



Morphological approaches as tools to study the development of male germ cells of *Crassostrea gasar* (Mollusca, Bivalvia)

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Abstract

Crassostrea gasar is a bivalve mollusk of economic interest in the Amazon estuary, because it displays good zootechnical performance and nutritional value. However, there is still a gap in the literature regarding studies on basic biology, especially spermatogenesis at the ultrastructural level. This study aimed to describe for the first time the male gonad development. Gonad fragments were analyzed using light microscopy and transmission and scanning electron microscopy. The ultrastructural analysis of the gonadal tubule showed two distinct cell populations: support cells and the cells of the spermatogenic lineage. Regarding the ultrastructural aspects, support cells were characterized by an irregular shape, sparse electron-dense cytoplasm, and a large nucleus with condensed chromatin; and two types of spermatogonia were seen (A and B); spermatocytes I where it was possible to characterize four stages of prophase I, namely leptotene, zygotene, pachytene, diplotene; spermatocytes II showing a reduction in cytoplasmic and condensed chromatin. Spermatids were classified into three stages of development: early, middle, and late. Mature spermatozoa are of the primitive type and have a cone-shaped head with a discoidal acrosome, a midpiece formed by four mitochondria, and a long flagellum. This information can help establish phylogenetic relationships between species of the genus *Crassostrea*. In addition, these findings can support conservation biology by developing and implementing biotechnological approaches, such as cryopreservation, and contributing to commercial-scale cultivation in the Amazon region.

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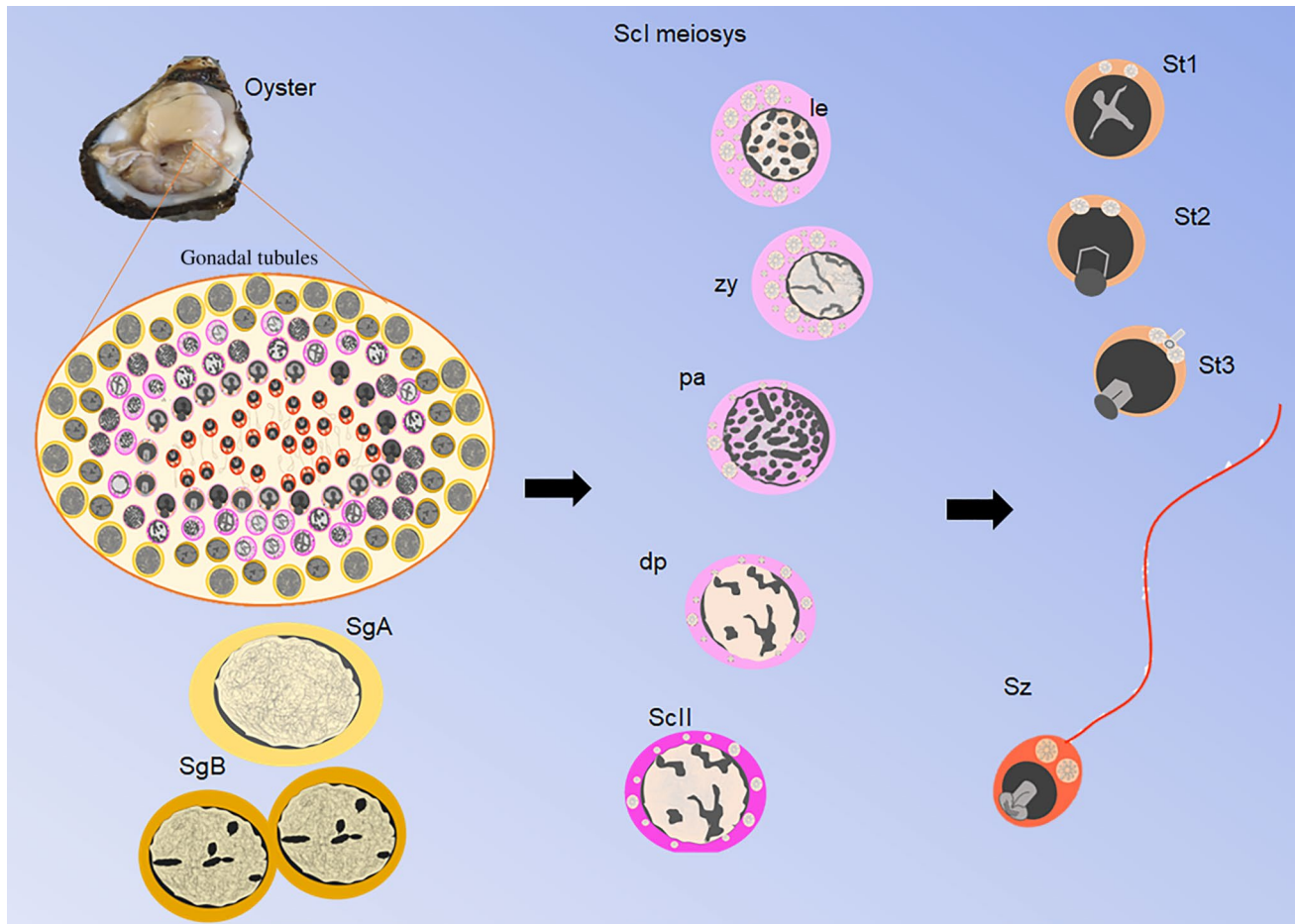
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Graphical Abstract



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Introduction

Reproductive biology is essential to assess a population's dynamics and species perpetuation (Gosling 2015; Watanabe et al. 2020). The knowledge of the male gonadal structure and spermatogenesis of bivalves has been important for understanding the species' reproductive biology and the mechanisms involved in sperm maturation (Healy et al. 2015; Gwo and Hsu 2020). Spermatogenesis is a cyclical process that starts from germ cells to the production of mature gametes, which are released into the environment (Mylonas et al. 2016; Nuurai et al. 2016).

Recently, these morphological data on spermatogenesis have established different reproductive strategies (Viana et al. 2022) and a broad understanding of phylogenetic and systematic relationships (Varela et al. 2007; Melo et al. 2010). However, studies addressing morphological differences are scarce for native species such as *Crassostrea*

gasar. These characteristics can help to understand the phylogenetic aspects and establishment of the species in a family.

The Ostreidae family has undergone a systematic review, which has seen a change in the name and clade of several species of economic importance (Liu et al. 2011; Salvi and Mariottini 2016; Kasmini and Batubara 2022). Thus, descriptive studies provide essential data regarding morphology, serving as a tool for taxonomy and phylogenetics. It is vital to keep databases up to date.

The oysters are dioecious, and have sexual reproduction with external fertilization, with no present sexual dimorphism (Christo and Absher 2006). According to Varela et al. (2007), there are three species of oysters on the Brazilian coast, *Crassostrea rhizophorae*, *Crassostrea gasar*, and *Crassostrea gigas*. In the northeast of the Brazilian state of Pará, the species *C. gasar* is dominant, because it is economically important for the Amazon region, generating

employment and income. There are several studies in the literature on the cultivation of *C. gasar* (Paixão et al. 2013; Oliveira et al. 2018; Pantoja et al. 2020; Lameira Silva et al. 2020).

For decades, histological and ultrastructural techniques have been used as tools to characterize spermatogenesis in several species of oysters over, mainly of the genus *Crassostrea*, such as *Crassostrea angulata* (Sousa and Oliveira 1994), *Crassostrea gigas* (Bozzo et al. 1993; Franco et al. 2008; Kim et al. 2010b; Yurchenko et al. 2010), and *Crassostrea virginica* (Eckelbarger and Davis 1996; O’Beirn et al. 1996). However, these aspects are not described in *Crassostrea gasar* (Deshayes, 1830) (sin. *Crassostrea tulipa* (Lamarck, 1819), an important species marketed in Brazil, especially in the Amazon region. There are still gaps in the description of spermatogenesis of *C. gasar*. Thus, the aim of the present study was to describe the development of germ line and support cells, and to characterize the cell types of the germ line.

Materials and methods

Collection of specimens

We collected 30 specimens of *C. gasar* at 3 months of age according to the de Souza Sampaio et al. (2019) cultivated in Augusto Corrêa, Pará, Brazil in 2019 (0° 52' 54" S and 46° 26' 54" W), thereby considered adults. Subsequently, the specimens were transported to the laboratory and then anesthetized with ice according to the protocol of Pantoja et al. (2020) for the removal and processing of gonadal tissue, stained with hematoxylin and eosin (HE) solution for sexual identification of oysters.

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM)

The fragments of gonads from 30 animals' males were fixed in Karnovsky's solution (4% paraformaldehyde, 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4) for 24 h. After fixation, the fragments were washed in 0.1 M sodium cacodylate buffer, pH 7.4 and post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.4 for 2 h. For TEM analysis, the fragments were dehydrated in an ascending acetone series and embedded in Epon 812. Ultra-thin sections were cut in a microtome and contrasted with uranyl acetate and lead citrate, followed by an examination in a JEOL (JEM -100CX II) electron microscope. For SEM analysis, a male gonad section and 500- μ l aliquots of raw semen were fixed in Karnovsky's solution, and then, a drop of the fixed semen sample was placed on a poly-L-lysine-coated coverslip. The samples were dehydrated in a graded

ethanol series (30–100%) and critical-point dried using CO₂ and mounted on stubs, coated with gold, and examined using a LEO 1430 SEM.

Morphometry and statistical analysis

For morphometry only, male gonad in the mature stage of *C. gasar* were evaluated, because this stage has the largest seminiferous tubules, following criteria previously established (Pantoja et al. 2020). A total of 10 specimens were analyzed, and for each specimen, the mean diameter of the 10 seminiferous tubules was measured. In each specimen, the mean diameter ($n = 100$) for each germ cell type (spermatogonia A and B, spermatocytes, spermatids, and spermatozoa) were analyzed. Only cells that contained a nucleus were measured, serial sections were cut, and the slides were evaluated under a photomicroscope with the software NIS-elements BR (4.00.07-bit), and measurements were made at 40 \times magnification. Each cell was overlaid with two dashed lines crossing at right angles in the middle of the cell, and the length of the segment of the line over that diameter of the cell was measured. The mean length of the two measurements was taken as the approximate diameter of the cell. The means were analyzed using a Student's parametric test with ($p < 0.05$). All analyses were performed using the program Statistica 2.0.

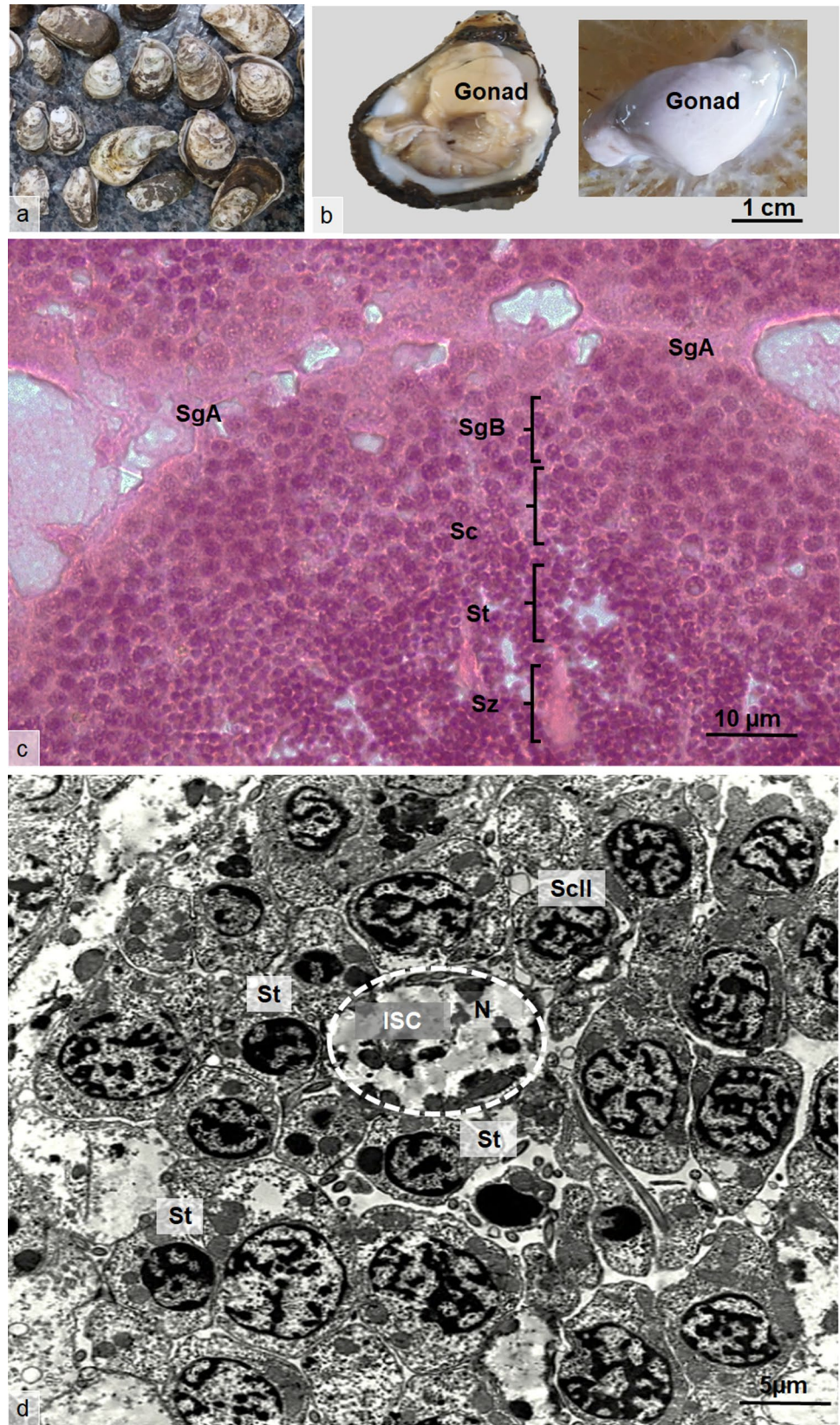
Results

Gonadal morphology and cellular organization in males of *C. gasar*

Specimens of *C. gasar* (Fig. 1a) at this advanced stage of spermatogenesis showed gonadal tissue (Fig. 1b) with a diffuse whitish aspect, consisting of numerous gonadal tubules, located between the mantle and the digestive gland. Microscopically, the gonadal tubules are enclosed by a thin layer of connective tissue, surrounded by hemocoel (Fig. 1c).

The gonadal tubules presented two distinct cell populations, the support cells and the germ cells. The support cells or intragonadal somatic cells (ISC) (Fig. 1d) were characterized by an irregular shape, sparse electron-dense cytoplasm and a large nucleus with condensed chromatin and by being in close contact with the germ cells (Fig. 1c, d). Germ cells were represented by spermatogonia (A and B), primary spermatocytes (in four phases of meiosis), secondary spermatocytes, spermatids (early stage, middle stage, and late stage) and spermatozoa, which are organized in a stratified manner and presented specific characteristics throughout gonadal development.

Fig. 1 Morphology of reproductive system in *Crassostrea gasar*. **a** Specimens of *C. gasar* of various sizes. **b** Location of the male gonadal tissue. **c** Cross-section of the gonadal tubule showing the layered organization of different cell populations. **d** TEM photomicrographs of sperm gonadal tubule showing an intragonadal somatic cell nucleus (white dashed line) surrounded by germinal epithelium. *ISC* intragonadal somatic cell (white dashed line), *SgA* spermatogonia A, *SgB* spermatogonia B, *Sc* spermatocyte, *St* spermatid, *Sz* spermatozoa



Ultrastructure of male germ cells

The germinal epithelium comprised spermatogenic cells in different stages that were evidenced by the nuclear characteristics, chromatin condensation patterns, and cytoplasmic content. They have morphometry (pseudo- $F = 1.23$; $p \leq 0.05$); a progressive and significant reduction was observed in the diameter of the nucleus of germ cells with statistic values, which occurred throughout spermatogenesis (Table 1).

The spermatogonia have a larger diameter of the cell nucleus and were classified into two types: type A (SgA) and type B (SgB) (Fig. 2a, b). SgA cells were found as individual cells directly attached to the wall of the gonadal tubule, and have electrolucent cytoplasm with several mitochondria and nucleus of large diameter ($5.05 \pm 0.1 \mu\text{m}$), spherical shape, fine chromatins. Meanwhile, SgB cells were found in successive cell layers and had smaller cell nucleus diameter ($4.51 \pm 0.2 \mu\text{m}$) compared to SgA, electron-dense cytoplasm with the presence of mitochondria and several round granulo-fibrillar dense bodies.

After successive mitotic divisions, SgB gives rise to primary spermatocytes (ScI) characterized by chromatin condensation ($3.94 \pm 0.2 \mu\text{m}$). Based on the chromatin arrangement, it was possible to distinguish ScI in four phases of prophase I: leptotene (le), where there were reduced cytoplasm, condensed nuclear chromatin, and little evidence of nucleolus (Fig. 2c, e); Zygotene (zy) the nuclear envelope

and long, thin filamentous chromatin; Pachytene (pa) characterized by bulky nucleus, thick chromatin filaments; Diplotene (dp) showing thick, irregular chromatin migrating to the nuclear membrane.

Secondary spermatocytes (ScII) were smaller than ScI and presented cellular bridges and cytoplasm with some proacrosome vesicles, mitochondria at a cell pole, which is the possible viewer of a centriole in each cell, and a nucleus with thick chromatin (Fig. 2d, e).

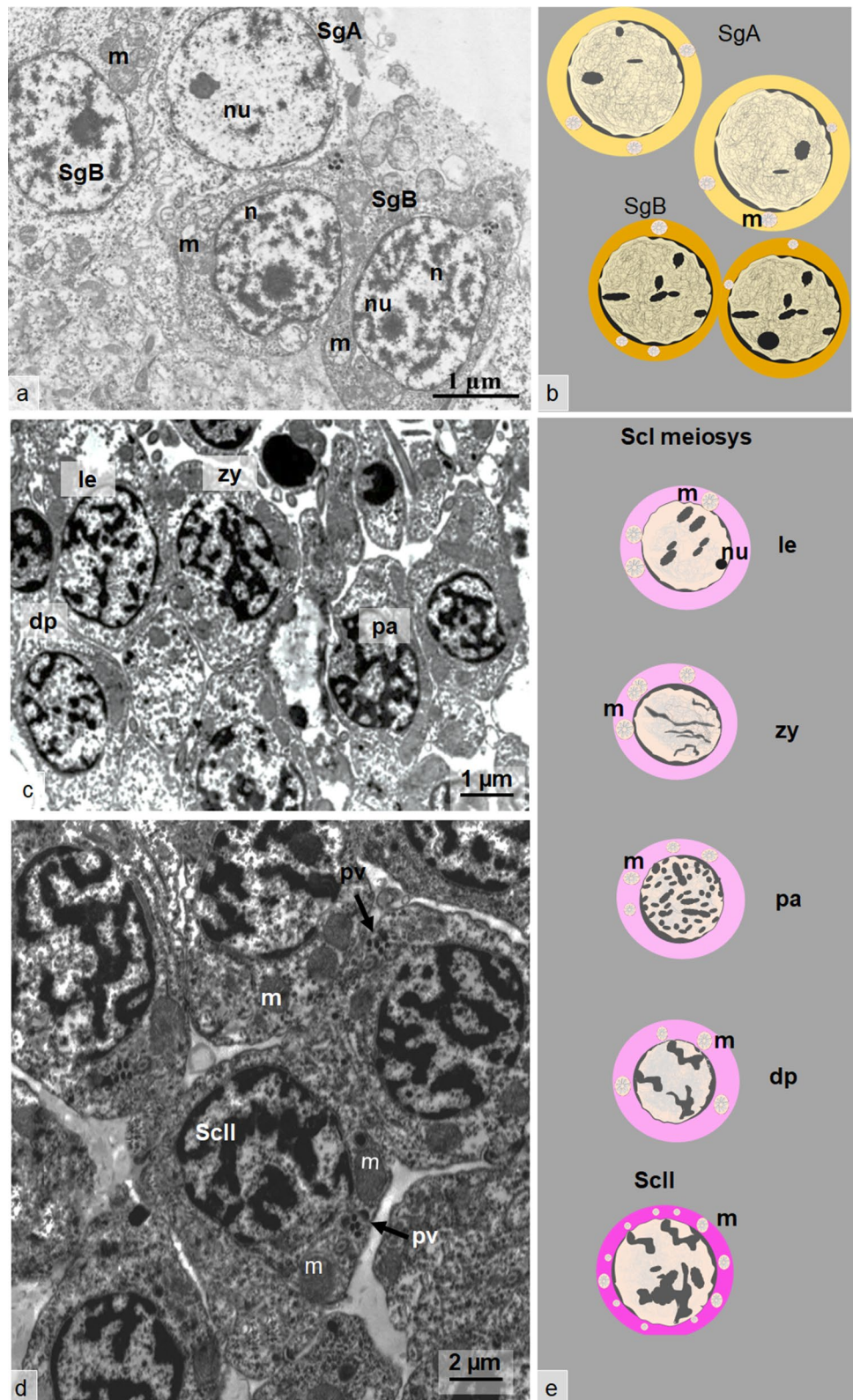
According to their shape and localization, the spermatids were subdivided into three subtypes: early stage (St1), middle stage (St2), and late stage (St3) (Fig. 3a–d). In the early stage (St1) ($3.53 \pm 0.2 \mu\text{m}$), the cytoplasm contains several electron-dense granules, two pairs of mitochondria, a large spherical acrosomal vesicle, along with the centriole, the nucleus contains thick electron-dense heterochromatin materials with a small amount of euchromatin (Fig. 3b). Middle stage (St2) (Fig. 3c) presents a nucleus containing densely packed heterochromatin, acrosomal vesicles with the rounded shape at the cell pole, while mitochondria remain at the posterior end of the cell ($3.29 \pm 0.2 \mu\text{m}$). In the late stage (St3) with reduced cytoplasm and well-defined cellular compartments, acrosomal vesicle, subacrosomal space containing electron-dense granules, a nucleus with condensed chromatin ($3.06 \pm 0.1 \mu\text{m}$), the subacrosomal space is apparent between the acrosomal vesicle and the nucleus, the proximal and distal centrioles, as well as the flagellum, are located at the caudal end of the cell (Fig. 3d).

Table 1 Mean (\pm SD) diameters (μm) of the male germ cells of *Crassostrea gasar*

Male gametes	Characteristics		
	Size (μm)/shape	Nucleus	Cytoplasm
Spermatogonium A	5.05 ± 0.1^a	Euchromatic with small blocks of heterochromatin nucleolus	Low electron dense
Spermatogonium B	4.51 ± 0.2^b	Patches of heterochromatin	Several mitochondria
Spermatocyte	3.94 ± 0.2^c	Chromosomes move to be aligned at the equatorial	Numerous mitochondria Proacrosomal vesicles around the chromosome Proximal centriole
Early spermatid	3.53 ± 0.2^d	Heterochromatin patches	Single large spherical acrosomal vesicle Mitochondria grouped
Middle spermatid	3.29 ± 0.2^e	Dense Chromatin patches	Slightly indented Acrosomal vesicle
Late spermatid	3.06 ± 0.1^f	Condensed chromatin	Cup-like structure of acrosomal vesicle Subacrosomal space Spherical mitochondria Proximal centriole Distal centriole
Spermatozoa	1.56^g	Barrel-shaped with condensed chromatin	Cup-shaped acrosome Axial rod Subacrosomal materials Proximal centriole Distal centriole

a–g in the same column indicates a statistically significant difference cells ($p < 0.05$)

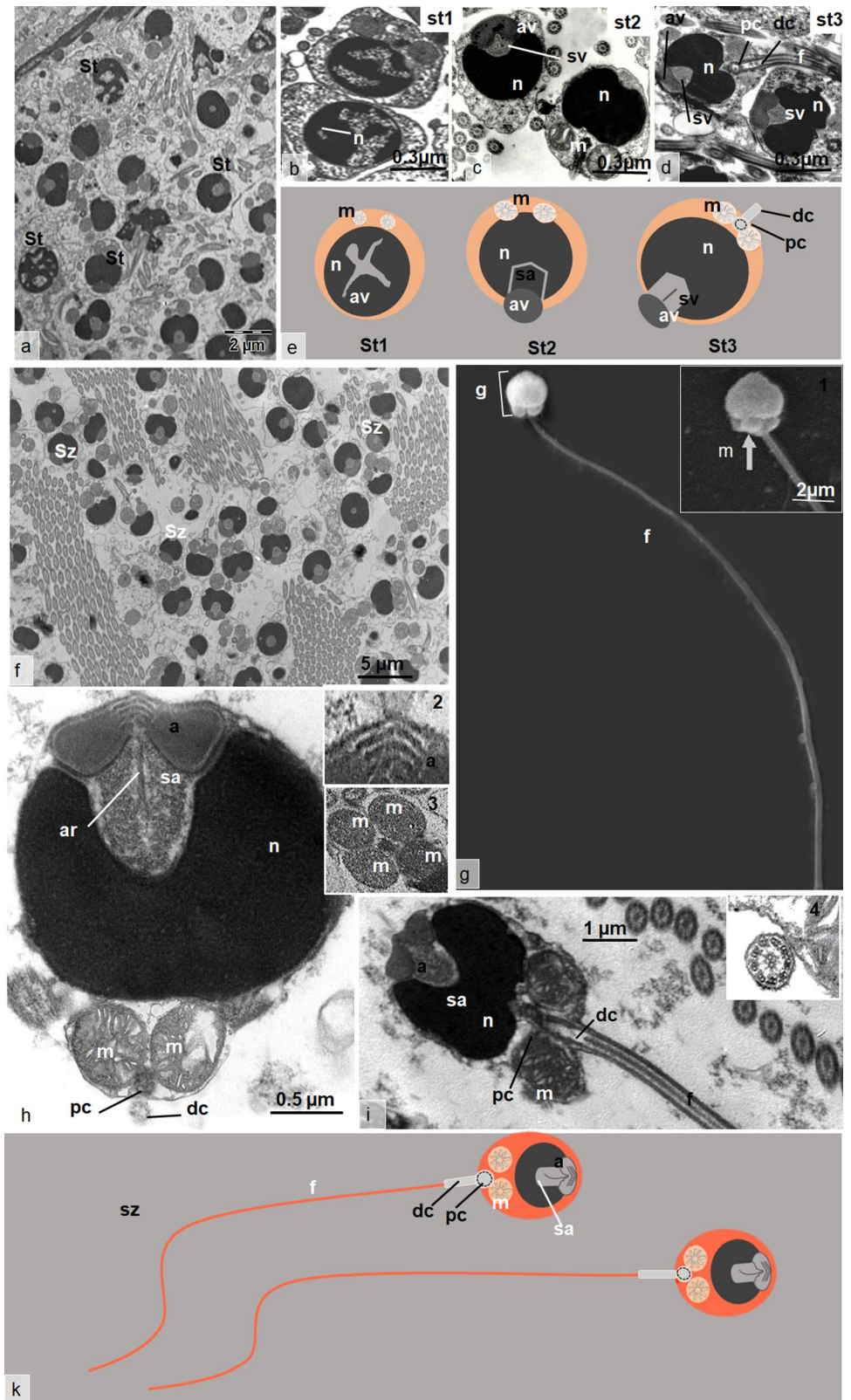
Fig. 2 TEM micrographs of the germinal epithelium in the male of *C. gasar*. **a** Type A spermatogonia on the periphery of the gonadal tubule, Type B spermatogonia. **b** Schematic drawing of cells Type A spermatogonia and Type B spermatogonia. **c** Primary spermatocytes at different stages of meiosis. (*le*) Leptotene-reduced cytoplasm and heterochromatin. (*zy*) Zygotene. (*pa*) Pachytene-Bulky nucleus, visible filamentous chromatin. (*di*) Diplotene Organization of chromatin aggregation near the nuclear membrane. **d** Secondary spermatocyte. **e** Schematic drawing of cells spermatocytes primary and secondary. *SgA* spermatogonia A, *SgB* spermatogonia B, *Scl* primary spermatocyte, *le* leptotene, *zy* zygotene, *pa* pachytene, *dp* diplotene, *SclI* secondary spermatocyte, *m* mitochondria, *n* nucleus, *nu* nucleolus, dense round granulofibrillar bodies (black arrow)



Spermatozoa (1.56 μm) were found in the lumen of the tubule gonadal (Fig. 3f) and had ovoid heads (Fig. 3g, inset 1). In the upper central piece of the acrosome, there were

transverse bands with three or four layers of structures (Fig. 3 h, inset 2). In the subacrosomal space, electron-dense granules containing axial rod and condensed chromatin were

Fig. 3 TEM micrographs of the germinal epithelium in the male of *C. gasar*. **a–e** Spermatids. **a** Spermatids at different stages of development within the gonadal tubule. **b** Early stage spermatid with the single large acrosomal vesicle, condensed chromatin. **c** Middle-stage spermatid—scant round-shaped acrosome vesicle at one cell pole, condensed nucleus. **d** Late-stage spermatid—sparse cytoplasm, defined acrosomal and subacrosomal vesicles; distal centriole at the posterior end of the cell and proximal centriole present, the beginning of flagellum formation. **e** Schematic drawing of cells St1, St2, and St3. **f–i**. TEM and SEM photomicrographs of the morphology of spermatozoa in *C. gasar*. **f** Mature sperm in the lumen of gonadal tubule. **g, h** Cone-shaped head of spermatozoa with the proximal piece of the sperm head showing acrosome with three or four transverse bands of whorl-like structure in the anterior region, subacrosomal space containing axial rod, barrel-shaped condensed nucleus, the intermediate piece with four mitochondria, proximal and distal centriole. Inset 1. Detail of the head of spermatozoa. Inset 2. Detail of acrosome with three or four transverse bands of whorl-like structure. Inset 3. Transverse section of the four mitochondria. Inset 4. Transverse section of the microtubule. **k** Schematic drawing of spermatozoa. *St1* early stage spermatid, *St2* middle-stage spermatid, *St3* late-stage spermatid, *Sz* spermatozoa, *m* mitochondria, *n* nucleus, *av* acrosome vesicle, *sa* subacrosomal area, *ar* axial rod, *a* acrosome, *pc* proximal centriole, *dc* distal centriole, *f* flagellum



demonstrated (Fig. 3h, white arrow). In the middle piece of the spermatozoa, we observed four round mitochondria with tubule-vesicular cristae in an electron opaque matrix

homogeneously distributed, enclosed the proximal and distal centrioles (Fig. 3i, k, inset 3). In the flagellum, the main piece was characterized by the arrangement of nine pairs of

peripheral microtubules and a central pair constituting the 9 + 2 structure (Fig. 3 i, Inset 4).

Discussion

In the present study, we characterized the gonadal tissue of *C. gasar* males. A pattern of organization of the germinal epithelium was observed inside the gonadal tubule, consisting of ISC and germline cells. This arrangement describes a centripetal development, where spermatogonia are adhered to the wall of the gonadal tubule, forming the first layers of cells. These germ cell distribution patterns look those of other bivalves, *C. cortezensis* (Rodríguez-Jaramillo et al. 2008), *C. gigas* (Franco et al. 2008; Kim et al. 2010a, b), and *C. virginica* (Hillman and Galtsoff 1965; O’Beirn et al. 1996). Therefore, the organization and reduction of the diameter of the cell nucleus of germ cells in the gonadal tubule can follow a pattern conserved mainly in bivalve mollusks.

C. gasar we found that ISC is distributed among germ cells, their location and cell pattern are like those observed in other mollusk species (Eckelbarger and Davis 1996; Erkan and Sousa 2002; Franco et al. 2011). The ultrastructural organization of ISC is compatible with the functions ensured by Sertoli cells in vertebrate and can assist the development of germ cells, serve as support, protection, and nutrition (Watanabe et al. 2020). In addition to promoting phagocytosis of residual bodies discarded by spermatids at the end of spermiogenesis (Franco et al. 2011; Kobayashi et al. 2020).

Spermatogonia have been classified into different types, based on location, cell size, and presence of nucleoli, and are the largest cells in the gonadal tubules of *C. gasar*, as in other studies of bivalves (Franco et al. 2008; Kim et al. 2010a, b; Yurchenko and Vaschenko 2010). In the present study, there was spermatogonia A, which exhibits large nuclei containing a low amount of euchromatin and several cytoplasmic granulo-fibrillar electron-dense bodies and in close relationship with mitochondria, similar features were observed in other bivalves, *Amiantis umbonella* (Al-Mohanna et al. 2001) and *C. gigas* (Franco et al. 2008). While Spermatogonia B exhibits large nuclei containing a relatively high amount of euchromatin with a prominent nucleolus and observed the presence of several dense round granulo-fibrillar bodies in the cytoplasm, where the presence of this structure can be identified as nuage, the observation of this material in spermatogonia is common in these cell types in spermatogonia has been described in *S. forskali* and *C. angulata*, the origin of these materials being related to mitochondria (Reunov et al. 2000).

The spermatocyte I of *C. gasar* showed alterations in the nucleus, with gradual condensation of chromatin and disappearance of the nucleolus, especially in the phase of

pachytene, related to genetic recombination between homologous chromosomes. These characteristics were like those described in *C. angulata* (Sousa and Oliveira 1994) and *C. virginica* (Eckelbarger and Davis 1996).

In spermatocyte II, the chromatin filaments are more condensed and located in the central region of the nucleus; this cell type is widely described in mollusks (Reunov and Hodgson 1994; Yurchenko et al. 2021, 2022). However, it is rarely observed in vertebrates, since it is a rapid phase transition during meiosis (Mazzoni et al. 2014; Gonçalves et al. 2019). The pro-acrosomal vesicle in the spermatocytes of *C. gasar*, as well as observed in other species of the genus, as found in *C. angulata* (Sousa and Oliveira 1994), *C. gigas* (Franco et al. 2008), and *C. virginica* (O’Beirn et al. 1996).

Based on acrosome formation and chromatin condensation, *C. gasar* spermatids were classified as early stage, medium stage, and late stage. The early stage spermatid has diffused homogeneous nuclear chromatin and bridging interconnections in the cytoplasm. A similar appearance has previously been found in *C. gigas* (Franco et al. 2008; Yurchenko et al. 2010). In middle-stage spermatids, the acrosomal vesicle is located at the cell pole, exhibiting more condensed chromatin. The late-stage spermatid comprises three distinct compartments: the acrosomal vesicle, the subacrosomal region and the nucleus, and the beginning of flagella formation. In contrast, there are reports in mollusks that the formation of flagella occurs in spermatocytes, as evidenced in *C. virginica* (Eckelbarger and Davis 1996) and *Pitar rudis*, *Chamelea gallina* (Erkan and Sousa 2002). Therefore, the process of flagella formation can occur at different stages of spermiogenesis which may occur later in *C. gasar*.

C. gasar spermatozoa has an ovoid head with a cone-shaped acrosome. In addition, we evidenced transverse bands that alternate in electron-dense (dark) and electron-lucid (light) areas in the upper region of the acrosome. Bivalves spermatozoa probably have less vigorous movements and do not need long distances to find oocytes for fertilization. According to literature suggest that these structures may play a signaling role, releasing mature spermatozoa into the external environment (Eckelbarger et al. 1990; Reunov and Hodgson 1994; Yurchenko et al. 2021).

The middle piece has four round mitochondria with tubule-vesicular cristae, similar to that seen in spermatozoa from other oysters, such as *C. gigas*, *Saccostrea commercialis*, and *C. angulata* (Kim et al. 2010a, b; Nurai et al. 2016; Sousa and Oliveira 1994), in contrast that observed in *Crassostrea virginica*, *Crassostrea rhizophorae* that exhibit more than the four conventional mitochondria (Eckelbarger et al. 1990; Eckelbarger and Davis 1996; Machado and Passos 2022) and other bivalves with a different number of mitochondria. We believe that the variation in the number of mitochondria in the middle piece of the spermatozoa may

be related to the energy consumption of each species, since this characteristic differs within the genus.

In the present study, a long flagellum was observed, the central piece has the typical shape of the axoneme 9 + 2, and the distal centriole is oriented on the same axis; similar features were observed all the oysters studied (Kim et al. 2010a, b; Healy et al. 2015; Yurchenko et al. 2021). Thus, the success of external fertilization can be ensured by the great quantity of gametes released into the external environment.

The morphometry of the nuclei of male germ cells in *Crassostrea gasar* is similar other studies with bivalves, *Saccostrea forskali*, *Saccostrea commercialis*, and *Crassostrea virginica* (Nuurai et al. 2016; Healy and Lester 1991; Yurchenko et al. 2010). The male germ cells in bivalves are small compared to vertebrates (Santos et al. 2001; Lo Nostro et al. 2003). In oysters, there is a continuous decrease in the size of germ cells during spermatogenesis.

In this study, we identified specific cellular aspects for *C. gasar*, a reduced number of mitochondria in the spermatozoa and long flagellum. These characteristics may be of help in phylogenetic study and the reproductive biology of bivalve mollusks, mainly species of socioeconomic interest.

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Author contributions JP, MF, IV, RO, ZN, GS, and RR wrote the main manuscript text. JP and RO prepared the samples. JP, RO, and MA performed scanning electron microscopy. JP, IV, and RO prepared the figures. All authors reviewed the manuscript.

Data availability Data will be available upon request.

Declarations

Conflict of interest The authors declare no competing interests.

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