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Histological and Ultrastructural Analysis of Spermatogenesis in *Gelastocoris flavus flavus* (Heteroptera: Nepomorpha)

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Abstract

Studies on the ultrastructural aspects of spermatogenesis and, specifically, the structure of sperm in aquatic Heteroptera are scarce. Therefore, the objective of this study was to analyse of the histology and ultrastructure of spermatogenesis. Semi-fine sections of the testicles of adult male *Gelastocoris flavus flavus* were stained with toluidine blue or impregnated with silver ions, and ultra-fine sections were analysed by transmission electron microscopy. The ultrastructural features observed during spermatogenesis of the species showed the presence of several small mitochondria uniformly distributed in the cytoplasm of cells in prophase I. These mitochondria then came together to form fewer, larger structures, which converged and formed the mitochondrial complex. Later, this mitochondrial complex was divided into two structures, termed mitochondrial derivatives, which were arranged bilaterally to the axoneme. The axoneme showed a flagellar pattern of 9+9+2. A vesicle was observed that originated in the early stages of spermiogenesis and was composed of many argyrophilic granules that united to form a single structure. This vesicle contained some highly stained structures in its interior. Thus, this paper describes histological and ultrastructural characteristics during spermatogenesis, contributing to the reproductive knowledge of these aquatic Heteroptera.

Keywords: Argyrophilic granules; Mitochondrial derivative; Axoneme; Nucleolus

Introduction

In both vertebrates and invertebrates, studies addressing the ultrastructural aspects of spermatogenesis and, specifically, the structure of the sperm comprise a vast and growing body of literature. In insects, the study of the ultrastructural aspects of spermiogenesis and sperm began in the 1970s, when the methods for electron microscopy of biological material were optimised [1-3], but in Heteroptera, studies are still scarce. Some ultrastructures involved in spermatogenesis, such as the acrosome, axoneme, mitochondria and chromatoid body, are described in the literature.

In most species, the acrosome (Ac) is an organelle essential for fecundation [1]. In addition to fecundation, the acrosome is also related to the sperm's ability to remove the protective mechanism on the surface of ovule and/or to digest barriers to the migration of sperm into the female reproductive tract [4]. Acrosomes originate from the Golgi complex [5,6].

Research on the structural organisation of the axoneme (Ax) has received valuable contributions from numerous observations made in the sperm flagellum of *Drosophila* [7-9]. In most insects, the organisational pattern of the axoneme follows the pattern 9+9+2, which is the usual arrangement of 9+2 microtubules surrounded by nine additional accessory microtubules [1,2,10]. In some mosquitoes, the pattern 9+9+1 is common, whereas in mayflies, the pattern 9+9+0 predominates [1]. There are also patterns that are considered aberrant; for example, in dipterous of the *Cecidomyiidae* family, the schema is 13+0 [11].

Another ultrastructure often analysed during spermatogenesis is the mitochondria, which assume different morphologies. In insects, the regularity in the mitochondrial shape is particularly notable [1]. During spermatogenesis, the mitochondria undergo metamorphosis, where the typical structure of mitochondria is completely modified. In the initial stages of differentiation, a complex fusion process occurs, and mitochondria rearrange, forming the mitochondrial complex. This complex is often termed "Nebenkern," especially in older studies [1,2,12-14]. As spermatogenesis continues, the mitochondrial complex divides into two mitochondrial derivatives (MD), which are positioned bilaterally to the axoneme during the spermatozoid stretching process [15].

During differentiation, the mitochondrial derivatives are filled along their length by a protein structure that is organised in a paracrystalline pattern, and many species of insects accumulate these structures in mitochondria during spermiogenesis [1,10,16,17]. This paracrystalline structure is also known as a crystalloid and is formed by a protein rich in proline [18]. In Heteroptera (*Pentatomidae*, *Reduviidae* and *Gerridae* family), two or three crystalline bodies within the mitochondrial derivatives have been observed [19].

Various functions have been suggested for the mitochondrial

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derivatives of sperm in insects; for example, they may participate in the control and regulation of flagellar movement or shape, or they may be involved in the storage and release of the energy required for flagellar motility [5,20,21]. However, Perotti [22] contradicted this last statement, relating the mitochondrial derivatives to paternal cytoplasmic inheritance and presuming that the material stored in the MDs may be involved in the activation and nutrition of the oocyte after fertilisation.

In addition to the structures mentioned previously, the cytoplasm of germ cells contains a build-up of a material termed a chromatoid body (CB) or "nuage" [23]. The CB is a macromolecular complex that is thought to play a coordinating role in the post-transcriptional control of gene products in haploid male germ cells and also to function as a centre for the determination of mRNA [23-25].

Some authors believe that the CB originates from a material between clusters of mitochondria that is present in the cytoplasm of germinal cells [26]. However, more recent studies describe the origin as coming from the fragmentation of nucleolar material during the process of spermatogenesis [27-34]. Other structures that have been studied are the nucleolus and other structures that are impregnated with silver ions. The size of the nucleolus is related to the biosynthetic activity of the cell; therefore, the size and number of nucleoli and pre-nucleolar bodies depend on the metabolic and functional characteristics of the cells [35-42].

In most species of arthropods, the nucleoli dissociate at the diplotene stage or diakinesis. Therefore, the corpuscles impregnated by the Ag-NOR technique, which are specific proteins associated with rRNA, are not visible from metaphase to telophase I. These markings reappear at the beginning of spermatid formation, indicating that the transcriptional functions of ribosomal RNA have resumed, and they finally disappear at the end of spermatid formation [43,44]. However, the literature reports some exceptions; for example, in *Asellus aquaticus* (Isopoda) and species of the genus *Rhodnius* (Hemiptera), Ag-NOR corpuscles were visualised during the entire process of spermatogenesis [42,43]; in *Callicrania seoanei* (Orthoptera), Ag-NORs were observed in interkinesis until pro-metaphase II [45]; and in *Triatoma infestans* and *T. sordida* (Hemiptera), the markings were present until metaphase I [35]. Furthermore, in *Carlisis wahlbergi* (Heteroptera, Coreidae), nucleolar semi-persistence (the presence of nucleolar corpuscles during metaphase) was observed until metaphase II [46], whereas for *Acanthocoris sordidus* (Heteroptera, Coreidae) and *Coptosoma punctissimum* (Heteroptera, Plataspidae), the nucleoli were detected in metaphase plates of primary and secondary spermatocytes [47].

With the objective of broadening the information known histological and ultrastructural about aquatic Heteroptera spermatogenesis, we analysed the species *Gelastocoris flavus flavus*.

Material and Methods

Testicles of *G. f. flavus* males were extracted and immediately fixed in Karnovsky fixative solution for a period of 3 to 48 hours. Samples were then washed twice in Millonig buffer, and post-fixation was performed with Osmium Tetroxide 1% Millonig (1:3) for 2 hours in a refrigerator. The material was washed thoroughly with bidistilled water and then dehydrated in a series of solutions with increasing concentrations of acetone until a concentration of 100% was reached. A pre-infiltration in araldite: acetone (1:1) was performed overnight at room temperature. Afterwards, infiltration in araldite was performed for 2 hours at 37 °C and was then continued for 48 hours at 60 °C. Semi-fine and ultra-fine sections were obtained using a Leica Ultracut UCT ultramicrotome. The semi-fine sections were stained with toluidine blue pH4.0 [48] or silver ions (Ag-NOR) (Howell and Black [49], with modifications). The ultra-fine sections were collected on *grids* and then contrasted with 2% uranyl acetate for 20 minutes [50], followed by 2% lead citrate solution with 1 N sodium hydroxide for 6 minutes [51]. The results of transmission electron microscopy were documented by electron micrographs obtained from a CM100 Philips transmission electron microscope at the Center of Electron Microscopy, Institute of Biosciences, Botucatu, UNESP – IBB Campus Botucatu. São Paulo, Brazil.

Results

The images obtained from semi-fine sections and ultramicrographs showed several rounded chromatoid bodies (CBs) with different sizes in prophase I (Figures 1a, 2a and 2b). These were located near pore complexes of the nuclear envelope (Figure 2d and 2e). Small mitochondria were also observed that were uniformly dispersed within the cytoplasm (Figures 1a and 2a-c). The mitochondria were starting to come together to form larger structures (Figure 1c) and were moving to one side of the cell (Figures 1c-1e and 2c), forming the mitochondrial complex (MC). The MC is a unique structure in that it is rounded and contains a tangle of mitochondria (Figure 1h and 1i). During the elongation of spermatids, the MC was divided in two sections, forming the Mitochondrial Derivatives (MDs), which were initially very close to each other and bilaterally flanked the axoneme (Ax) (Figures 1l, 2f and 2g). In a later stage, the MDs separated, with the Ax between them (Figures 1n-p and 2 f-i). At the end of the process, the MDs completely surrounded the Ax (Figure 2j). A crystalloid structure was also seen in the interior of the MDs (Figure 2f and 2g). In Figure 2k,

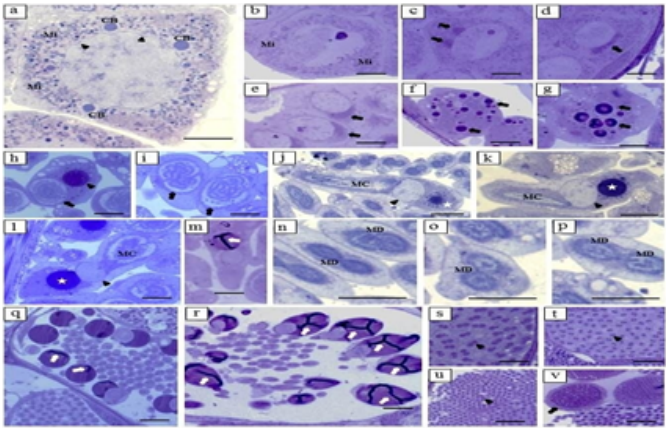

Figure 1: Semi-fine sections of *Gelastocoris flavus flavus* testicle stained with toluidine blue. **a)** Prophase I, showing the chromatoid bodies (CB) rounded, of varying sizes and with sharp metachromasia. Near the CB are pore complexes (arrowheads) and mitochondria (Mi); **b-c)** mitochondrial complex formation; in "b", mitochondria (Mi) are small, isolated and dispersed throughout the cytoplasm. In "c", mitochondria are beginning to unite on one side of the cell (arrows); **d,e)** mitochondrial complex formation (arrows); **f-g)** early spermatids with several argyrophilic granules of different sizes (arrows); **h)** union of argyrophilic granules forming a vesicle (arrowhead) and the mitochondrial complex (arrow); **i)** spermatids with the mitochondrial complex (arrows); **j-l)** spermatids in elongation showing the mitochondrial complex (MC), a highly stained vesicle (asterisk) and a vesicle without staining (arrowhead); **m)** vesicle containing a more intensely coloured region (arrow); **n-p)** cross-section of a tail showing the mitochondrial derivative (MD); **q-r)** longitudinal section of the head, containing a vesicle with more highly stained interior regions (arrows); **s-u)** cross-section of different regions of the tail showing the mitochondrial derivative (arrowheads); **v)** cyst with spermatozoids (arrow). Bars: 10 µm.

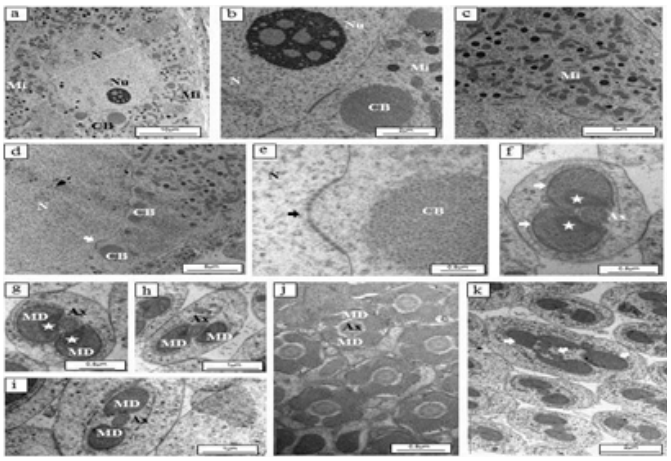

Figure 2: Electron micrographs of *Gelastocoris flavus flavus* testicular cells. **a)** Cell in prophase I showing the nucleolus (Nu), which is more electrodense than the other structures and has less electrodense interior regions (this region, in more detail, can be viewed in b); **a-c)** mitochondria (Mi) of different sizes and enlargements. Note that in "c", mitochondria are primarily located on one side of the cell, CB: chromatoid body, N: nucleus, Nu: nucleolus; **d,e)** the chromatoid body (CB) and the pore complex (arrows). In "e", these structures are shown at a higher magnification; **f)** cross section of tail, showing the mitochondrial derivatives (arrows) and the presence of interior crystalloids (asterisks) and the axoneme (Ax); **g-j)** tail formation process in relation to the mitochondrial derivative (MD) and the axoneme (Ax). In "g", the mitochondrial derivatives are close, and the axoneme is still moving to the central region of the tail; note the presence of crystalloids (asterisks); **j)** axoneme surrounded by the mitochondrial derivative; **k)** supernumerary elements (arrows). Bars: 10 µm.

supernumerary elements were observed (the presence of four Ax and two mitochondrial derivatives in the same tail). Another structure observed in the cell nucleus at prophase I was the nucleolus (Figure 2a and 2b), which was rounded and more electrodense than the other structures, with less electrodense interior regions. As shown in Figure 3a and 3b, a cell in prophase I was also observed with various markings that were silver-positive, small, rounded and dispersed throughout the cytoplasm. In the early stages of spermiogenesis, many argyrophilic granules could be seen that were strongly coloured and of different sizes (Figure 1f and 1g). They began to unite into a single vesicle, which was rounded and heavily stained (Figure 1h, k). In cells impregnated with silver ions, rounded structures of different sizes were observed; the structures united and formed a single structure that was also rounded and located adjacent to the MC (Figure 3c-h). Observation of the silver-impregnated sections revealed a more highly impregnated region inside the vesicle (Figure 3h-3m). As shown in Figures 1q, 1r and 3n, o longitudinal sections of the spermatid head were observed and showed the vesicle with markings. Toluidine blue staining indicated that the interior of the vesicle contained intensely stained filaments with an irregular morphology (Figure 1m and 1r). The organisational pattern of the axoneme was verified by transmission electron microscopy to have the 9+9+2 pattern (Figure 2j). As shown in Figure 1s-v, cysts containing sperms at different stages of development were observed.

Discussion

The literature regarding the ultrastructural aspects of *Heteroptera* spermatogenesis is extremely limited, yet understanding these aspects is of fundamental importance, because some structures cannot be analysed with light microscopy. In the species analysed in this study, argyrophilic granules were observed both by toluidine blue staining and by silver ion markings. These granules were present in the cytoplasm of prophase I and spermatids, and the granules came together to form a vesicle. Because these structures have not been described in other aquatic *Heteroptera*, more studies are necessary to determine the contents of these granules and their function. The vesicles observed in spermatids are most likely the nucleus and acrosome; however, it was not possible to determine the position of these structures in spermatids with the techniques used here. Further studies should be performed to determine accurate positions of the mentioned structures, both in spermatids and in sperm of *G. f. flavus*.

The acrosome, for example, was analysed in *Gerris najas* (Gerridae) [52], *Notonecta glauca* (Notonectidae) [53] and *Euchistus heros* [54], and it was observed that acrosome vesicles were formed from the Golgi complex. Changes in the form and degree of chromatin condensation were also observed. Initially, chromatin was dispersed and had a low electrodensity, but during the course of spermatogenesis, the chromatin assumed a compact fibril arrangement and developed a high electrodensity.

Acrosome enzymes are usually stored as proenzymes and are released and activated when the acrosome reaction occurs, thus participating in the event of fertilisation [55-58]. Souza and Itoyama et al. [59] analyzed the acromosomogensis in six species of *Heteroptera*, by means of Periodic Acid Schiff (PAS) and have found that, in general, the behavior of periodic acid Schiff-positive granules for all of the species analyzed is similar. In the beginning of spermiogenesis, there is a central granule that migrates to one of the extremities of the spermatid, and later, it becomes elongated and cannot be distinguished in the spermatozoa.

CBs of *G. f. flavus* were observed near the pore complexes of the nuclear envelope. This observation agrees with recent work in various species showing that CBs originate from the fragmentation of nucleolar material, such as, for example, in observations by Comings and Okada [27] and Andonov [29] in rats, by Anderson (1978) in the blue fox, by

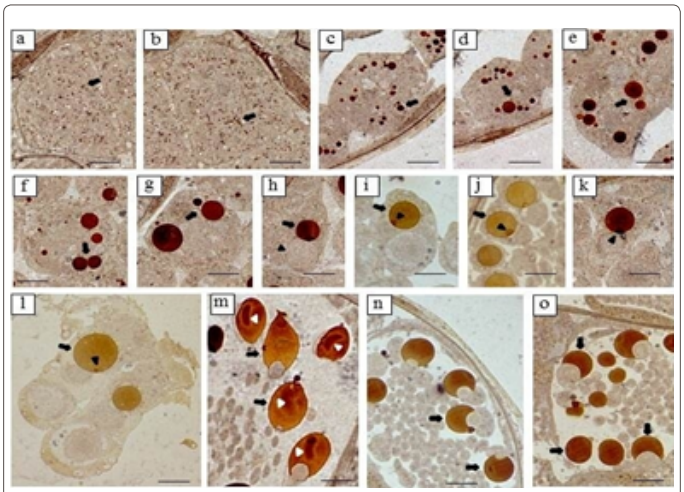

Figure 3: Semi-fine sections of *Gelastocoris flavus flavus* testicle impregnated with silver ions. **a,b)** Prophase I with several positive silver markings (arrows); **c-e)** spermatids with several silver-positive argyrophilic granules (arrows); **f,g)** spermatids showing silver-positive argyrophilic granules uniting and forming larger and rounded vesicles (arrows); **h)** spermatid with a silver-positive vesicle (arrow) and the mitochondrial complex (arrowhead); **i-m)** silver-positive vesicle (arrows) with stronger interior staining, which internalises with spermatid elongation (arrowheads); **n,o)** longitudinal section of the head of the spermatids, with a silver-positive vesicle (arrows). Bars: 10 µm.

Peruquetti et al. [30-32] in *Rattus novergicus, Mus musculus*, gerbils and amphibians, and in triatomines by Silistino-Souza et al. [34].

Initial analysis of CB in Hemiptera hematophagous suggest that the formation of this organelle starts in spermatocytogenesis [60]. Furthermore, the authors suggest that the nucleolar persistence phenomenon observed in these insects is essential for CB formation, which presents extreme importance during the spermiogenesis these vectors, since during this phase of spermatogenesis the nucleolus shows no transcriptional activity [61].

Regarding the axoneme, the flagellar pattern observed for *G. f. flavus* was 9+9+2 (9 accessories, 9 doublets and 2 central microtubules). Araujo et al. [19,62] observed that for some species of the Pentatomidae and Largidae family, the flagellum consists of an axoneme and two mitochondrial derivatives, and the axoneme follows a microtubule arrangement pattern of 9+9+2. This pattern was also described for other species of Heteroptera: Pentatomidae [63,64], Reduviidae [65,66], aquatic Heteroptera [67-69], Leptopodomorpha [70], Cimicomorpha, Gerromorpha, and Pentatomomorpha [19,71] and is thus the default for this suborder.

It is known that insect mutants may exhibit disorders in spermiogenesis [72,73] and that ambient conditions such as temperature, humidity and food availability may influence the normal process of spermiogenesis [74,75]. The presence of aberrant spermatids in insect spermiogenesis does not appear to be a rare event. Caetano [76], for example, described *Atta capigura* and *A. sexdens rubropilosa* (Formicidae) sperm with supernumerary elements. The supernumerary elements found in the *G. f. flavus* species may be caused by possible stress during collection, by the condition of the environment where they were collected or due to natural occurrence. However, more study is necessary to conclusively determine why occurs supernumerary elements during spermatogenesis.

Thus, this paper describes histological and ultrastructural characteristics during spermatogenesis, contributing to the reproductive knowledge of these aquatic *Heteroptera*.

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