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ORIGINAL ARTICLE

Rosa Guerra · Mário Sousa · Artur Torres
Elsa Oliveira · Luis Baldaia

Fine structure of acrosome biogenesis and of mature sperm in the bivalve molluscs *Glycymeris* sp. (Pteriomorphia) and *Eurhomalea rufa* (Heterodonta)

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Abstract Proacrosomal vesicles form during the pachytene stage, being synthetized by the Golgi complex in *Glycymeris* sp., and by both the Golgi and the rough endoplasmic reticulum in *Eurhomalea rufa*. During early spermiogenesis, a single acrosomal vesicle forms and its apex becomes linked to the plasma membrane while it migrates. In *Glycymeris* sp., the acrosomal vesicle then turns cap-shaped (1.8 µm) and acquires a complex substructure. In *E. rufa*, proacrosomal vesicles differentiate their contents while still at the premeiotic stage; as the acrosomal vesicle matures and its contents further differentiate, it elongates and becomes longer than the nucleus (3.2 µm), while the subacrosomal space develops a perforatorium. Before condensation, chromatin turns fibrillar in *Glycymeris* sp., whereas it acquires a cordonal pattern in *E. rufa*. Accordingly, the sperm nucleus of *Glycymeris* sp. is conical and elongated (8.3 µm), and that of *E. rufa* is short and ovoid (1.1 µm). In the midpiece (*Glycymeris* sp.: 1.1 µm; *E. rufa*: 0.8 µm), both species have four mitochondria encircling two linked orthogonal (*Glycymeris* sp.) or orthogonal and tilted (30–40°; *E. rufa*) centrioles. In comparison with other Arcoida species, sperm of *Glycymeris* sp. appear distinct due to the presence of an elongated nucleus, a highly differentiated acrosome, and four instead of five mitochondria. The same occurs with *E. rufa* regarding other Veneracea species, with the acrosome of the mature sperm strongly resembling that of the recent Mytilinae.

Keywords Mollusca · Bivalvia · Heterodonta · Pteriomorphia · Spermatogenesis

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R. Guerra
Department of Biology, Faculty of Sciences,
University of Valparaiso, Chile

M. Sousa (✉) · A. Torres · E. Oliveira
Laboratory of Cell Biology,
Institute of Biomedical Sciences Abel Salazar,
University of Porto,
Lg. Prof. Abel Salazar 2, 4099-003 Porto, Portugal
e-mail: msousa@icbas.up.pt
Fax: +351-22-2062232

L. Baldaia
Laboratory of Physiology and CIMAR,
Institute of Biomedical Sciences Abel Salazar,
University of Porto, Portugal

Introduction

In the Mollusca, the morphology of mature sperm has been used for tracing phylogenetic and taxonomic relationships between species (Popham 1979; Franzén 1983; Jamieson 1987; Rouse and Jamieson 1987; Healy 1995; Hodgson 1986, 1995; Morse and Zardus 1997; Kafanov and Drozdov 1998). The ultrastructural characteristics of spermatogenesis can also reveal important distinguishing features between the species, including the cell stage at which proacrosomal vesicles first appear, the mechanism by which the acrosomal vesicle migrates towards the apical pole of the cell, the morphological appearance and the chemical constitution of the acrosome subcomponents, the origin of the subacrosomal material, the pattern of chromatin remodeling during condensation, and the composition and structure of the midpiece (Sousa and Azevedo 1988; Sousa et al. 1989, 1995, 1998, 2000; Sousa and Oliveira 1994a, 1994b, 1994c; Kafanov and Drozdov 1998).

In the Veneroida, the specific study of the complete steps of spermatogenesis has been very limited, and includes the Lucinacea (Johnson et al. 1996), Leptonacea (Eckelbarger et al. 1990), Mactracea (Longo and Anderson 1969), Solenacea (Hodgson et al. 1987; Reunov and Hodgson 1994), Tellinacea (Sousa et al. 1989; Hodgson et al. 1990; Reunov and Hodgson 1994; Sousa and Oliveira 1994a), Corbiculacea (Konishi et al. 1998), and Veneracea (Reunov and Hodgson 1994). In the Arcoida, even fewer studies have been published (Reunov and Hodgson 1994; Suwanjarat 1999).

In the present study we describe the ultrastructural features of acrosome formation and of the mature sperm (primitive/ect-aquasperm type) in two species, *Glyc-*

ymeris sp. and *Eurhomalea rufa*. It is shown that sperm of *Glycymeris* sp. differ from those of other Arcoida species by the presence of a complex acrosomal vesicle substructure, a long nucleus, and four mitochondria in the midpiece. Similarly, sperm of *Eurhomalea rufa* present a completely different sperm structure in relation to the other Veneracea, being similar to species of the recent Mytilidae.

Methods

Specimens of *Glycymeris* sp, Da Costa, 1778 (Mollusca, Bivalvia, Pteriomorphia, Prionodonta, Arcoida, Limopsacea, Glycymerididae), were collected at depth regions (40–50 m) of the intertidal North Atlantic coast of Portugal. Specimens of *Eurhomalea rufa* (Mollusca, Bivalvia, Heterodonta, Veneroida, Veneracea, Veneridae, Tapetinae), were collected at Bahia Tongoy, IV region of the Pacific coast of Chile.

Small pieces of testis (<1 mm) were fixed with 2.5% glutaraldehyde buffered with 0.2 M sodium cacodylate, pH 7.4, for 2 h at 4°C and then rinsed in the same buffer. Specimens were post-fixed in 2% osmium tetroxide in buffer for 2 h at 4°C, dehydrated in an ethanol series followed by propylene oxide, and embedded in Epon. Ultrathin sections were cut in a Leica ultratome with a Diatome knife, collected in 300-mesh copper grids (Taab), double-stained with alcoholic concentrated (5%) uranyl acetate for 20 min followed by lead citrate (Reynolds) for 10 min, and studied at 60 kV in a transmission electron microscope JEOL 100 CXII (Sousa et al. 2000).

For scanning electron microscopy, isolated sperm pellets were diluted after post-fixation in 70% ethanol. From this suspension, 50 µl was dropped into a small round glass cover-slide, previously glued with nail polish to a cylindrical metal support. After air-drying, samples were rotary shadowed with gold and observed in a SEM-JEOL-35C at 25 kV (Center for Material Sciences, Faculty of Engineering, University of Porto, Portugal).

All chemicals were of analytical grade and were purchased from Merck.

Results

Glycymeris sp.

During the pachytene stage, the Golgi complex sheds small light vesicles which then fuse to form small dense proacrosomal vesicles (Figs. 1 and 2). At early spermiogenesis, a single acrosomal vesicle with homogeneous contents forms and attaches by its apex to the plasma membrane. After migrating towards the apical pole of the cell, the inner acrosomal vesicle membrane becomes coated by a dense layer. The acrosomal base then invaginates to create a subacrosomal space filled with an intermediate-dense fibrillar material. During this process, the nucleus elongates and chromatin becomes fine fibrillar before condensing (Fig. 3).

The mature spermatozoon of *Glycymeris* sp. is 95 µm long, and consists of a conical head (11.2–12 µm) and a flagellum (83 µm). The head contains a conical cap-shaped acrosomal vesicle (1.8 µm) which is connected to the plasma membrane by a dense material, a subacrosomal space filled with a fine fibrillar material, a dense conical and elongated nucleus (8.3 µm), and a midpiece (1.1 µm) with four mitochondria encircling two linked and orthogonal centrioles (Figs. 4, 5, and 6). The acrosomal vesicle shows a complex substructure (Fig. 6). It displays an apical saccular invagination filled with a dense material; a dense layer that internally coats the acrosomal inner membrane and ends in the apical pouch; an intermediate-dense matrix; and a central dense and double-layered band that surrounds the entire acrosomal vesicle and fuses with the apical pouch. From the inner surface of this band, small dense projections cross between each other in the middle region, establishing a central linkage between the outer and inner portions of the band.

Eurhomalea rufa

In this species as well, proacrosomal vesicles are formed during the pachytene stage. The dense material that fills the early proacrosomal vesicles is first observed inside the Golgi cisternae before being shed into small round vesicles (Fig. 7). These dense proacrosomal vesicles then intimately interact with rough endoplasmic reticulum cisternae (Fig. 8). After this, their contents differentiate into a dense and convex basal region, an apical fibrillar component, and a thin intermediate-dense band that internally coats the inner proacrosomal vesicle membrane (Fig. 9). During early spermiogenesis, a single acrosomal vesicle forms (Fig. 10). Its apex then attaches to the plasma membrane, and the inner acrosomal vesicle membrane becomes externally coated by a fine fibrillar material. Simultaneously, the dense basal material turns concave, decreases in thickness and spreads laterally, whereas the peripheral band expands apically to surround the entire acrosomal vesicle membrane (Fig. 10). After reaching the apical pole of the spermatid, the basal dense material extends apically (Fig. 11) and then retracts from the central basal region and becomes ring-shaped (Fig. 12). Thereafter, the dense component concentrates at the acrosomal vesicle base. During this process, it becomes surrounded by the peripheral intermediate-dense component and develops external indentations (Figs. 13, 14, and 15). Simultaneously, the apical fibrillar component becomes cord-like (Fig. 14) and then transforms into fine dense and concentric lamellae (Fig. 15). As the acrosomal vesicle base invaginates (Figs. 12, 13, 14, and 15), the subacrosomal space becomes filled with a fine fibrillar material, and the inner acrosomal vesicle membrane appears to be coated by a fine dense material at its basal half (Figs. 14 and 15).

The mature sperm of *Eurhomalea rufa* shows a conical head (5.1 µm in length), that comprises a midpiece (0.8 µm) with four mitochondria encircling two linked and tilted orthogonal centrioles, a short ovoid nucleus (1.1 µm), and a very long cap-shaped acrosomal vesicle (3.2 µm) whose wide subacrosomal material is filled with longitudinally oriented fine fibrils (Fig. 16).

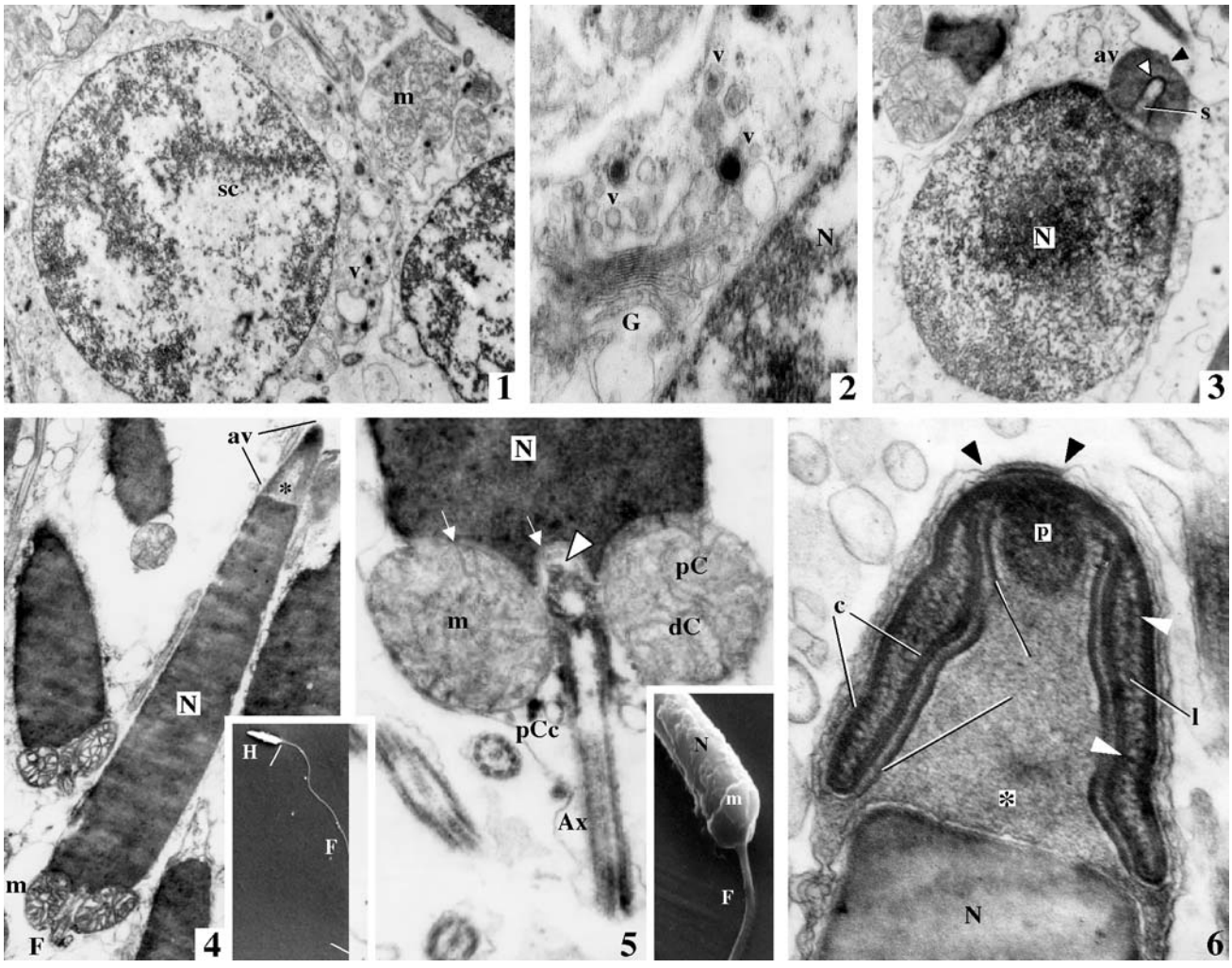

Fig. 1 Pachytene spermatocytes of *Glycymeris* sp. Note the presence of synaptonemal complexes (*sc*), mitochondria (*m*) and the small dense proacrosomal vesicles (*v*). Multiplication ×5,200

Fig. 2 Detail of a pachytene spermatocyte of *Glycymeris* sp. Note origin of proacrosomal vesicles (*v*) from the Golgi complex (*G*), *N* nucleus. Multiplication ×15,000

Fig. 3 Elongating spermatid of *Glycymeris* sp. In the nucleus (*N*), chromatin is turning fine fibrillar. The acrosomal vesicle (*av*) is linked to the plasma membrane by its apex (*black arrowhead*); its base is invaginated to form the subacrosomal space (*s*) and appears coated by a dense material (*white arrowhead*). Multiplication ×11,200

Fig. 4 Mature sperm of *Glycymeris* sp. The head (*H*) comprises a dense elongated nucleus (*N*), a cap-shaped acrosomal vesicle (*av*), the subacrosomal space (*), and a midpiece with mitochondria (*m*), *F* flagellum. Multiplication ×7,400; inset ×450

Fig. 5 *Glycymeris* sp. mature sperm midpiece with four mitochondria (*m*). The nucleus (*N*) shows mitochondrial and centriolar fossae (*white arrows*), the proximal centriole (*pC*) is linked to the nuclear envelope by a centriolar adjunct (*white arrowhead*); the distal centriole (*dC*) anchors to the plasma membrane by the pericentriolar complex (*pCc*) and gives rise to the axoneme (*Ax*) of the flagellum (*F*). Multiplication ×25,900, inset ×5,500

Fig. 6 *Glycymeris* sp. mature sperm acrosome. The inner acrosomal vesicle membrane is internally coated by a dense narrow layer (*i*) that ends into the dense apical pouch (*p*); the double-layered and circular dense band (*c*) fuses apically with the dense apical pouch, and from its inner surface small dense projections (*white arrowheads*) cross between each other in the middle region (*l*). The apex of the outer acrosomal vesicle membrane attaches to the plasma membrane through a dense material (*between black arrowheads*). Note absence of a perforatorium in the subacrosomal space (*), *N* nucleus. Multiplication ×49,200

Discussion

In the Arcoida, spermatogenesis has been studied in *Barbatia obliquata* and *B. foliata* (Arcacea, Arcidae), as also in *Anadara trapezia* and *A. granosa* (Arcacea, Anadaridae). In these species, and similarly to *Glycymeris* sp., proacrosomal vesicles are formed during the zygotene/pachytene stage and chromatin condenses homogeneously during spermiogenesis (Popham 1979; Reunov and Hodgson 1994; Suwanjarat 1999). Also in common with all Arcoida sperm, *Glycymeris* sp. shows absence of a perforatorium in the subacrosomal space, and two orthogonal centrioles in the midpiece. In this group, however, *A. trapezia* is an exception regarding the centrioles, which appear tilted. However, the mature sperm of those species are different from that of *Glycymeris* sp., as they

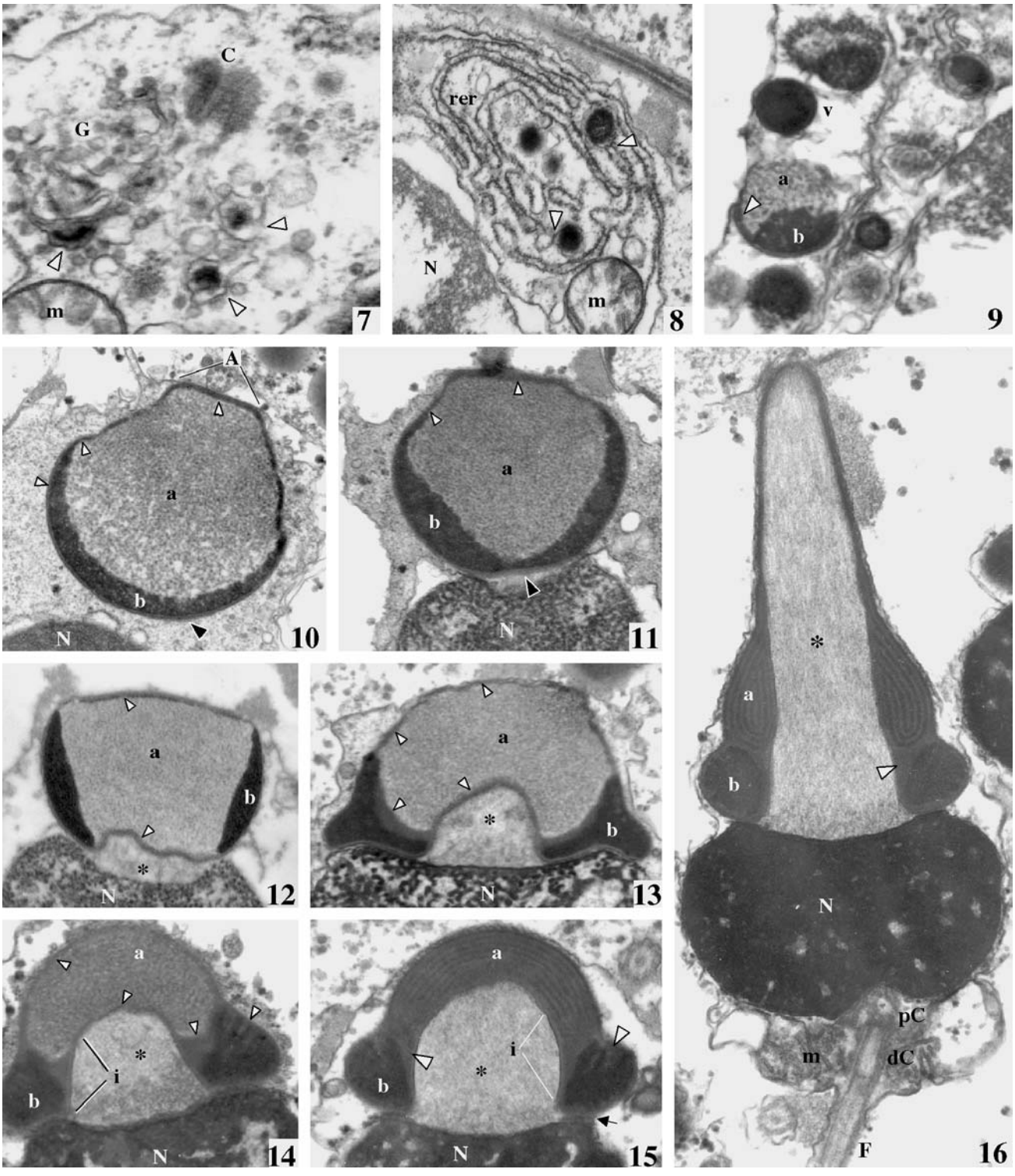

Fig. 7 Detail of a pachytene spermatocyte of *Eurhomalea rufa*. Note the Golgi (*G*) origin of proacrosomal vesicles (*white arrowheads*). *C* centriole, *m* mitochondria. Multiplication ×42,400

Fig. 8 Detail of a pachytene spermatocyte of *Eurhomalea rufa*. Note the intimate relation between proacrosomal vesicles (*white arrowheads*) and the rough endoplasmic reticulum cisternae (*rer*). *N* nucleus, *m* mitochondria. Multiplication ×24,000

Fig. 9 Detail of a pachytene spermatocyte of *Eurhomalea rufa*. Note differentiation of the contents of the early dense proacrosomal vesicles (*v*) into a basal dense and convex region (*b*), an apical fibrillar material (*a*), and a basal peripheral and intermediate-dense layer (*white arrowhead*). Multiplication ×25,300

Fig. 10 Transformation of the acrosome during spermiogenesis in *Eurhomalea rufa*. The apex of the acrosomal vesicle is linked to the plasma membrane (*A*) and its base is externally coated by a fine dense material (*black arrowheads*). The basal dense component (*b*) of the acrosomal vesicle turns concave. The peripheral intermediate-dense layer (*white arrowheads*) extends apically. *N* nucleus. Multiplication ×15,800

typically show a short barrel-shaped nucleus (2.2–2.5 vs 8.3 μm), a short conical acrosome (0.9–1.0 vs 1.8 μm), absence of internal differentiation of the acrosomal contents (vs a very complex arrangement of contents in *Glycymeris* sp.), and five mitochondria in the midpiece (vs four mitochondria in the midpiece of *Glycymeris* sp.). In relation to other Glycymerididae, *Glycymeris yessoensis* shows a similar elongated and conical nucleus, although slightly shorter than that of *Glycymeris* sp. (6.0 vs 8.3 μm), but the acrosomal vesicle is round, shorter (0.7 vs 1.8 μm) and far apart from the nuclear apex (Paschenko and Drozdov 1991).

In almost all Veneroida species, sperm are also of the primitive type, although including some nuclear and midpiece modifications (Lucinacea: Johnson et al. 1996; Leptonacea: Galeommatidae: Eckelbarger et al. 1990; Cardiacea: Sousa and Azevedo 1988; Tellinacea: Sousa et al. 1989; Dreissenacea: Denson and Wang 1994, 1998; Walker et al. 1996; Veneracea: Turtoniidae: Ockelmann 1964), as also a few examples of modified and dimorphic sperm (Lucinacea: Moueza and Frenkiel 1995; Leptonacea: Lasaeidae: Ó Foighil 1985a, 1985b; Leptonacea: Montacutidae: Ockelmann 1965; Corbiculacea: Komaru and Konishi 1996; Konishi et al. 1998).

Regarding spermatogenesis in the Veneracea, proacrosomal vesicles were described to derive from the Golgi complex during pachytene spermatocytes in *Tivela polita* (Reunov and Hodgson 1994), or during spermiogenesis in *Venerupis* sp. (Pochon-Masson and Gharagozlou 1970; Gharagozlou and Pochon-Masson 1971) and *Callista chione* (Nicotra and Zappata 1991). Conversely, in *Eurhomalea rufa*, proacrosomal vesicles, although also appearing during the pachytene stage, are the product of both the Golgi complex and the rough endoplasmic reticulum. Also unlike all other Veneracea, the sperm of *E. rufa* show a short nucleus and a long conical acrosome, whereas all other species exhibit a nucleus that is longer than the acrosome. In common with other Veneracea, sperm of *E. rufa* shows a perforatorium, although the absence of an axial rod was noticed in *T. polita* (Reunov and Hodgson 1994). Although the typical pattern in the Veneracea is the presence of five mitochondria in the midpiece, sperm of *E. rufa* show only four mitochondria, which is similar to *Venerupis* sp. (Pochon-Masson and Gharagozlou 1970; Gharagozlou and Pochon-Masson 1971) and *Turtonia minuta* (Ockelmann 1964). In comparison with other groups, the shape of the acrosomal vesicle and the internal differentiation of the acrosomal contents give *E. rufa* sperm a rather strange resemblance to the sperm of the recent Mytilinae (Kafanov and Drozdov 1998; Reunov et al. 1999). The major differences between sperm of *E. rufa* and those of the recent Mytilinae are the presence of only four mitochondria, the flattened nucleus, and the absence of a narrow axial rod.

In relation to other members of the order Veneroida, proacrosomal vesicle formation during the leptotene stage was described in *Divariscintilla* sp. (Eckelbarger et al. 1990), and at the zygotene/pachytene stage (as in *Eurhomalea rufa*) in *Solen* sp. (Solenacea) (Hodgson et al. 1987; Reunov and Hodgson 1994), *Donax* sp. (Tellinacea) (Hodgson et al. 1990; Reunov and Hodgson 1994; Sousa and Oliveira 1994a), and *Corbicula fluminea* (Corbiculacea) (Konishi et al. 1998). However, in all these species the origin of proacrosomal vesicles is the Golgi complex, and thus the active participation of the rough endoplasmic reticulum in this process is here first described in the Veneroida for *E. rufa*. Another original finding in *E. rufa*, which is here also first described in the Veneroida, is the precocious differentiation of proacrosomal vesicle contents during the premeiotic stage. In *E. rufa* and *Glycymeris* sp., the acrosomal vesicle migrates towards the apical pole of the cell with its apex linked to the plasma membrane. During this event, it acquires a thin basal external coat that defines the basal pole of the acrosomal vesicle and corresponds to the precursors of the subacrosomal material. A similar situation has only been previously described in species from Tellinacea, *Donax trunculus* and *Scrobicularia plana* (Sousa et al. 1989; Sousa and Oliveira 1994a).

◀ **Fig. 11** Transformation of the acrosome during spermiogenesis in *Eurhomalea rufa*. The base of the acrosomal vesicle is externally coated by a fine dense material (*black arrowheads*). The basal dense component (*b*) extends apically. The peripheral intermediate-dense layer (*white arrowheads*) extends apically. *N* nucleus. Multiplication ×19,400

Fig. 12 Transformation of the acrosome during spermiogenesis in *Eurhomalea rufa*. The basal dense component (*b*) of the acrosomal vesicle then retracts. The peripheral intermediate-dense layer (*white arrowheads*) extends apically to coat the entire acrosomal vesicle membrane. *N* nucleus. Multiplication ×20,800

Fig. 13 Transformation of the acrosome during spermiogenesis in *Eurhomalea rufa*. The basal dense component (*b*) of the acrosomal vesicle then retracts into a basal ring. The peripheral intermediate-dense layer (*white arrowheads*) enlarges at the base to surround the dense ring. *N* nucleus, * subacrosomal space. Multiplication ×21,300

Fig. 14 Transformation of the acrosome during spermiogenesis in *Eurhomalea rufa*, showing the basal ring with outer indentations; the apical fibrillar region (*a*) acquires a cord-like pattern. The peripheral intermediate-dense layer (*white arrowheads*) enlarges at the base to surround the dense ring. As the subacrosomal space (*) forms, the inner acrosomal membrane acquires a fine dense coat (*i*). *N* nucleus. Multiplication ×22,000

Fig. 15 Transformation of the acrosome during spermiogenesis in *Eurhomalea rufa*, showing the basal ring with outer indentations; the apical fibrillar region (*a*) finally develops concentric lamellae. The peripheral intermediate-dense layer (*white arrowheads*) enlarges at the base to surround the dense ring. As the subacrosomal space (*) forms, the inner acrosomal membrane acquires a fine dense coat (*i*). The base of the acrosomal vesicle is linked to the nuclear envelope by a dense material (*black arrow*). *N* nucleus. Multiplication ×22,800

Fig. 16 Mature spermatozoon of *Eurhomalea rufa*. The wide subacrosomal space is filled with a fine fibrillar material that is longitudinally oriented (*). The highly elongated cap-shaped acrosomal vesicle is differentiated into an intermediate-dense matrix (*white arrowhead*), a basal dense region (*b*), and a neck and apical region containing concentric lamellae (*a*). *N* nucleus, *m* mitochondria, *pC* proximal centriole, *dC* tilted distal centriole, *F* flagellum. Multiplication ×25,400

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