The ultrastructure of the sperm and motile spermatozeugmata released from the freshwater mussel *Anodonta grandis* (Mollusca, Bivalvia, Unionidae)

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Mature sperm are released from *Anodonta grandis* as multiple spherical spermatozeugmata, which are complexes measuring 40–50 μm and containing up to 2800 sperm when fully populated. The 1.5 × 4.0 μm cylindrical sperm head is anchored in the spherical spermatozeugma periphery, with the 35 μm long flagella projecting perpendicular to the surface. Rotational and progressive motility is achieved by an apparently asynchronous but sequential flagellar beat. An acellular 80-nm lamina around the spherical spermatozeugma encircles a central fluid-filled sphere called the globe. Each sperm head is embedded in a chamber formed by extensions of the 80-nm lamina and in contact with the sperm plasmalemma in a flagellar collar posterior to the 5 mitochondria at the base of the sperm nucleus. A proximal centriole located in a fossa in the nucleus base is connected to a distal centriole by a dense amorphous matrix. The distal centriole is locked to the flagellar collar via 9 striated, bifurcated pericentriolar processes. The anterior part of the sperm contains an acrosome-like region containing 0.1 μm diameter membrane-bound vesicles. The spermatozeugma complexes may survive up to 24 h and may serve to transport sperm to the gill chamber of the female mussel.

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Les spermatozoïdes à maturité d'*Anodonta grandis* sont libérés sous forme de complexes multiples sphériques de 40–50 μm de spermatozeugmas contenant jusqu'à 2800 spermatozoïdes à leur capacité maximale. La tête cylindrique du spermatozoïde, de 1,5 × 4,0 μm, est ancrée à la périphérie du spermatozeugma sphérique par son flagelle de 35 μm qui est perpendiculaire à la surface. Les battements apparemment asynchrones mais successifs des flagelles assurent une motilité progressive et rotatoire à la structure. Une couche acellulaire de 80 nm sur le spermatozeugma sphérique entoure une sphère centrale fluide appelée globe. Chaque tête de spermatozoïde est ancrée dans une enceinte formée par les prolongements de la lamina de 80 nm et est en contact avec le plasmalemme spermatique dans un collier flagellaire postérieur aux cinq mitochondries sises à la base du noyau du spermatozoïde. Un centriole proximale, situé dans une fosse à la base du noyau, est relié à un centriole distal par une matrice amorphe compacte. Le centriole distal est rattaché au collier flagellaire par 9 prolongements péricentriolaires striés et ramifiés. La partie antérieure du spermatozoïde comporte une région semblable à un acrosome contenant des vésicules de 0,1 μm de diamètre entourées d'une membrane. Les complexes de spermatozeugmas peuvent survivre jusqu'à 24 h et assurent probablement le transport des spermatozoïdes jusque dans la chambre branchiale de la femelle.

[Traduit par la Rédaction]

Introduction

Numerous previous reports have described the basic structure of the spermatozoa of different freshwater mussels (Franzén 1955; Trimble and Gaudin 1975; Popham 1979; Peredo et al. 1990; Rocha and Azevedo 1990). Most of these sperm are of the primitive type, with a conical head, 4 or 5 mitochondria in the midpiece, and an acrosomal region that is either reduced or absent (Franzén 1955; Popham 1979; Peredo et al. 1990). Structural characteristics of sperm from both freshwater and marine bivalve species are often used to define phylogenetic relationships and cladistics trees.

In the case of freshwater species, the sperm are reportedly spawned free into the environment regardless of whether the female mussel broods the embryos. To date, only limited reports of freshwater bivalves spawning specialized spermatozeugmata have been published (Utterback 1931; Edgar 1965; Pekkarinen 1991). As a result, the structure of the spermatozeugmata, the morphology of the sperm, and the containment of the sperm in these structures have been largely overlooked. By far the best documented examples have been in the marine oysters, *Ostrea* sp. (Coe 1931; Menzel 1955; Ó Foighil 1989). There have been no ultrastructural studies of spermatozeugmata from freshwater bivalves, however. The possible function of the spermatozeugmata as storage containers providing prolonged viability for the sperm versus their function in transporting the sperm has also been debated (Mann 1984; Ó Foighil 1985*a*, 1985*b*).

In the present paper, the structure of the mature sperm and the unique manner in which they are individually encased in the spermatozeugmata are described for the first time in *Anodonta grandis*. This is the first report detailing the ultrastructure of a spermatozeugma sphere in a freshwater bivalve.

Materials and methods

Animals

Mature *Anodonta grandis* were collected in freshwater ponds near Baton Rouge, Louisiana, during August and September and taken to the laboratory. They were maintained in small aquaria in artificial pond water containing 20 mM NaCl, 0.2 mM KCl, 0.5 mM $CaCl_2$, and 2 mM $NaHCO_3$ at an approximate pH of 7.4.

Electron microscopy

Spawned spermatozeugmata were collected from the aquarium water by gentle hand centrifugation of 15-mL batches. Samples were fixed for 2–4 h in 3% glutaraldehyde in 0.1 M cacodylate buffer, rinsed in buffer, and postfixed in 0.5% osmium tetroxide for 30 min. Following postfixation, samples were rinsed 3 times in 0.1 M cacodylate buffer and dehydrated in a graded acetone series. Dehydrated samples were either embedded in a low-viscosity epoxy resin (Spurr 1969) or prepared for scanning electron microscopy as

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described below. Embedding in Spurr's epoxy resin was facilitated by centrifuging aliquots of the fixed samples in a BEEM (Ernest F. Fullam, Inc., Latham, N.Y.) capsule on a hand-crank centrifuge to concentrate the specimen in the tip of the mold. Sections were cut with glass knives on a Reichert Ultracut, stained with methanolic uranyl acetate and Reynolds' lead citrate, and observed with a JEOL 100c transmission electron microscope.

For scanning electron microscopy, samples were fixed in glutaraldehyde and postfixed in osmium tetroxide as described above. Following dehydration in a graded acetone series, samples were critical-point dried, mounted on copper strips, coated with 10 nm of gold–palladium, and observed with the SEM function of a JEOL 100c electron microscope.

Samples of ovulated eggs released from a gently split section of the female gonad were also fixed as described above for the sperm and prepared for scanning electron microscopy.

Histochemistry

Live samples of *A. grandis* sperm released from the spermatozeugmata were incubated for 20 min in 0.1% acridine orange in artificial pond water. In addition to specifically staining regions containing nucleic acids a bright apple green, acridine orange differentially localizes in acrosomes of many animal sperm and fluoresces orange (Bishop and Smiles 1957). Stained sperm were examined on a Nikon Diaphot inverted microscope equippped with an epifluorescence illuminator and fluorescent lenses. A FITC filter block was used for the excitation and barrier filters.

Results

Specimens of *Anodonta grandis* brought into the laboratory in mid-September were observed to spawn spontaneously into the aquariums in which they were being held. The spawns consist of multiple motile spermatozeugmata as described in greater detail below. In brief, each of the spermatozeugmata consisted of an acellular sphere comprising (*i*) a central region herein called a globe, surrounded by an 80-nm layer called a lamina; (*ii*) radially arranged chambers enclosed by an 80-nm lamina and containing a single sperm head; and (*iii*) a laminar (80 nm) covering over each chamber in contact with the sperm plasmalemma in the region of the midpiece collar.

When observed at low magnification with a dissection microscope, the cloudy spawn contained large numbers of large motile spheres 40–50 μm in diameter. At higher magnification, each sphere contained numerous sperm at the periphery of the sphere. Each sperm appeared to have its head embedded in the sphere. The anterior of the sperm pointed towards the center of the sphere, while the flagellum extended away from the sphere surface. Flagella were beating rapidly at all times, causing partially populated spheres not only to move progressively but also to tumble.

Observed with phase-contrast microscopy, the flagella can be distinguished at the leading edge of the sphere (Fig. 1, upper left quadrant, showing the flagella crisscrossed). Synchrony of the flagellar beat could not be established in preliminary slow-frame video analysis of the swimming spermatozeugma complexes, and any apparent coordinated movement may be a result of nearest neighbor interference.

Some spermatozeugmata were observed to be fully populated with sperm, although most were only partially populated. The partially populated nature of the spermatozeugmata (SS) is particularly evident in scanning electron micrographs (Fig. 2). In partially populated SS, the sperm tended to be oriented predominantly to one side of the complex, consistent with the ability of the spermatozeugma complex to be progressively motile.

The head of the sperm can be observed just beneath the surface layer of the spermatozeugma complex, with the flagella extending out from the surface (Fig. 3). Individual sperm released from the spermatozeugma complex had a 1.5 × 4.0 μm cylindrical head (Fig. 4) with 5 mitochondria located at the base of the sperm. A single flagellum approximately 35 μm long extended from the posterior and, surrounded at the base of the sperm head by a thick collar.

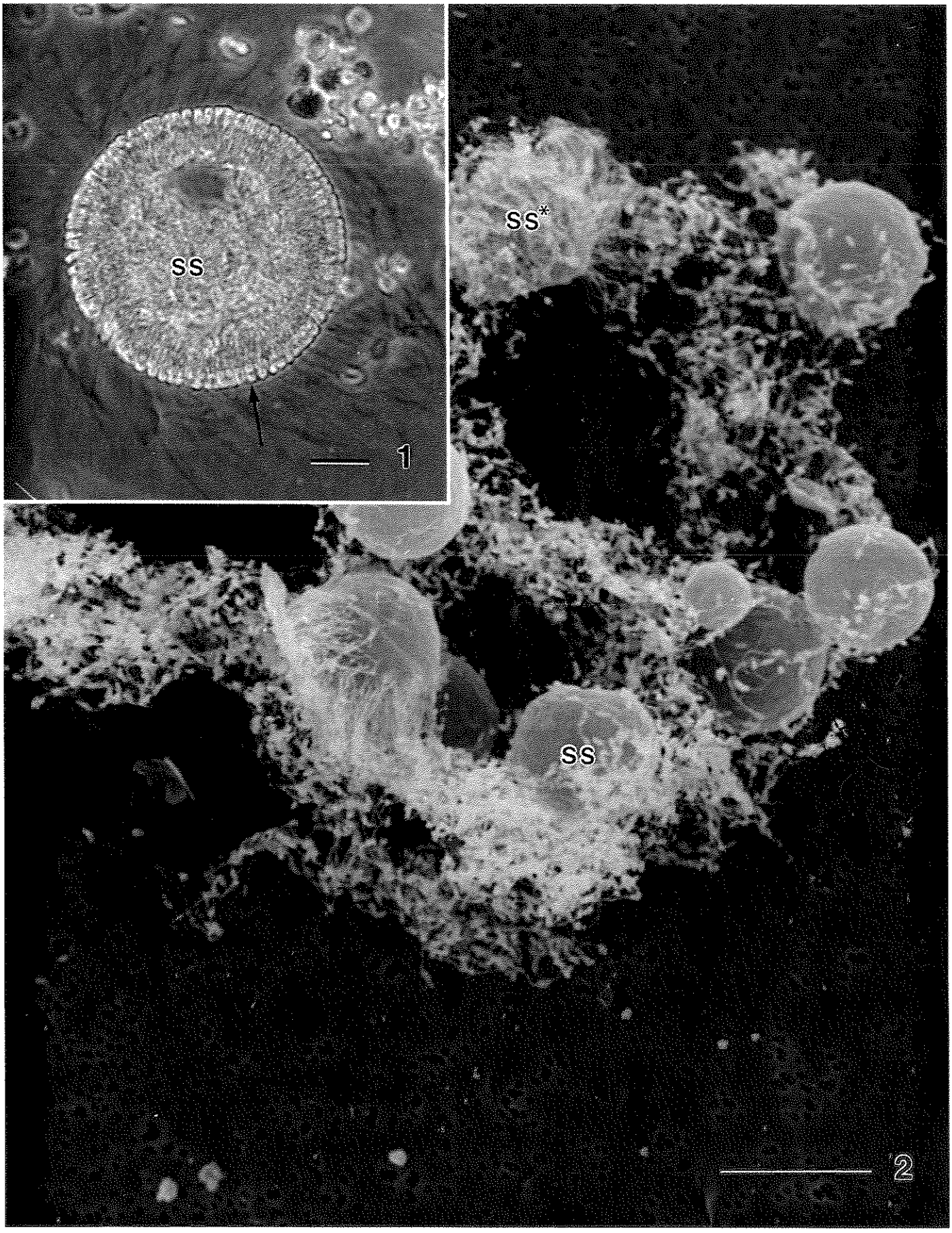
As observed with transmission electron microscopy (Fig. 5), the sperm heads were embedded in the periphery of the spermatozeugmata. The incompletely populated spermatozeugma complex shown in Fig. 5 is partially collapsed from the fixation and dehydration process and flocculent material is visible in the collapsed interior of the sphere. The inner layer of the spermatophore core persists even after all the sperm are released from the complex and may exist for several minutes to several hours after the sperm have been released (Fig. 7), before disintegrating. The individualization of the compartmented sperm is apparent in Fig. 6. The acellular spermatozeugma lamina appears to interconnect the sperm by a continuity with the sperm plasmalemma in the collar region of the sperm. Compartmentalization is complete and no cytoplasmic bridges were observed interconnecting the sperm.

Staining with the vital stain acridine orange indicated that the head region of the sperm is largely occupied by the nucleus (not shown). Although the nucleus was bright green when sperm were stained with acridine orange, differential staining of the anterior region containing the vesicles was not apparent. It is possible, however, that staining in the apical region was not resolvable with light microscopy techniques, or that intense fluorescence from the staining of the nucleus obscured any staining that may have been present. The nucleus is observed as a large, lightly staining region in transmission electron micrographs. Little or no cytoplasmic space is observed in the region between the sperm plasmalemma and the nuclear membrane in electron micrographs. Anterior to the nuclear region was a small central apical cap containing numerous small vesicles (Fig. 8). The exact number of membrane-bound vesicles is unclear but is at least four (Fig. 9). The area containing the vesicles is not distinctly delineated in scanning electron micrographs (not shown).

At the posterior end of the sperm, elaborate organization of the proximal and distal centrioles was characterized by (*i*) a connecting piece anchoring the proximal centriole in a fossa at the base of the nucleus; (*ii*) dense amorphous material in which the proximal and distal centrioles are embedded and connected; and (*iii*) nine striated, bifurcated pericentriolar elements anchoring the distal centriole to the

FIG. 1. Phase micrograph of a swimming spermatozeugma complex (SS) recently released from an adult male. The arrow points to an individual sperm head. Scale bar = 10 μm. FIG. 2. Low-magnification scanning electron micrograph of a fully populated (SS*) and several partially populated spermatozeugmata (SS). Scale bar = 50 μm.

FIG. 3. Scanning electron micrograph of a partially populated spermatozeugma shows the sperm heads embedded in the periphery of the sphere (arrows) Scale bar = 10 μm. FIG. 4. Scanning electron micrograph showing the typical cylindrical morphology of the sperm head typical of primitive-type spermatozoa. N, nuclear region; M, mitochondria; C, flagellar collar; F, flagella. Scale bar = 1.0 μm.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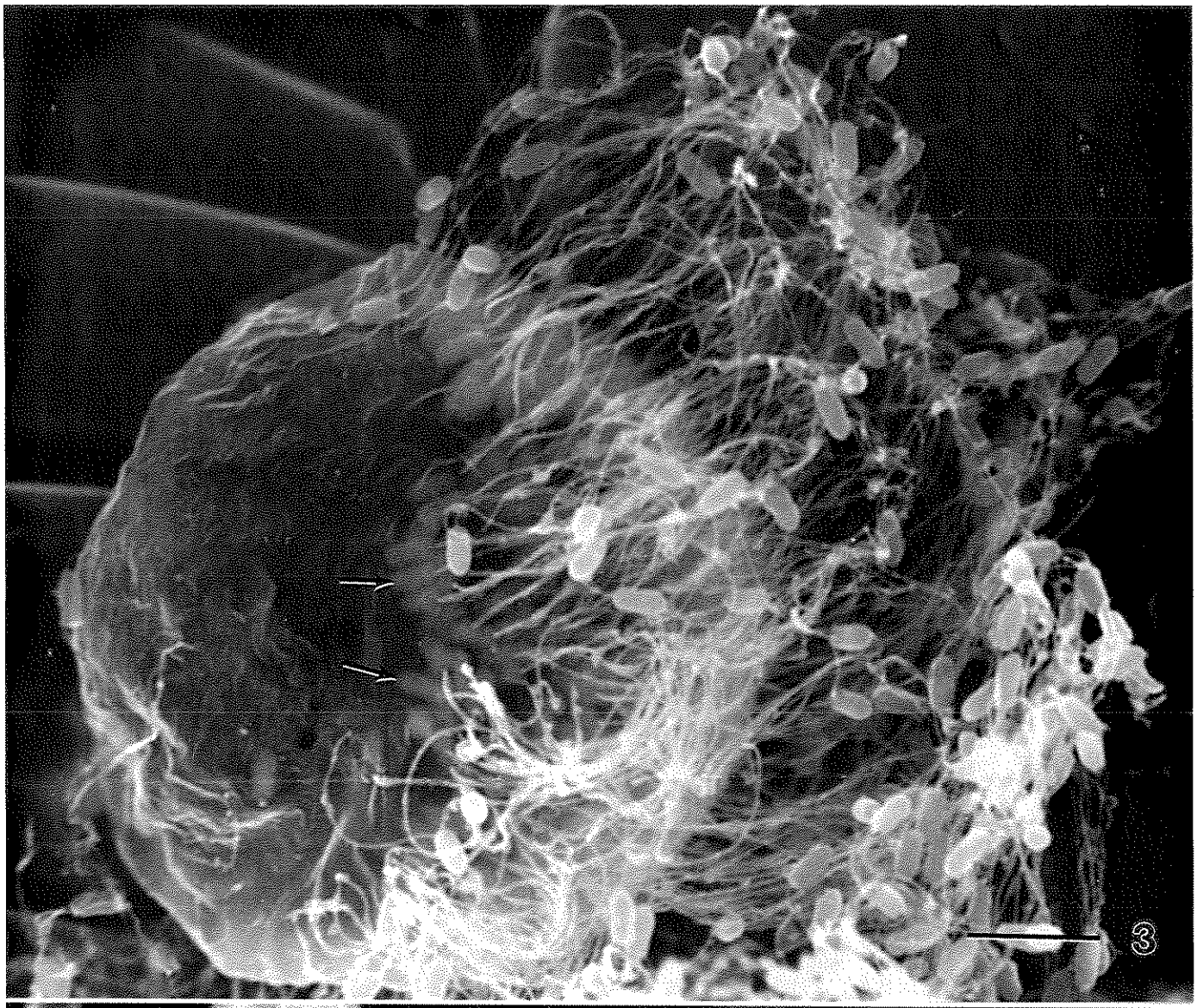
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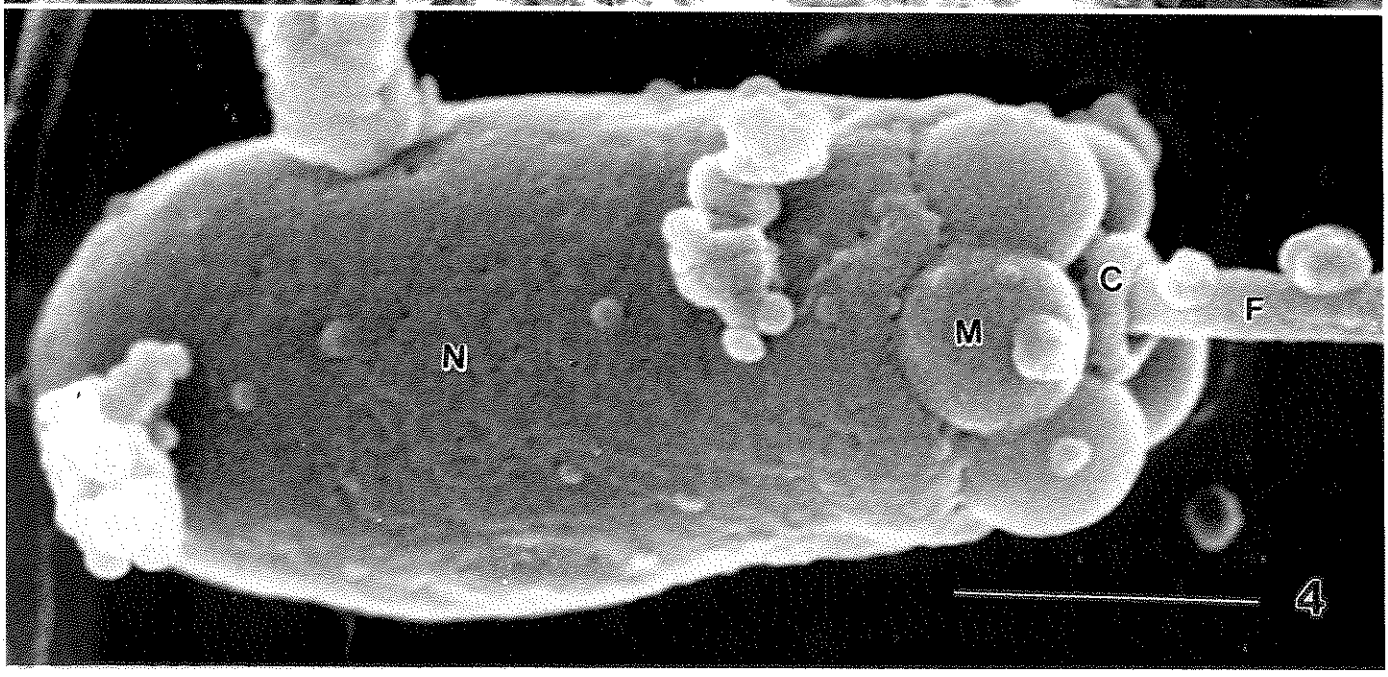
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collar at the base of the sperm head (Figs. 10, 11, 13). Periodicity of the striations was approximately 8 nm in both the bifurcated and non-bifurcated segments of the pericentriolar processes. Both centrioles display a characteristic nine-triplet pattern.

In more posterior sections of the distal centriole, the bifurcated pattern of the pericentriolar process was evident. At this level, the typical nine-triplet pattern was replaced by nine doublets and a central pair of tubules (Fig. 13). The bifurcated ends of the pericentriolar processes were embedded in the dense material of the collar region as the flagellar apparatus leaves the base of the sperm. Tangential sections of the collar region also revealed a striated appearance similar to that observed in the pericentriolar processes (Fig. 11). It is particularly important to note the close association between the sperm plasmalemma and the envelope of the spermatozeugma sphere (Figs. 6, 11, 12).

Posterior to the collar of the sperm head, a typical plasmalemma surrounded the 9 + 2 flagella, but the nine outer doublets remained attached to the flagellar membrane by bifurcated amorphous material (Fig. 14). In the most distal sections, the 9 + 2 arrangement was preserved and typical dynein arms, nexin, and radial spokes were evident (Fig. 15). In addition, the central pair of tubules appeared to be connected at all levels in the flagella by an arc of dense material.

Although sperm–egg interactions were not directly observed in these studies, a brief description of the gross morphology of ovulated eggs of this species will aid in the discussion of sperm function. A large, highly refractile egg envelope was evident surrounding the 140–150 μm diameter egg in phase-contrast micrographs (Fig. 16). Egg placement in the resulting perivitelline space was eccentric and the cell appears to remain attached at one point to the egg envelope by means of a small protuberance extending through the egg envelope. In phase-contrast micrographs, a prominent germinal vesicle was observed, which remains in a more translucent area of the oocyte cytoplasm. The cytoplasm appeared to be unequally distributed, with a denser, more heavily pigmented area close to the point of attachment of the egg to the egg envelope.

The attachment site of the egg to the egg envelope was also apparent in scanning electron micrographs (Fig. 17). A small circular 20 μm diameter pedestal was observed projecting through one point of the egg envelope and extending 3–4 μm above the external surface of the envelope. The surface of the egg envelope in the immediate vicinity of the projection was covered in a network of fibrils. Exogenous materials appeared to adhere readily to the protuberance but not to the surrounding egg envelope areas. These eggs were not followed for an extended period of time, but the egg morphology described above in phase-contrast micrographs remained consistant for approximately 3 h prior to fixation for scanning electron microscopy.

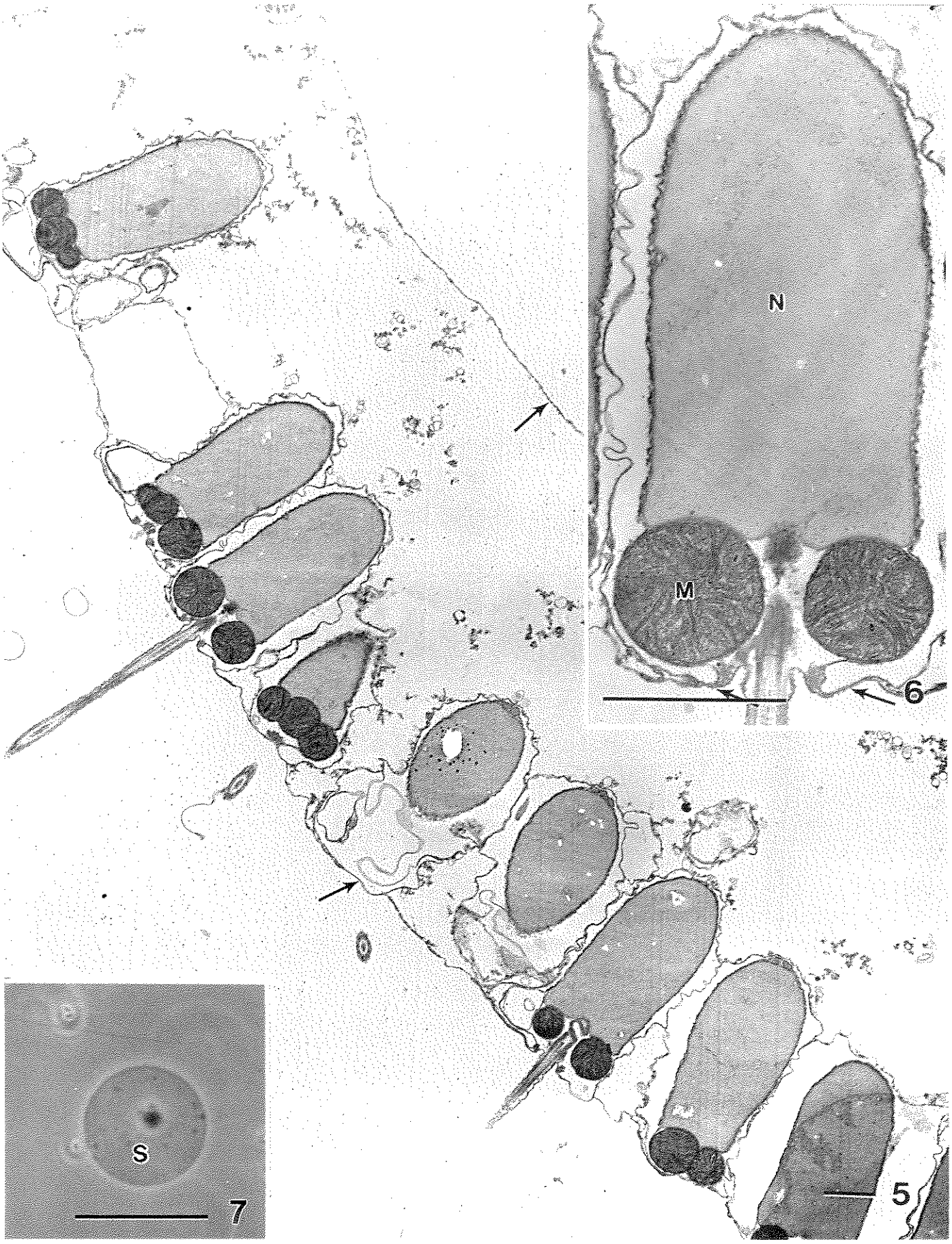
Discussion

The morphology of mature sperm of *Anodonta grandis* closely resembles that of other mature unionid sperm reported in the literature (Peredo et al. 1990; Trimble and Gaudin 1975; Rocha and Azevedo 1990). The basic ultrastructure of *A. grandis* sperm is typical of primitive-type flagellated sperm consisting of (*i*) a cylindrical head containing a condensed nucleus, an acrosome-like region, mitochondria, and centrioles; (*ii*) a 9 + 2 flagella arrangement elaborated from the distal centriole; and (*iii*) an elaborate pericentriolar complex associated with the distal centriole and connected to the flagellar collar. Of unique and special importance are (*i*) the packaging of the sperm into spherical spermatozeugmata; (*ii*) motility of the spermatozeugmata, utilizing the flagellar beat of the sperm; and (*iii*) anastomosis of the spermatozeugma "membrane" and the collar region of the individual sperm. These features are especially intriguing, since they may all relate to the coordinated transport of the sperm to the female mussel.

A unique feature of the spermatozeugmata described in this paper is the clear and complete containment of each individual sperm head in a compartment at the periphery of a noncellular "gelatinous" sphere. It is also apparent from the current study that the individual sperm heads do not retain any cytoplasmic bridges between them, and the sperm can be released individually from the sphere over a period of time. The envelopes embedding the sperm heads and composing the sphere as a whole are simple and singular, as opposed to the multilaminar arrangement observed by Ó Foighil (1985*a*). The nonmembranous envelope enclosing these spheres is also in intimate contact with a specialized region of the collar of the sperm where striated pericentriolar processes terminate. The suggested contractile nature of similar structures in both

FIG. 5. Low-magnification transmission electron micrograph (TEM) showing a portion of a partially populated spermatozeugma with embedded sperm heads. Note the thin envelope surrounding the sphere of the spermatozeugmata, as indicated by the arrows. Scale bar = 1.0 μm. FIG. 6. This higher magnification TEM illustrates a single sperm head encapsulated in an individual compartment at the periphery of the spermatozeugma. Of particular note is the connection between the flagellar region of the sperm head and the nonmembranous material composing the spermatozeugma sphere. The arrows point to a region near the collar of the sperm where the laminar structure of the spermatozeugma sphere appears to anastomose with the sperm collar. N, sperm nucleus; M, mitochondrion. Scale bar = 1.0 μm. FIG. 7. A depopulated spermatozeugma sphere is translucent but usually contains one or more dense areas within it. These spheres spontaneously degenerate within minutes to hours after complete depopulation. Scale bar = 50 μm.

FIG. 8. At the anterior end of the sperm, TEM reveals several vesicles (*) between the nuclear membrane and the plasmalemma of the sperm. N, nucleus; A, acrosome-like region; the arrow points to the envelope of the spermatozeugma. Scale bar = 0.1 μm. FIG. 9. Frontal section through the anterior tip of the sperm revealing at least four vesicles in the acrosome-like region. The section through this region of the sperm is a tangential cut and as such does not necessarily section the vesicles equatorially, therefore the vesicles (*) do not appear as large as those indicated in Fig. 8. The arrow points to the sphere envelope. Scale bar = 0.1 μm. FIG. 10. Longitudinal section through the sperm illustrating the relation between the proximal centriole (PC), distal centriole (DC), collar region (arrows), and flagella. Note the dense material connecting the proximal and distal centrioles and extending into the nuclear fossa (arrowhead). N, nucleus; M, mitochondrion. Scale bar = 1.0 μm. FIG. 11. Tangential section through the pericentrolar processes attaching to the collar region, revealing the striated nature of the bifurcating processes. The processes extend from the distal centriole into the collar region and terminate in a multilaminar structure associated with the connection between the sperm flagellar collar and the spermatozeugma. DC, distal centriole; M, mitochondrion; the star indicates the bifurcating process; the arrow points to the pericentriolar process; the arrowheads point to the multilaminar structure terminating the sperm collar. Scale bar = 1.0 μm.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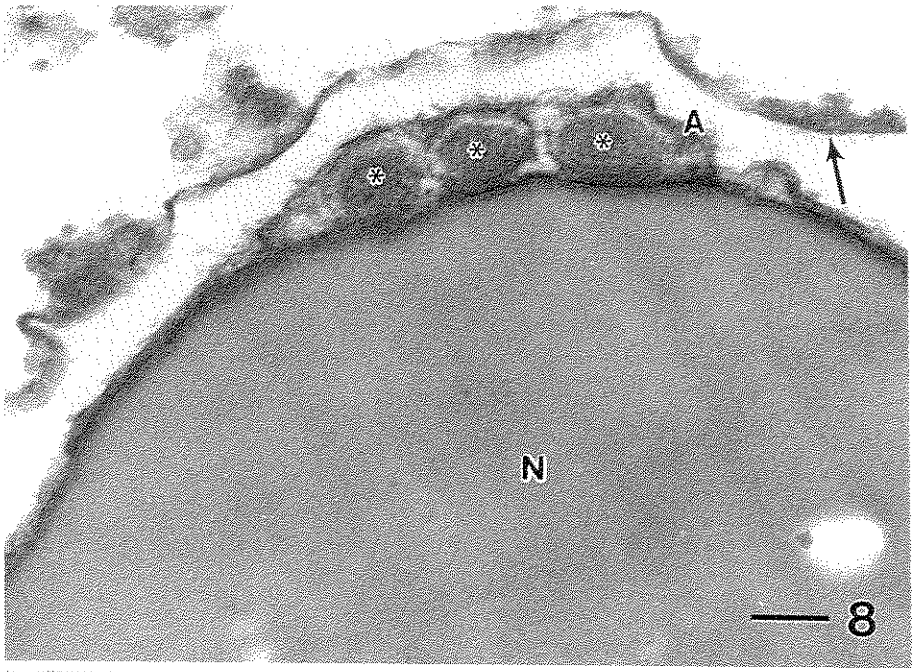
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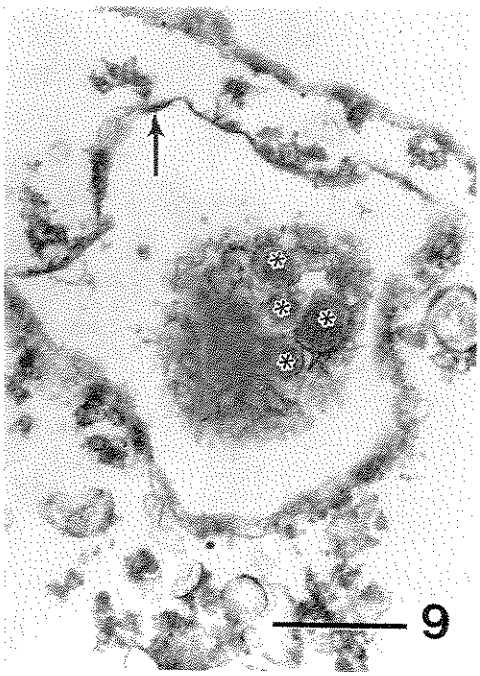
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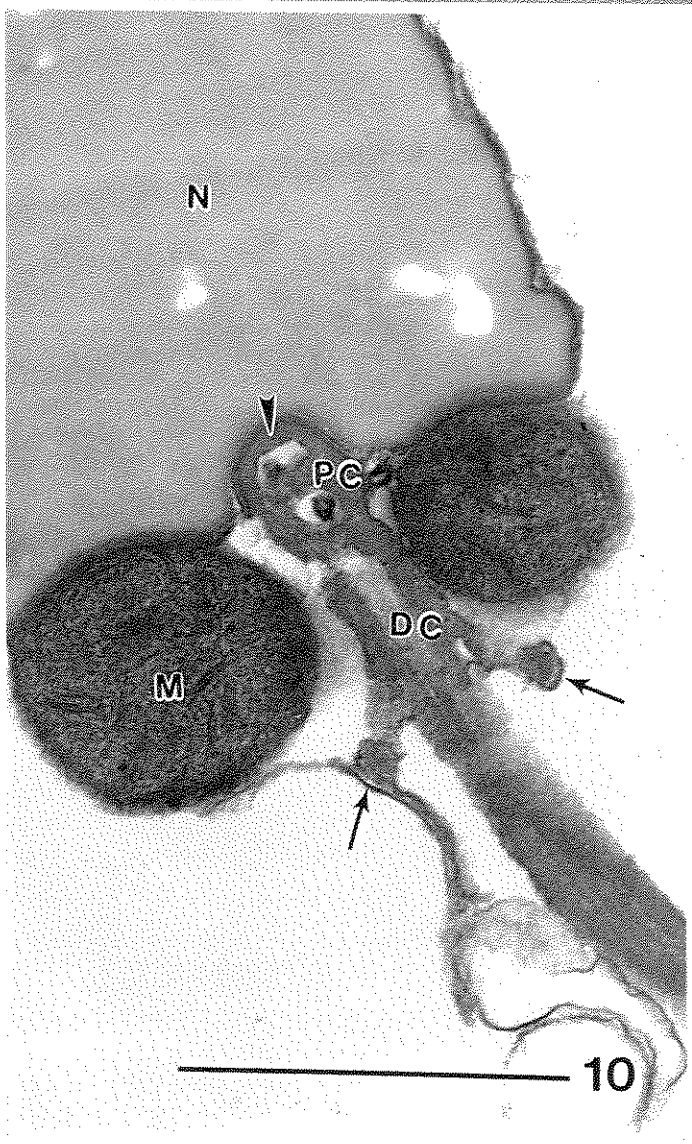
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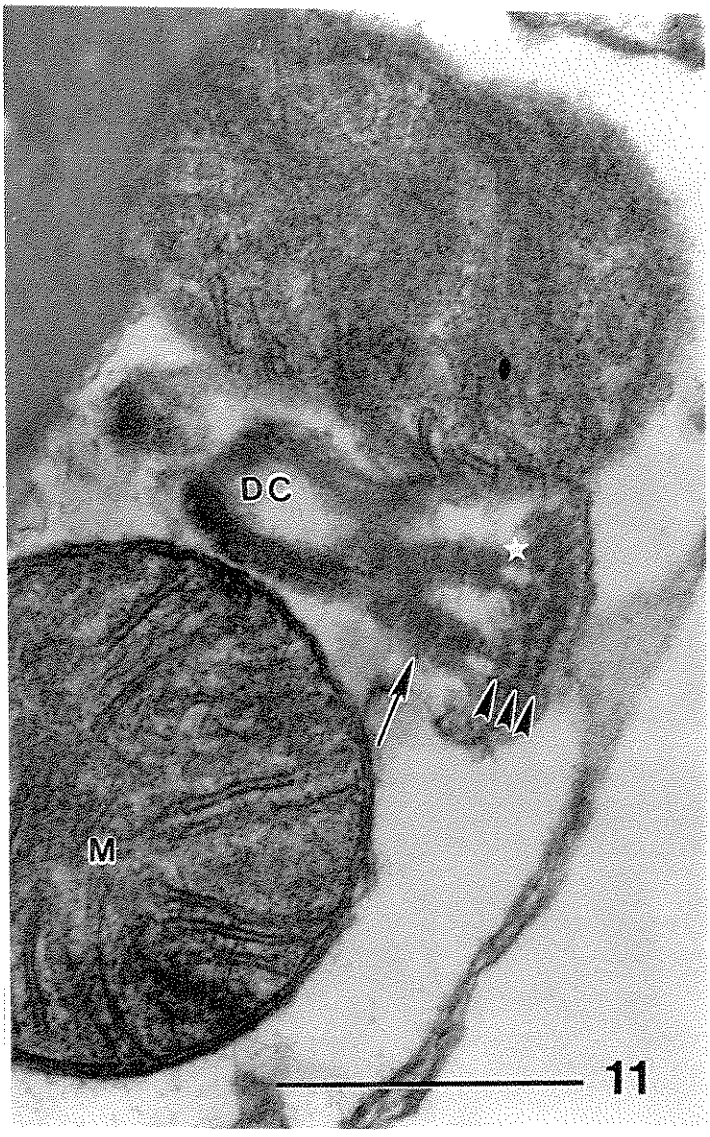
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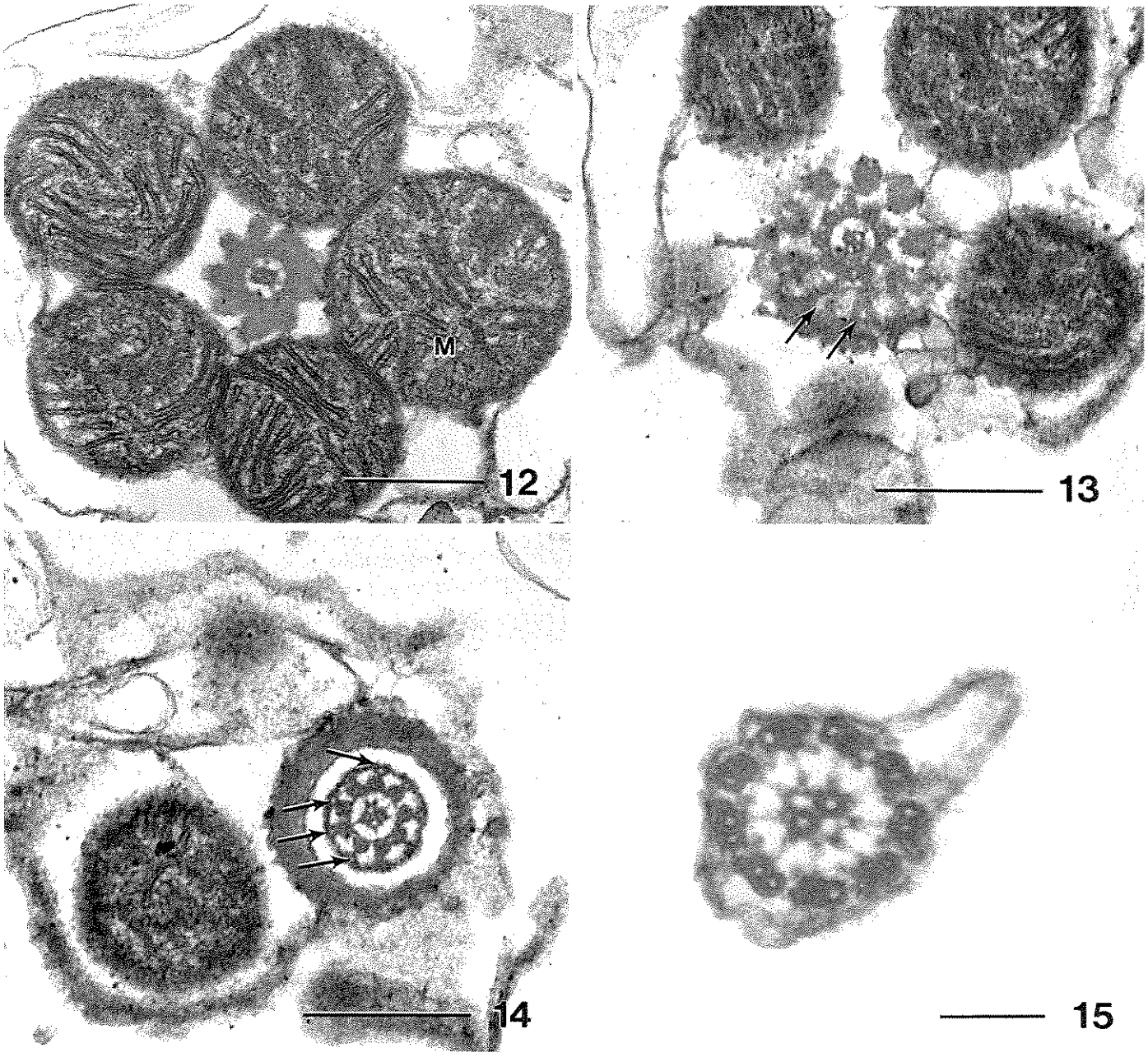

FIG. 12. The midpiece region containing 5 mitochondria (M) surrounding the distal centriole – flagellar origin. Scale bar = 0.5 μm. FIG. 13. Towards the collar region, the bifurcations of the pericentriolar processes become apparent (arrows) and the distal centriole is replaced by the configuration of a 9 + 2 axoneme. Scale bar = 0.5 μm. FIG. 14. Below the collar region, the nine outer doublets of the axoneme are attached to the flagella membrane with short bifurcated processes (arrows). Scale bar = 0.5 μm. FIG. 15. Distal to the sperm head, the flagella have a typical axoneme arrangement consisting of 9 + 2 axoneme, dynein arms, and radial spokes connecting the inner pair to the outer doublets. Scale bar = 0.1 μm.

cnidarian sperm (Kleve and Clark 1976, 1980) and algal ciliary rootlets (Salisbury and Floyd 1978) is an indication that similar striated structures in sperm may serve a far more important function than simply as an anchoring device, as suggested by other authors.

The basic ultrastructural morphology of *A. grandis* sperm is characteristic of and consistent with that observed in other freshwater bivalve species, including (but not limited to) *Anodonta cygnea* (Rocha and Azevedo 1990), *Diplodon chilensis* (Peredo et al. 1990), *Legumia subrostrata* (Trimble and Gaudin 1975), and *Hyriopsis schlegelii* (Higashi 1964). The observations reported here extend our morphological data base on sperm morphology in freshwater bivalve species. In contrast to many other species (Popham 1979; Franzén 1983; Ó Foighil 1985*b*), *A. grandis* sperm contain 5 mitochondria in their midpiece rather than the usual 4. Nevertheless, in general, other authors consider that these types of sperm retain a primitive morphology indicative of species where fertilization occurs in the protective environment of the gill suprabranchial chamber or mantle cavity of the female and the eggs are subsequently brooded (Popham 1979). The rather diminished size of the acrosomal region has been suggested as an

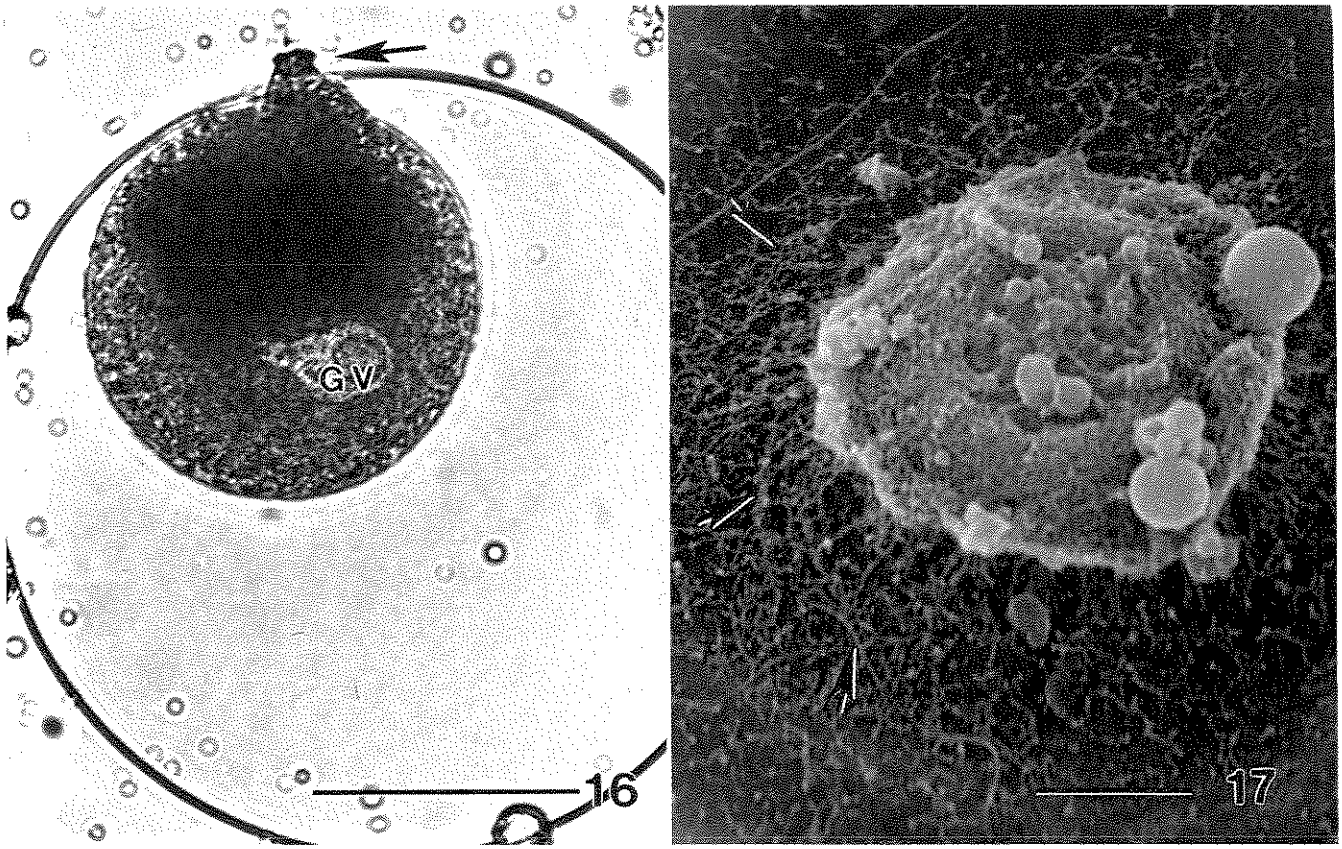

FIG. 16. Phase-contrast micrograph of an ovulated egg, showing a large egg envelope with the egg eccentrically placed in the perivitelline space. An attachment site between the egg and the envelope is apparent (arrow). A germinal vesicle in the cytoplasm is displaced from the site of egg to egg envelope attachment by a dense cytoplasmic region. Scale bar = 100 μm. FIG. 17. Scanning electron micrograph of the egg envelope attachment, revealing an area elevated above the envelope surface. The surface in the immediate vicinity of the elevated area is covered in a meshwork of fibrillar structures (arrows). The protrusion appears to be somewhat adherent and debris is observed both on the pedestal and in the immediately surrounding region. No other areas of the external egg coat demonstrated this "sticky" nature. Scale bar = 1.0 μm.

adaptation in species where sperm are not challenged with thick layers of jelly or substantial egg envelopes (for review see Franzén 1983; Mackie 1984).

Studies on sperm interaction with the *A. grandis* egg have not been reported to date. Based on limited observations of ovulated eggs and the diminished size of the acrosomal region and acrosomal vesicles in *A. grandis*, however, the most likely site of sperm interaction is at the site of egg protrusion through the egg envelope, as was reported in the related unionid *Unio elongatulus* (Focarelli et al. 1988; Focarelli et al. 1990). Formation of a cytoplasmic pedestal projection associated with a specialized region of the egg envelope has been described in *U. elongatulus* during oogenesis and this may act as a specialized region for sperm entry (Focarelli et al. 1990). The striking morphological similarity of the pedestal region in *A. grandis* and *U. elongatulus* suggests that the region may also be important for sperm–egg interaction in *A. grandis*. Forcarelli et al. (1988) contend that the entrance site is not the point of cytoplasmic protrusion but rather the differentiated region of the vitelline coat around the pedestal. Clearly, additional observations of controlled sperm–egg interactions are required to resolve this question.

Packaging of sperm into a spermatozeugma/spermatophore for transfer to a female is not an unusual phenomenon in invertebrate taxa (Fretter 1953; Werner 1973; Buckland-Nicks and Chia 1977; Rice 1978; Itaya 1979; Hadfield and Hopper 1980; Mann 1984; Ferguti et al. 1989; Healy 1990), but the occurrence of true spermatozeugmata is best known in the molluscs. Although packaging of sperm into spermatozeugmata or spermatophores for transfer to a female is documented in many species, the active and progressive motility of a spermatozeugma complex, in contrast, appears to have been described only briefly in the marine oyster *Ostrea* sp. (Coe 1931; Ó Foighil 1989) and five species of freshwater bivalves. Edgar's (1965) observations on spermatozeugmata in *Anodontoides ferussacianus*, Utterback's (1931) descriptions of hollow globular masses of sperm discharged by *Lampsilis ventricosa*, *Lasmigona complanata*, and *Utterbackia ohiensis*, and Pekkarinen's (1991) report on sperm balls in *A. anatina* are the only other reports of spermatozeugmata in freshwater molluscs. None of these previous studies of freshwater animals has addressed the ultrastructural features of the individual sperm containment in the spermatozeugmata.

Sperm morulae, which have been reported as abnormal or aberrant spermatogenic stages that rarely occur during the period of "normal" sperm production (for review see Heard 1975), may be analogous with sperm balls or spermatozeugmata according to Mackie (1984) and Pekkarinen (1991). In fact, the appearance of so-called sperm morulae in the testis may be related to the formation of the motile spermatozeugmata, but convincing evidence linking the two structures

has yet to be presented. In the present study, some observations of earlier spermatogenic stages were made and sperm morulae consisting of dense-staining spherical packets of pie-shaped wedges were observed. These packets were abundant several weeks prior to the spawning of *A. grandis*, but were significantly less frequent in recently spawned males. Indeed, it is unclear at this time whether the syncytial masses reported as sperm morulae (for reviews see Coe 1931 and Heard 1975, for example) are actually spermatogenic stages or, instead, contribute in some way to the production of the spermatozeugmata. In contrast to the "sperm balls" reported by Pekkarinen (1991), which apparently occur after the peak reproductive season and therefore may represent abnormal spermatogenesis, release of spermatozeugmata in *A. grandis* occurs only during the peak reproductive season. In fact, some investigators have used the appearance of spermatozeugmata in the testis of males as an early indicator of eminent production of a brood in the gill of a female (T.H. Dietz and H. Silverman, personal communication). Further extensive investigation is required to clarify the production and role of the spermatozeugmata.

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