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Ultrastructure of the gonad and gametogenesis in the eastern oyster, *Crassostrea virginica*. II. Testis and spermatogenesis

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Abstract The ultrastructural features of the testis and spermatogenesis have been described from the eastern oyster, *Crassostrea virginica* (Gmelin, 1791). The testis is a diffuse organ consisting of branching acini containing differentiating sperm in a variety of stages. Spermatogonia are located nearest the outer wall of the acinus, while spermatocytes and spermatids are positioned nearer the lumen. Mature spermatozoa are all confined to the central region of the acinus. The acinus is surrounded by an intermittent layer of myoepithelial cells and is bathed by a fluid-filled hemocoel. Vesicular connective tissue (VCT cells) fills the region between adjacent acini, and the cells contain glycogen granules and lipid droplets. Each acinus is divided radially into subcompartments that are partially separated by pleomorphic accessory cells which remain in close contact with sperm until late stages of development. Sperm are similar to those described in other oysters, except that five midpiece mitochondria were observed in some sperm rather than the usual four, and the acrosomal vesicle lacked the "whorled" substructure described in some other oyster sperm. We suggest that the neutral term "accessory cells" be applied to bivalve testicular somatic cells until more detailed studies are available to justify the use of "Sertoli cell" and other descriptive terms which have previously been adopted from other taxa only distantly related to bivalves.

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Introduction

Spermatogenesis and mature sperm morphology has been documented to varying degrees in many species of bivalve molluscs using both light and electron microscopy (reviewed by Gaulejac et al. 1995). The sperm of more than 70 species have been described from 10 orders and nearly 30 families. Sperm ultrastructure has long been viewed as a tool in assessing phylogenetic relationships in the Metazoa through the use of spermiocladistic analysis (see Jamieson 1987, 1991). In the Mollusca, sperm morphology has been used increasingly in assessing long-standing taxonomic problems (Popham 1979; Healy 1983, 1988, 1995, 1996; Koike 1985; Hodgson and Bernard 1986).

Ultrastructural observations of mature spermatozoa have been conducted on many species in the Bivalvia (reviewed by Sousa and Oliveira 1994) including those of several oyster species (Galtsoff and Philpott 1960; Daniels et al. 1971; Brandriff et al. 1978; Gutierrez et al. 1978; Osanai and Kyozuka 1982; O'Foighil 1989; Healy and Lester 1991; Komaru et al. 1994). However, far fewer studies have critically examined the ultrastructural features of spermatogenesis in the F. Ostreidae. Comprehensive studies of spermatogenesis have been restricted to a few species in the families Mytilidae (Longo and Dornfield 1967; Hodgson and Bernard 1986) and the Pectinidae (Dorange and Le Pennec 1989) and a single study within the Ostreidae (Sousa and Oliviera 1994). No ultrastructural study of spermatogenesis has been reported on the eastern oyster, *Crassostrea virginica*.

While descriptions of bivalve spermatozoa are plentiful, broader ultrastructural studies of the bivalve testis are surprisingly rare. During spermatogenesis, sperm are closely associated with accessory cells which presumably play an important role during sperm differentiation, but the terminology used to describe these cells is confusing. We attempt to resolve this confusion and

to indicate what studies are needed to assess the function of the accessory cells.

The present study is the first to describe the ultrastructural features of the testis and spermatogenesis in the eastern oyster, *Crassostrea virginica*, a commercially important species cultivated along the eastern seaboard (Loosanoff and Davis 1963).

Materials and methods

Eastern oysters, *Crassostrea virginica* (Gmelin, 1791), were collected and conditioned as previously described (Eckelbarger and Davis 1996). Preparation of tissues from males for light and transmission electron microscopy was also described previously (Eckelbarger and Davis 1996).

Results

General morphology of the mature testis

The general morphology of the testis of *Crassostrea virginica* is similar to that for the ovary (Eckelbarger and Davis 1996). It consists of a series of highly branching acini lying within a matrix of support cells called the vesicular connective tissue, or VCT cells. The VCT cells are ultrastructurally identical to those described in the ovary but are less abundant in the testis.

Each acinus is radially subdivided into a variable number of subcompartments that partially isolate groups of developing sperm cells. Within each subcompartment, germ cells are distributed in a centripetal pattern from the acinus wall to the lumen (Fig. 1a, b). Spermatogonia are positioned nearest the inner wall of the acinus, spermatocytes and spermatids are located closer to the acinus lumen, and mature sperm are largely confined to the central lumen. Accessory cells are closely associated with all sperm stages except the mature spermatozoon. In addition to VCT cells, each acinus is surrounded by a connective tissue compartment, the hemocoel, a fluid-filled space that contains amoeboid hemocytes. It is also partially surrounded by an intermittent, single layer of squamous myoepithelia cells which forms a partial barrier between the germinal epithelium and the hemocoel. Sperm cells in adjacent subcompartments are partially segregated by myoepithelial and pleomorphic accessory cells (Fig. 1a, b, e). Amoeboid hemocytes with large cytoplasmic vacuoles are also occasionally observed in this region (Fig. 2a). The myopeithelial cells contain elongated nuclei, occasional electron dense granules, and dilated cisternae of rough endoplasmic reticulum (RER) (Fig. 1c). The accessory cells are distributed both between and within acinal subcompartments in close association with developing sperm cells (Fig. 1a, b, e). The accessory cells are amoeboid, and the cytoplasm contains an irregular nucleus, mitochondria, a few RER cisternae and scattered lipid droplets (Fig. 1a; 2b, c). Due to a relative scarcity of cytoplasmic ribosomes, they stain very lightly in contrast to the more darkly staining cytoplasm of adjacent sperm cells. Occasional desmosomes were observed between germ cells and auxiliary cells (Fig. 1e insert).

Stages of spermatogenesis

Spermatogonia are large cells (4 to 5 μm diam) each with a spherical nucleus and a single nucleolus (Fig. 1d). They are confined to the outer region of the acinus. Their cytoplasm is largely devoid of organelles except for scattered mitochondria and a nucleus containing sparse heterochromatin. Primary spermatocytes are slightly smaller cells (3 to 4 μm) that are distinguished by nuclei with more abundant and more darkly staining heterochromatin (Figs. 1e; 2b). The cytoplasm of early spermatocytes contains a few electron-dense, proacrosomal granules which become more abundant in later stages of development (Figs. 2d, e) Late stage primary spermatocytes are frequently observed undergoing mitotic division, and the proacrosomal granules tend to cluster around the chromosomes (Fig. 2d). Cytokinesis is incomplete, and the resulting spermatids remain attached by way of intercellular bridges (Fig. 3a).

Early spermatids possess a single axoneme with associated proximal and distal centrioles and a single Golgi complex at the presumptive posterior end of the cell (Fig. 3a, b). Their nuclei contain relatively condensed heterochromatin with scattered electron-opaque regions. As spermiogenesis progresses, the nuclear chromatin continues to condense, and the posterior region of the organelle invaginates to form a shallow nuclear fossa into which the proximal centriole inserts (Fig. 3c). Several spherical mitochondria are positioned lateral to the centrioles, and a single acrosomal vesicle rests at the presumptive anterior pole of the cell. The acrosomal vesicle is initially oval in shape but gradually assumes a cap-like form with a slightly pointed anterior prominence and a sharply invaginated posterior face (Fig. 3d). The acrosomal contents have a relatively uniform electron density. The mature spermatozoon has a nucleus that is wider than long, with a prominent anterior invagination containing a finely granular subacrosomal material (Fig. 3e). The subacrosomal material contains a central filamentous axial rod composed of anterio-posterior-oriented filaments. Transverse sections through the midpiece indicate that in most cases, four mitochondria surround the centrioles. On rare occasions, five mitochondria were observed (Fig. 3f). The sperm flagellum has the standard 9 + 2 pattern of microtubules.

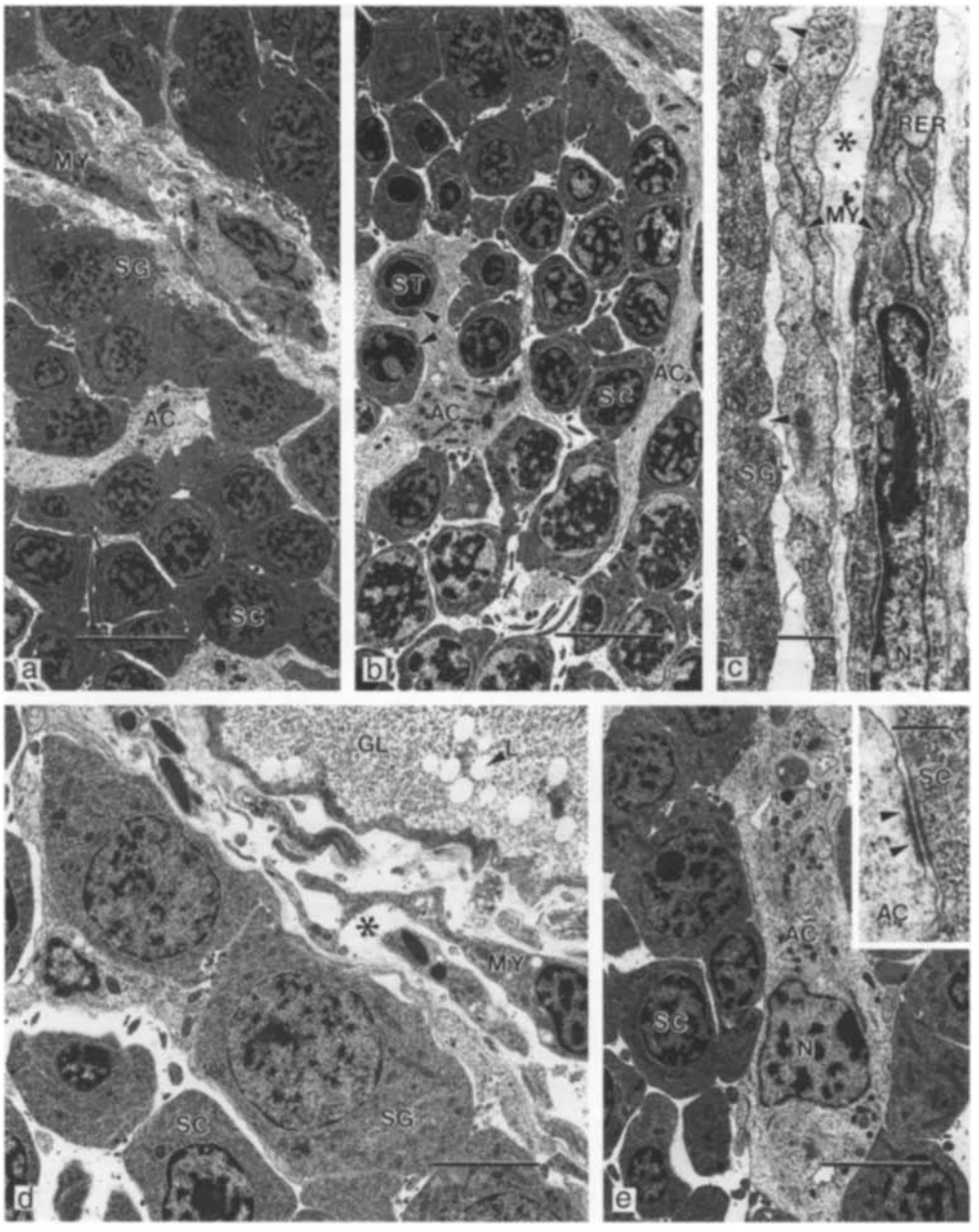

Fig. 1 *Crassostrea virginica.* **a** Two adjacent subcompartments of acinus showing developing sperm (*AC* accessory cells; *MY* myoepithelial cells; *SC* spermatocytes; *SG* spermatogonia). Scale bar = 4.0 μm. **b** Outer region of subcompartment showing spermatocytes (*SC*), spermatids (*ST*), and accessory cells (*AC*). Scale bar = 4.0 μm. Note that one accessory cell surrounds two adjacent spermatids (*arrowheads*). **c** Outer wall of acinus showing myoepithelial cells (*MY*) in hemocoel (*) adjacent to spermatogonium (*SG*). Note coated pits along surface of spermatogonium (*arrowheads*). (*N* nucleus; *RER* rough endoplasmic reticulum) Scale bar = 1.0 μm. **d** Spermatogonia (*SG*) in outer wall of acinus. Note adjacent VCT cells containing glycogen (*GL*) and lipid droplets (*L*) (*MY* myoepithelial cells; *SC* spermatocytes; * hemocoel). Scale bar = 2.0 μm. **e** Spermatocytes (*SC*) adjacent to accessory cell (*AC*) (*N* nucleus). Scale bar = 3.0 μm. *Insert*: higher magnification showing cell junctions (*arrowheads*) between spermatocytes and accessory cells. Scale bar = 0.25 μm

Discussion

Early investigations of bivalve sperm ultrastructure demonstrated the taxonomic value of comparative studies (Franzén 1970, 1983; Popham 1979), and such studies are now widely used in taxonomic analyses (reviewed by Healy 1995). The "primitive" sperm show sufficient structural variability that they are useful in taxonomic studies. Hodgson and Bernard (1986) and Healy (1989), for example, showed that different subclasses of bivalves each have unique acrosomal morphologies. The number of mitochondria in the sperm midpiece tends to be stable within any given family or superfamily varying from a maximum of 14 in the mytiloid *Modiolus difficilis* (Drozdov and Reunov 1986) to a minimum of 4 (common to many bivalve families) (reviewed by Healy 1989, 1995).

The ultrastructure of *Crassostrea virginica* sperm was first documented by Galtsoff and Philpott (1960). Sperm have now been described in several oyster species including *Crassostrea angulata* (Gutierrez et al. 1978; Sousa and Oliveira 1994), *Crassostrea gigas* (Brandriff et al. 1978; Osanai and Kyozuka 1982; Bozzo et al. 1993; Komaru et al. 1994) and *Saccostrea commercialis* (Healy and Lester 1991; Molinia and Swan 1991). Although the number of oyster species examined is small, their sperm structure shows general consistency

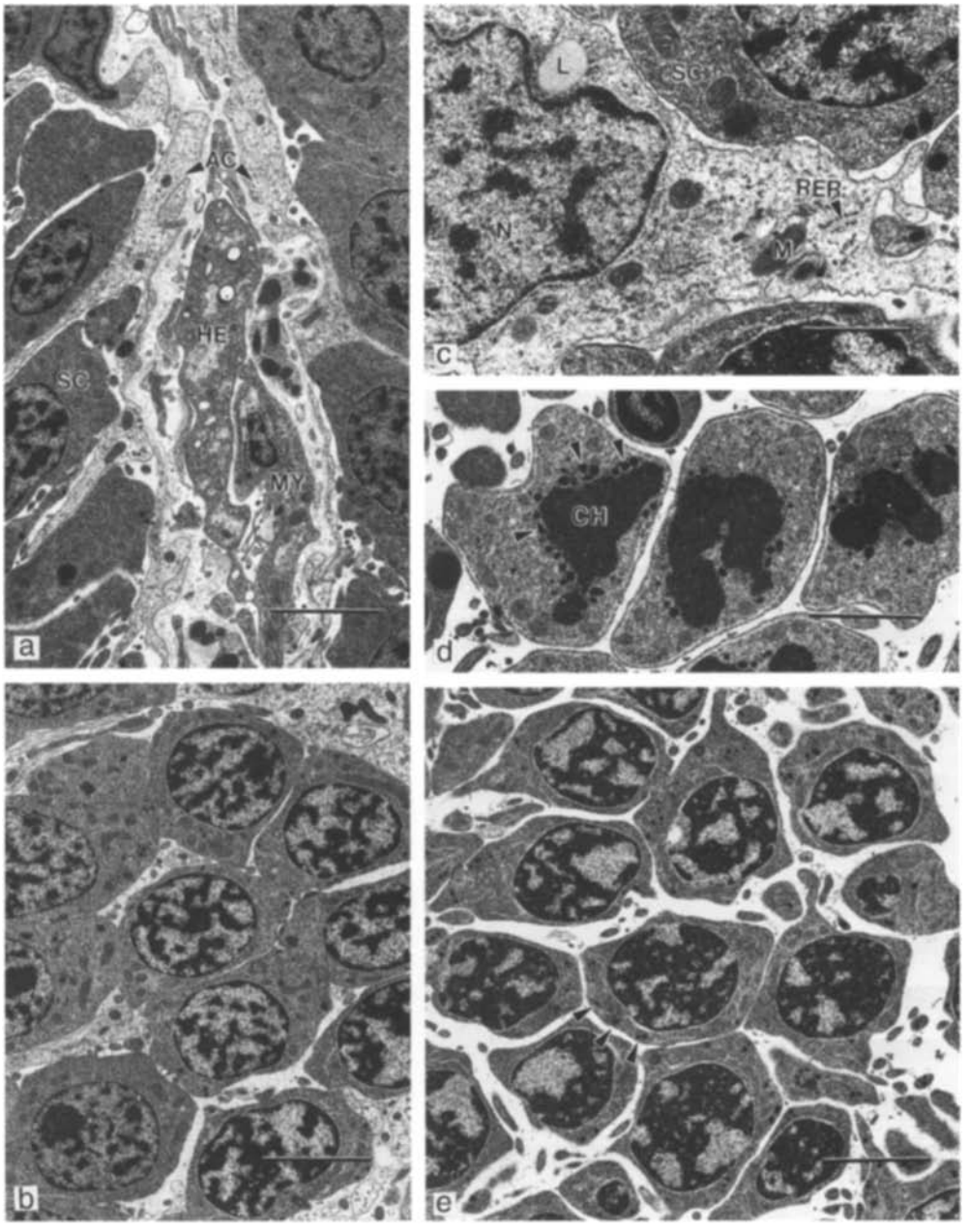

Fig. 2 *Crassostrea virginica.* **a** Adjacent subcompartments of acinus separated by a variety of somatic cells including a hemocyte (*HE*), myoepithelial cell (*MY*) and accessory cells (*AC*). **b** Spermatocytes. **c** Spermatocytes. Higher magnification of accessory cell cytoplasm containing nucleus (*N*), RER cisternae (*RER*), lipid droplet (*L*) and mitochondria (*M*) (*SC* spermatocyte). **d** Mitotically dividing spermatocytes containing chromosomes (*CH*) and surrounding proacrosomal vesicles (*arrowheads*). **e** Early spermatids (*Arrowheads* proacrosomal granules). All scale bars = 2.0 μm

with respect to the presence of a broad, cup-shaped acrosome, subacrosomal material including an axial rod, a relatively spherical nucleus and four pericentriolar mitochondria. These features, combined with a constant chromosome number of $2n = 20$ (Nakamura 1985), have suggested to some authors that the F. Ostreidae is a cohesive taxonomic unit (Healy and Lester 1991). While the present study confirms most of the ultrastructural features described in *C. virginica* sperm in earlier studies (Galtsoff and Philpott 1960; Daniels et al. 1971), it is the first to report more than four mitochondria in the sperm midpiece of any oyster sperm. The sperm of species within the Ostreoidea have previously all been reported to have four mitochondria (Healy 1989), but the present study suggests that some variation occurs. Intraspecific variation in mitochondrial number from 4 to 5 has also been reported within the Pterioidea, Veneroidea, and Cardiodea (Healy 1989).

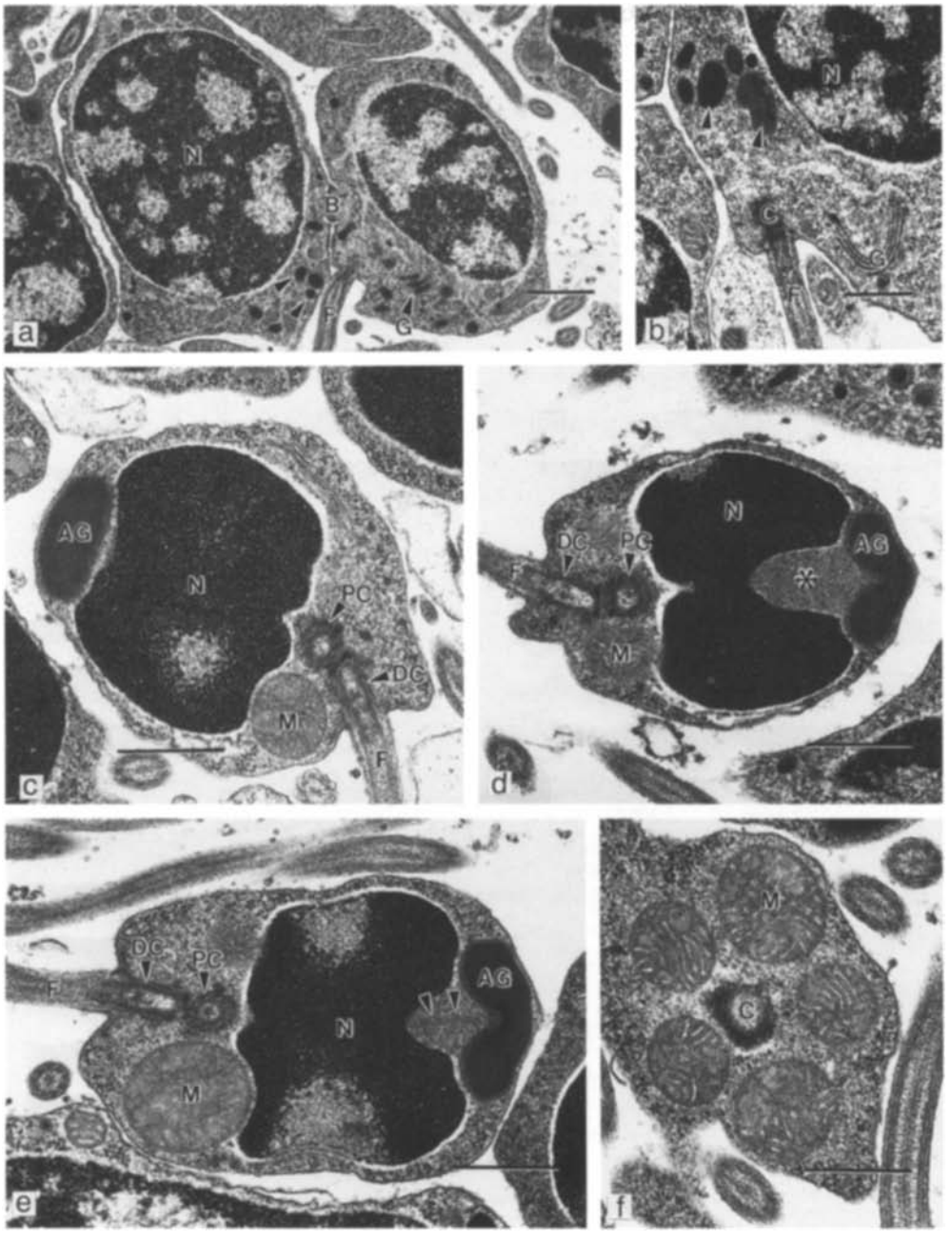

Fig. 3 *Crassostrea virginica.* **a** Spermatids connected by intercellular bridge (*B*) (*F* flagellum; *G* Golgi complex; *N* nucleus; *arrowheads* proacrosomal vesicles). **b** Spermatid showing flagellum (*F*), centriole (*C*), Golgi complex (*G*), proacrosomal vesicles (arrowheads), and nucleus (*N*). **c** Spermatid showing early acrosomal granule (*AG*), condensed nucleus (*N*), posterior mitochondrion (*M*), proximal centriole (*PC*) and distal centriole (*DC*), and flagellum (*F*). **d** Spermatid showing acrosomal granule (*AG*), subacrosomal material (*), nucleus (*N*), posterior mitochondria (*M*), proximal centriole (*PC*) and distal centriole (*DC*), and flagellum (*F*). **e** Mature spermatozoon with cap-like acrosomal granule (*AG*), subacrosomal material with antero-posterior-oriented fibrils (*arrowheads*), nucleus (*N*), mitochondria (*M*), proximal centriole (*PC*) and distal centriole (*DC*), and flagellum (*F*). **f** Transverse section through midpiece of spermatozoon showing central centriole (*C*) and five surounding mitochondrial profiles (*M*). All scale bars = 0.5 μm

Until recently, acrosomal vesicle formation in molluscs was believed to occur in two distinctly different ways. The first is observed in internally fertilizing species of cephalopods, prosobranch, opisthobranch, and pulmonate gastropods, in which a single acrosomal vesicle is synthesized by the Golgi complex. In the second, observed in most bivalves, the Golgi complex produces numerous proacrosomal vesicles which initially diffuse throughout the cytoplasm and later coalesce to form the definitive acrosomal vesicle (Healy 1989). While this may be common to many bivalve species, limited studies of acrosome formation in *Pecten maximus* (F. Pectinidae) (Dorange and Le Pennec 1989), *Scrobicularia plana* (F. Tellinidae) (Sousa et al.

1989), and *Callista chione* (F. Veneridae) (Nicotra and Zappata 1991) suggest that the Golgi complex may form only a single acrosomal vesicle in a manner similar to other molluscs. Recently, Healy (1989) described a third method in *Neotrigonia* spp. (F. Trigonidae) in which the Golgi produces a mature acrosomal complex consisting of up to three vesicles. Variability is also reported among species with respect to when the proacrosomal vesicles first appear during spermatogenesis. For example, proacrosomal vesicles were first observed in the spermatogonial stage in *Crassostrea angulata* and *Ostrea edulis* (F. Ostreidae) (Sousa and Oliveira 1994) but not until the spermatid stage in *Perna perna* (F. Mytilidae) (Bernard and Hodgson 1985), *Pecten maximus* (Dorange and Le Pennec 1989) and *Brachiodontes variabilis* (Mytilidae) (Al-Hajj 1990). While we did not confirm their presence at the spermatogonial stage in the present study, proacrosomal vesicles were common in *C. virginica* spermatocytes. The above studies collectively show that the mechanisms of acrosomal vesicle formation in mollusc sperm are diverse and that no single mechanism characterizes bivalve sperm.

There are species within the Bivalvia that have a prominent, reduced, or temporary acrosome or no acrosome at all in their sperm (reviewed by Peredo et al. 1990). In ostreids, the mature sperm acrosome is a prominent organelle that is superficially similar among the five species examined (Healy and Lester 1991). The acrosome of *Saccostrea commercialis* sperm, however, reportedly has an anterior banded substructure consisting of alternating electron dense and electron lucent regions (Healy and Lester 1991). Other authors have noted that the acrosome of *Crassostrea gigas* sperm exhibits unique electron-lucent whorls (Brandriff et al. 1978). Healy and Lester (1991) suggested that a similar "whorled" substructure might occur in *C. virginica.* Sousa and Oliveira (1994) recently reported "semi-circular dense lamellae" in the anterior acrosomal vesicle of the sperm of *C. angulata* similar to that reported in *S. commercialis.* We did not observe any banding pattern in the sperm acrosomal vesicle of *C. virginica.*

Silver staining has been used to demonstrate argyrophilic characteristics in the acrosomes of bivalves, including the oyster *Crassostrea angulata* (Sousa and Oliveira 1994), which can be useful in distinguishing between species with identical sperm ultrastructural morphologies. Regional differences in silver staining may reflect differences in the chemical composition of the acrosomal material, and these differences have been used to distinguish between closely related species (e.g. Eckelbarger and Grassle 1987). It is unclear what significance can be applied to differences in acrosomal morphology in bivalves. However, it has been proposed that variation in acrosome and sperm head morphology reflects differences in the egg envelope (Galangau 1969; Popham 1979; Franzén 1983), or correlations between elongated nuclei and the evolution of large, yolky eggs (Franzén 1983; Moueza and Frenkiel 1995).

Bivalve testes contain accessory (somatic) cells that may play some role in sperm maintenance and nutrition. For example, in an ultrastructural study of spermatogenesis in three species of galeommatoidid bivalves (Eckelbarger et al. 1990), two types of accessory cells were described. The first, a pleomorphic "follicle cell" was confined to the outer wall of the testicular acinus and contained glycogen and lipid deposits. These cells resemble the VCT cells we recently described from *Crassostrea virginica* (Eckelbarger and Davis 1996). The second was a phagocytic cell that was scattered throughout the acinus in close association with developing sperm. This cell does not resemble anything previously reported in other bivalve species. To our knowledge, this is the only example in which two accessory cells have been described from the testis of a single bivalve species.

Workers have described bivalve accessory cells using a variety of terms including "nutritive cells" (Coe 1943), "nutritive phagocytes" (Loosanoff 1937; Xiang and Yongqiang 1989), "phagocytic cells" (Rocha and Azevedo 1990; Eckelbarger et al. 1990), "follicle cells" (Galtsoff 1964; Eckelbarger et al. 1990), "Leydig cells" (Andrews 1979), "nutritive globulocytes" (Motavkine and Varaksine 1983), "auxiliary cells" (Dorange and Le Pennec 1989; Gaulejac et al. 1995), "Sertoli cells" (Pipe 1987) or "Sertoli-like cells" (Dorange and Le Pennec 1989), and "support cells" (Sousa et al. 1989). Few studies have documented the ultrastructural features of testicular accessory cells in bivalves, and most descriptions are too cursory to be useful in assessing homology. However, we believe that some attempt should be made to reassess and standardize the terminology used to describe these cells.

Differences have been reported with respect to the presence or absence of cell junctions between accessory cells and developing sperm in bivalves. Accessory cells were observed to be connected to adjacent germ cells via desmosomes in the testes of *Scrobicularia plana* (Sousa et al. 1989), *Anodonta cygnea* (Rocha and Azevedo 1990), *Pinctada margaritifera* (Thielley et al. 1993), and *Crassostrea virginica* (present study). Tight junctions were reported in *Pecten maximus* (Dorange and Le Pennec 1989), and septate junctions in *Mytilus edulis* (Pipe 1987). No junctions were observed between germ cells and accessory cells in *Pinna nobilis*, and neither of the two types of accessory cells described in the three galeommatoidid species formed cell junctions with germ cells (Eckelbarger et al. 1990). These observations show that the interaction between germ cells and accessory cells varies significantly in different species suggesting that they may play different physiological roles.

Sousa et al. (1989) described very active, phagocytic "support cells" from the testis of *Scrobicularia plana*, which are autolysed at the end of spermatogenesis. Similarly, Eckelbarger et al. (1990) and Rocha and Azevedo (1990) described irregularly shaped "phagocytic

cells" in the testes of several galeommatoidid bivalves and the unionid *Anodonta cygnea*, respectively, that appeared to be principally involved with engulfment and digestion of residual sperm cells. In *Pinctada margaritifera* (Thielley et al. 1993) and *Pinna nobilis* (Gaulejac et al. 1995), the authors described "auxiliary cells" with pseudopodia-like projections between germ cells that appeared to serve a resorptive function near the end of spermatogenesis. One common feature of these accessory cells is that they appear to have a phagocytic or resorptive function, but the respective ultrastructural descriptions of these cells differed significantly among the studies, and conflicting data on the presence or absence of junctional complexes between accessory cells were reported. This suggests that there may be more than one type of accessory cell in the bivalve testis.

The auxiliary cells we observed in the testis of *Crassostrea virginica* are ultrastructurally most similar to those described in *Scrobicularia plana* (F. Tellinidae) (Sousa et al. 1989). In both species, the cells extend from the wall of the acinus to the lumen and have cytoplasm largely devoid of ribosomes. The remaining ultrastructural descriptions of testicular accessory cells (cited above) are so limited, it is impossible to determine if they are homologous. Accessory cells also appear to vary ultrastructurally during spermatogenesis, so different authors may have described a single cell type during different stages of ontogeny. Until more information on their function is available, testicular somatic cells should probably be assigned the neutral term "accessory cells".

In summary, spermatogenesis in *Crassostrea virginica* is similar to that described in other bivalve species, particularly, other members of the Ostreidae. Mature sperm morphology is generally typical of that reported from other members of the Ostreidae, except that we observed some spermatozoa with five midpiece mitochondria rather than four and we did not observe a banding pattern in the acrosomal vesicle of the kind recently reported for other bivalve species. The testis of *C. virginica* contains an intra-acinal accessory cell the function of which is presently unknown. Bivalves as a whole may have more than one type of accessory cell in their testes, but the large number of terms used to describe them is unwarranted. The use of the neutral term "accessory cell" is advisable until additional information is available.

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