by Patterson (1946) is too simple. New terminology is necessary to distinguish the different phenomena embraced under this concept. We propose the terms "sperm sac", "mating plug", and "true insemination reaction" to categorize the different structures formed in the female vagina after copulation.

Sexual selection and sperm competition theory might help us to understand the presence of such different postmating phenomena. Behavioral studies currently are being pursued to explore female choice and male competition strategies that might relate to these postmating phenomena in the different species. Our structural analysis raises many interesting questions. Can we predict analogous behaviors in species that share similarities at the structural level? Can knowledge of the structural composition help us to predict how the female manipulates the male ejaculate? Can we interpret male strategies based on the structure and composition of the ejaculate inside the female?

Acknowledgements. We thank Galya Orr for expert technical assistance with electron microscopy, Dr. David Morton for assistance with electrophoresis work, Craig Jacobs, Juan Caldas and Chip Hedgcock for photographic assistance, and Eric Dyreson and Kiyomi Morino for their discussions of the manuscript. Leona Mukai provided the *D. melanogaster* strain. This study was supported by a graduate trainee grant from the Center for Insect Science and the Du Pont Aid in Education program to H.A.P.

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