ULTRASTRUCTURAL ASPECTS OF SPERMIOGENESIS AND SYNSPERMIA IN THE BROWN SPIDER *LOXOSCELES INTERMEDIA* (ARANEAE: SICARIIDAE)

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SHORT RUNNING TITLE
SPERMIOGENESIS AND SYNSPERMIA IN *Loxosceles intermedia*

ABSTRACT

This study reports ultrastructural and cytochemical aspects of spermiogenesis and synspermia in the brown spider *Loxosceles intermedia*. The roundish early spermatids are initially interconnected by cytoplasmic bridges, forming groups of four cells. During spermiogenesis, these cells pass through a series of modifications: (1) progressive nuclear condensation brings chromatin into a fibrillar arrangement; (2) the nucleus becomes long and asymmetric, with a short post-centriolar elongation; (3) formation of the long, cone-shaped acrosome and the F-actin acrosomal filament; (4) establishment of the implantation fossa and the 9×2+3 pattern flagellum, which extends away from the sperm cell body. Eventually, the entire cell undergoes twisting and folding resulting in a synspermium, containing four sperm cells in which the flagellum and nucleus are delimitated by plasma membrane, as individualized structures, but remain involved by the fused remaining cytoplasm and plasma membrane. Reaching the vas deferens, the synspermia are surrounded by a basic glycoproteic secretion. Synspermia are considered a derivative character, probably developed in this Sicariidae species, as well as in other Haplogynae, as an adaptation to improve the reproductive strategy.

1. INTRODUCTION

Ultrastructural differences of almost all sperm cell components, such as the acrosomal complex, acrosomal filament (also called the perforatorium) or nucleus shape, have been the focus of many investigations on spiders (Alberti, 1990).

Besides sperm ultrastructure, in spiders, the mode of organization and transfer of sperm cells also varies, with phylogenetic implications (Michalik et al., 2003, 2004a). The four sperm-transfer forms are present in haplogyne spiders: coenospermia, cleistospermia, spermatophore and synspermia. Coenospermia – many sperm cells surrounded by a secretion sheath – is considered a plesiomorphic character (Alberti, 1990, 2000; Michalik et al., 2003, 2004b). Synspermia, in which several fused sperm cells share a common cytoplasm enclosed by a membrane and surrounded by a secretion sheath, is considered a highly derivative character (Michalik et al., 2004a). This condition has been described for *Dysdera*, *Dasumia*, and *Harpactea* (Dysderidae), *Segestria* (Segestriidae) and *Scytodes* (Scytodidae) (Alberti and Weinmann, 1985; Alberti, 1990, Michalik et al., 2004a). Although synspermia occurs in all the cited families, some differences have been registered, e.g. the number of fused spermatozoa inside the synspermia is variable within the Dysderidae (two for *Harpactea* and three or four for *Dysdera*) and, for members of this family, large vesicular areas are seen during the cyst formation – a fact not observed in synspermia of other groups (Michalik et al., 2004a). The haplogyne group, Sicariidae, was cited by Alberti (1990) as presenting synspermia in the form of sperm transference. The author refers to unpublished data.

The brown spider *Loxosceles intermedia* Mello-Leitão 1934 (Araneae, Sicariidae) /Platnick, 2005/ is widely distributed in the urban environment of Curitiba, Southern Brazil. Aspects of its reproductive behavior, such as courtship and mating, were described by Fischer (1996), but no reference was made to morphological or ultrastructural details.

In the present study, ultrastructural aspects of spermiogenesis, sperm cells and transfer form are described for *L. intermedia*, using ultrastructural and cytochemical

techniques. These observations will lead to a better knowledge of the ultrastructure of sperm cells, as well as the precise arrangement of the synspermia in this species of Sicariidae. The data will contribute to the discussion of the phylogenetic implications and possible evolutionary significance of the peculiarities presented by these reproductive features.

2. MATERIAL AND METHODS

Adult males of *Loxosceles intermedia* were collected from houses in Curitiba, Paraná, Brasil ($25^{o}25'40''$ S/ $49^{o}16'23''$ W).

2.1. Light Microscopy

Specimens were etherized and dissected for isolation of the testes and vasa deferentia, which were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4, for 2 h at room temperature. The pieces were dehydrated using graded ethanol series and embedded in JB4 historesin. Sections of 5 μm were stained with hematoxylin–eosin and also submitted to PAS and ninhydrin–Schiff reactions for neutral carbohydrate and basic protein detection, respectively. Photomicrographs were taken with a Leitz photomicroscope.

For F-actin localization, testes and vasa deferentia portions were fixed in 4% paraformaldehyde prepared in PBS (2 h at room temperature), washed several times in PBS, permeabilized in Triton X–100 0.1%, and treated with TRITC–phalloidin (Sigma) and DAPI (4',6-diamidino-2-phenylindoledihydrochloride; Invitrogen–Molecular Probes). Samples were analyzed under a confocal microscope (Confocal Radiance 2,100; Bio-Rad, Hercules, CA, USA) coupled to a Nikon-Eclipse E800 with plan–apochromatic objectives (Sciences and Technologies Group Instruments Division; Melville, NY, USA). Micrographs were taken with the aid of the Laser Sharp program.

2.2. Electron microscopy

Animals were dissected and testes, ducts, ampulla and vasa deferentia (Fig. 1a) prefixed in 2.5% glutaraldehyde, 2% paraformaldehyde in 0.2 M cacodylate buffer pH 7.4 for 3 h at 4 ^{0}C.

2.2.1. Transmission electron microscopy

After pre-fixation and several washes in the same buffer, the samples were post-fixed in 1% OsO_4 (1 h at room temperature) and treated with 2% uranyl acetate. Tissues were dehydrated using a graded ethanol series and embedded in Spurr's resin. Semi-thin sections were placed on glass slides and stained with toluidine blue. Ultrathin sections (70 nm) were stained with 2% uranyl acetate and lead citrate. Semi- and ultrathin sectioning was done on a Leica ultracut ultramicrotome. Electronmicrographs were obtained using a JEOL-JE1200 EXII transmission electron microscope (operating at 80 kV) and GATAN-MULTISCAN 600 W software.

2.2.2. Scanning electron microscopy

After fixation, tissues were dehydrated in a graded ethanol series then critical-point dried. The dried testes and vasa deferentia were cut into small pieces and placed on a metallic holder, sputter-coated with gold and analyzed in a JEOL JSM-6360LV scanning electron microscope.

3. RESULTS

3.1. Male reproductive tract

3.1.1. Gross anatomy

Loxosceles intermedia testes consist of a long pair of cylindrical bodies, which extend between the genital opening and the spinneret base, ventrally in the opisthosoma (Fig. 1a). Extensions of the midgut gland surround the testes more dorsally making their localization difficult during dissection, when the incision is made at the dorsal side of the abdomen.

A thin duct followed by an ampulla connects the proximal portion of each testis to the corresponding vas deferens (Fig. 1a). The duct always appears empty, whereas the ampulla, the wall of which is composed of an epithelial secretory tissue, contains sperm cells and the product of secretion (data not shown). The vasa deferentia consist of very thin, long and coiled tubes that run anteriorly towards the lungs, turn and fuse to form an ejaculatory channel, which reaches the epigastric furrow lying more posteriorly (Fig. 1a). A single layer of epithelial cells with a secretory function forms the vas deferens wall (Fig. 5c). The secretion is PAS and ninhydrin–Schiff positive, indicating basic, glycoproteic composition. At the final vas deferens a secretion sheath is deposited around each synspermium unity (Fig. 6d), and the conjunct synspermia + secretion is released at copulation.

3.1.2. Testis histology

A single layer of flattened cells, rich in F-actin (data not shown), resembling myoid cells, covers each testis (Fig. 1b), the wall of which is composed of two kinds of cells: somatic and germinative lineage cells (spermatogonia, spermatocytes, spermatids and sperm cells). In contact to the basal lamina, somatic cells are large, irregular, with a basal nucleus and many extensions, between which lie the germinative lineage cells. Spermatogonia are present in the basal region of the germinative tissue and in contact with somatic cells. Spermatocytes are found in the mid layer (site of meiosis), while the spermatids and sperm cell cysts (with mid-

spermatids, late-spermatids, and sperm cells) are located at the ad-luminal position of this tissue (Fig. 1b).

3.2. Spermiogenesis

Early spermatids are spherical cells and appear in groups of four units, interconnected by cytoplasmic bridges (Fig. 1d). This cell aggregation is observed from the first division of meiosis, when the spermatocytes remain interconnected to each other (Fig. 1c). The spermatids have a large roundish nucleus, in which the chromatin is uniformly distributed in a thin sparkled granular arrangement (Fig. 1d).

Chromatin condensation occurs heterogeneously inside the spermatid nucleus and this process is concomitant to the change in shape of the nucleus and the whole cell (Figs. 1e–g). Condensation starts with chromatin being organized in a fibrillar pattern at the region where the implantation fossa (a posterior nuclear indentation) develops later (Fig. 1e). Simultaneously the spermatid nucleus changes in an asymmetrical way, resulting in an elliptical structure with a large posterior region, and a short postcentriolar elongation (Fig. 5a), which partially covers the proximal portion of the axoneme. Another nuclear indentation, which contains the acrosomal filament (Fig. 1g), develops at the anterior region, continuing as the nuclear channel. This is a long and thin channel that takes a twisted course around the periphery of the elliptical nucleus (Figs. 1e, f and 2b), extending towards the postcentriolar elongation. The acrosomal filament appears in transmission electron microscopy as a filamentous structure (Fig. 2c) and its F-actin composition is revealed by the TRITC-phalloidin treatment (Fig. 5a).

The condensed chromatin fibrils, which seem to extend from the inner nuclear membrane at the posterior region of the nucleus and where the nuclear channel runs, are in a parallel arrangement. The remaining chromatin initially shows a loose pattern (Fig. 1e) but, progressively, all the nuclear chromatin assumes a highly organized arrangement in which the fibrils are parallel and in close apposition (Fig. 1f) until complete condensation occurs (Fig. 1g). From the beginning of nuclear condensation

in early spermatids, a microtubular manchette surrounds the nucleus (Fig. 2a), disappearing at the end of spermiogenesis (Fig. 1g). A close correlation is observed between the position of the microtubules outside the nuclear membrane and the apparent point of contact between chromatin fibrils and the inner nuclear membrane (Fig. 2b).

The centrioles, oriented at right angles, are seen in early spermatids near the Golgi apparatus (data not shown). During spermiogenesis, the centrioles migrate posteriorly where they occupy the forming implantation fossa, and eventually are organized in tandem position (Fig. 2e). The *L. intermedia* axoneme, which shows the $9 \times 2+3$ pattern, typical for spiders, (Fig. 2d) originates from the distal centriole, emerging from the implantation fossa, and extending away from the cell body (Fig. 2e). The three central microtubules emerge from the central portion of the flagellar basal body, which external tubules are continuous with the nine peripheral pair of microtubules of the axoneme (Fig. 2f). During the change in shape of the spermatid nucleus close to the implantation fossa region, many vesicles are present and coalesce to form the flagellar tunnel, which holds the proximal flagellum (Fig. 2e). No mitochondria are seen around the proximal portion of *L. intermedia* axoneme (Figs. 2e, f and 3b).

3.3. Synspermia

Synspermium aggregation in *L. intermedia* occurs, concomitantly to spermiogenesis, inside the testicle germinative tissue (Figs. 3a–e). During differentiation, early spermatids, initially connected by narrow cytoplasmic bridges and surrounded by extensions of the somatic cells (Figs. 1d and 3a), become closer and pass through a consolidation of the fusion process, resulting in one syncytium composed of four cells. The spermatids progressively change their morphology: the cytoplasmic bridges seem to widen (Fig. 3a) and, while the acrosomal and flagellar regions become individualized by the cytoplasmic membrane of each cell (Figs. 2c, e and 3b), the remaining cytoplasm accumulates in large quantity between the partially

individualized portions of the late spermatids (Fig. 3b). This individualization is maintained even in mature synspermia as the ones observed in the vasa, as clearly shown by scanning electron microscopy images (see Figure 6b). Comparing this with Figs. 3b and 6a, which show the synspermium still in the testicular lumen, we observe that the head and tail of the sperm cells are individualized in both situations. The plasma membrane covering the inside structures doesn´t allow the visualization, by SEM, of the acrosome over the nucleus but the shape clearly corresponds to the inferred from ultrastructural analysis. The synspermia are seen as round structures lacking the remaining cytoplasm (Fig. 6b) removed by dessication and fracturing of the material during preparation for SEM. Fig. 4 represents a model for the arrangement of synspermia, based on ultrastructural details obtained in this work. In Fig. 4a we depict the synspermium as the late spermatids at the end of spermiogenesis, showing the remaining cytoplasm connecting each cell and thus maintaining the syncitial arrangement. The enlarged cell bridges are at the posterior region of the cells (see Fig. 3 b). This situation remains in the mature synspermium, with just some folding and twisting of the individualized heads and tails of the sperm cells, that bring all the components closer, with the remaining cytoplasm occupying the periphery and involving all the individualized structures. Those remain delimitated by plasma membrane as represented in Fig. 4b based on evidence observed in synspermia as in Figs. 3d, 3e, and 3f. Fig. 4c represents the 3D image of the synspermium as suggested by SEM images.

The remaining cytoplasm is neither expelled nor phagocytosed by the somatic cells, but finally surrounds the main components of the late spermatids at the end of spermiogenesis, resulting in a synspermium (Figs. 3c, e, f), in which the cells are partially individualized but still contain a relatively large amount of common cytoplasm surrounded by a common plasma membrane. The sperm cells change their shape, in which the entire cell body twists slightly, the acrosome region folds and the remaining common cytoplasm encircles the whole conjunct. A lateral twist allows the late spermatids to be organized, as a group, in a round arrangement, in which the cells are in close apposition (Figs. 4c and 6a), still connected to each other through the

common cytoplasm around them. The acrosomes are folded to the inside of the conjunct and the flagella are accommodated laterally to each cell body, in a sinuous arrangement. Sections of synspermia show clearly the presence of plasma membrane around the axoneme, evidence that the flagellum sectioned many times due to spiralization, is still individualized (Fig. 3d). Evidence that the nuclei are also individualized and have not coiled into the cytoplasm is the presence of the plasma membrane delimitating each of them and the corresponding acrosome (Figs. 2c and 3e). The cytoplasm surrounding nuclei and sections of flagella corresponds to folds of the remaining cytoplasm and its plasma membrane. At the periphery of the synspermium, the remaining cytoplasm containing vesicles, inclusions, PAS positive components and some mitochondria is evident (Figs. 3c, e, f).

Synspermia are released into the testicle lumen, where they are surrounded by a light PAS positive secretion (Fig. 5b), and then carried on to the ampulla (data not shown) and the vas deferens (Figs. 5c and 6b, c, and d), where they are immersed in a basic glycoprotein secretion (Fig. 5c). At the distal portion of the vasa deferentia each synspermium is surrounded by a multilayered secretion sheath apparently secreted by the epithelial cells (Fig. 6d).

4. DISCUSSION

The gross anatomy of the genital apparatus of *L. intermedia* is partially in agreement with descriptions of other groups of Araneae (Bücherl, 1951, for specimens of the genus *Grammostola*; Michalik et al., 2003, for *Wandella orana* (Filistatidae); Michalik et al., 2004a for *Dysdera crocata, D. erythrina, D. ninnii, Harpactea arguta, H. piligera, Dasumia taeniifera* (Dysderidae)).

In *L. intermedia*, the paired testes are non-coiled and tubular structures connected to the vas deferens by a thin duct, followed by an ampulla. These structures have not been described for other species of spiders and are probably species-specific characteristics. The ampulla and the vas deferens epithelia produce a glycoproteic

secretion that surrounds the synspermia and has certainly a function in maintaining synspermia integrity, once they are retained inside the male genital tract for an undetermined period of time, until copulation.

Chromatin condensation occurs heterogeneously from the flagellum to the acrosome, from the posterior to the anterior region of the cell, as described for *W. orana* (Michalik et al., 2003). The chromatin fibrils extend from the inner nuclear membrane, as described for *Pisaurina* sp. by Reger (1970), initially in the basal indentation and then also from the nuclear channel internal membrane. In *L. intermedia,* chromatin condensation occurs in a very organized form, resulting in apposed fibrils, disposed parallel to the long axis of the nucleus. In a number of studies (Michalik et al 2004a; Alberti and Weinmann, 1985), late spermatid nuclei are shown with some nucleoplasmic spaces inside the condensed chromatin, a fact not observed in *L. intermedia*.

During chromatin condensation a microtubular manchette surrounds the *L. intermedia* nucleus. A close correlation between the positioning of the chromatin fibrils at the periphery of the nucleus and the microtubules outside is observed in our preparations. The role of microtubules in chromatin condensation might be explained by their function as a cytoskeleton, i.e. either a mechanical influence on the elongation of structures (Dallai, 1970; Kato et al., 2004), or a relation between the microtubular manchette and the nuclear architectural organization in differentiating and maturating cells (Baluska et al., 1997). However, the molecular aspects of the relationship between chromatin and microtubules in *L. intermedia* spermiogenesis are still obscure.

The acrosomal filament, a common structure in invertebrate sperm cells, is formed and positioned concomitantly to the nuclear channel and acrosome establishment in *L. intermedia.* During nuclear shaping, this component, composed of F-actin, is positioned in a spiral form inside the nuclear channel. It also extends inside the subacrosomal space, protruding toward the acrosome and forcing it to assume a cone shape, as described for other spiders (Alberti, 1990). In *L. Intermedia,* F-actin is already present at the completion of spermatogenesis as evidenced by labeling with phalloidin, a small and stable compound with strong F-actin binding affinity

(Verderame et al, 1980). A wide variety of species have globular-actin in the acrosome complex that changes the configuration to F-actin at fecundation (De Rosier and Tilney, 1991), such as in sea urchin (Colwin and Colwin, 1963). There is still no detailed description of the fecundation process in spiders and, thus, no suggestion of a possible role for the acrosomal filament in it.

The post-centriolar elongation, resulting from asymmetrical elongation of the spermatid nucleus, overlaps the proximal portion of the axoneme in *L. intermedia* as a short and broad structure. This contrasts to the description for other species of spiders (Bücherl, 1951; Alberti and Weinmann, 1985, for Dysderidae, and Oonoppidae, and Scytodidae species; Michalik et al., 2003, for *W. orana;* Michalik et al., 2004a, for Dysderidae spiders) as being a very long, coiled structure, which projects from the nuclear body and runs alongside the proximal portion of the axoneme.

L. intermedia sperm cell shows a 9×2+3 flagellar pattern, common for spiders (Rosati et al., 1970), in which inner and outer dynein arms are present. It is known that these structures, formed by dynein molecules, are dependent on ATP energy sources for flagellar movement (Gibbons, 1981). An interesting fact, however, is the absence of mitochondria in the flagellar region, reported to occur only in Mesothelae sperm cells (Rosati et al., 1970), at the middle piece. There is no evidence of the metabolic pathway used by the sperm cells of *L. intermedia* to obtain energy for movement. Mitochondria observed in the periphery of the remaining cytoplasm do not appear to be a possible source of energy for flagellar movement, due to their position away from the flagellar proximal region.

Sperm cell aggregation in specialized transfer forms – synspermia, coenospermia, and cleistospermia – is common within Araneae (for a review, see Alberti, 1990, 2000). These structures represent phylogenetic characteristics within spider families, and synspermia is considered an apomorphic character (Alberti, 1990, 2000; Michalik et al., 2004a). Synspermia is reported to be unique to spiders and is shown by members of different haplogyne families: Scytodidae, Segestriidae (Alberti and Weinmann, 1985), Dysderidae (Alberti and Weinmann, 1985; Michalik et al., 2004a), and Sicariidae (Alberti, 1990, cited as unpublished data). Although synspermia

may be a synapomorphic character for those groups, differences between the number of cells in the synspermium and the occurrence or not of large vesicles were reported. In *L. intermedia*, four spermatid forms, resulting from the maintenance of cytoplasmic bridges after the first meiotic division, are present in each synspermium. Michalik et al. (2004a) reported the involvement of vesicular areas in synspermia formation in Dysderidae spiders. The occurrence of vesicular areas and their involvement in the organization of the synspermium is not conspicuous in *L. intermedia.*

Alberti (1990) defines synspermia as a condition in which several spermatids are fused in a syncytium and surrounded by a common sheath. In *L. intermedia* synspermia, the general appearance is similar to that described above and comparable to observations on other haplogyne spiders on the aspect of cell aggregation. We suggest, based on ultrastructural evidences, that the arrangement of the cells in the synspermium is different in *L. intermedia* comparing to other species. The coiling of nucleus and axoneme described for *S. senoculata* (Alberti & Weinmann, 1985), resulting in a retraction of these structures into the cytoplasm and the appearence of vesicles representing the empty membranes, is not seen in *L. intermedia*. Rather, the presence of plasma membrane surrounding the axoneme, nucleus and the acrossome even in the mature synspermia observed in the distal vasa, are strong evidence that parts of each sperm cell remain partially individualized even after twisting and folding. Our model predicts that synspermium formation in *L. intermedia* involves the progressive expansion of the remaining cytoplasm and cytoplasmic bridges between the spermatids, which are undergoing spermiogenesis. The remaining cytoplasm of each spermatid, which is neither expelled nor phagocytosed by the somatic cells, surrounds the entire conjunct. During this process, the spermatids remain individualized in their acrosomal and flagellar extremities by the cytoplasmic membrane of each cell.

We propose that the organization of the remaining cytoplasm is as described above, and partial individualization of the sperm cells is the most probable arrangement in *L. intermedia.* The role of all the remaining cell components is not understood and all explanations are still highly speculative. We suggest the possibility

that the remaining cytoplasm surrounding the sperm cells might serve as protection during transference to the female, being an easily disposable material when fertilization signals are released.

A general view of spermiogenesis, sperm cell characteristics and a model for the synspermium formation are described in the present work for *L. intermedia, a* spider of the Sicariidae family, for which no detailed description of these features has been previously published. Our findings demonstrated that this species presents sperm cells with ultrastructural features considered plesiomorphic characteristics for haplogyne spiders (Alberti, 1990; Michalik et al., 2003): a thin, long cone-shaped acrosomal vesicle that covers the subacrosomal space and houses the acrosomal filament, which extends inside the nuclear channel to the postcentriolar elongation. The occurrence of the highly derivative character synspermia suggests that *L. intermedia* has, as other Haplogynae, evolved an efficient reproductive strategy by developing a special sperm cell transfer form. This could not only protect these cells from environmental hazards but also prevent premature capacitation, delaying exposure in the female environment, maintaining synchrony with female reproductive physiology and, thus, improving reproductive success.

Further investigations are being conducted on the final transformation of sperm cells, when stored in the spermatheca and released at fertilization, to better understand the benefits and advantages of this form of sperm cell transfer.

ACKNOWLEDGMENTS

REFERENCES:

Alberti, G., 1990. Comparative spermatology of Araneae. Acta Zoologica Fenica 190, 17-34.

Alberti, G., 2000. In: Jamieson, B.G.M., (Ed.), Chelicerata, Progress in Male Gamete Ultrastructure and Phylogeny, vol. 8. Oxford and IBH Publishing Co., pp. 311 – 388.

Alberti, G., Weinmann, C., 1985. Fine structure of spermatozoa of some Labidognath spiders (Filistatidae, Segestriidae, Dysderidae, Oonopidae, Scytodidae, Pholcidae; Araneae; Arachnida) with remarks on spermiogenesis. Journal of Morphology 185, 1-35.

Baluska, F., Volkmann, D., Barlow, P.W., 1997. Nuclear components with microtubule-organizing properties in multicellular eukaryotes: functional and evolutionary considerations. International Review of Cytology, A survey of Cell Biology, Kwang W. Jeon (Ed), 175, 91-135.

Bücherl, W., 1951. Estudos sobre a biologia e a sistemática do gênero *Grammostola* Simon, 1892. Monografias do Instituto Butantan, São Paulo, Brasil.

Colwin, A.L., Colwin, L.H., 1963. Role of the gamete membranes in fertilization in *Saccoglossus kowalevskii* (Enteropneusta): I. The acrosomal region and its changes in early stages of fertilization. The Journal of Cell Biology 19, 477-500.

Dallai, R., 1970. The spermatozoon of arthropoda XI. Further observations on Collembola. In: Comparative spermatology. Edited by Baccio Baccetti. Proceedings of the International Symposium, held in Rome and Siena, 1-5 July 1969 Accademia Nazionale Dei Lincei – Rome Academic Press, New York, London, pp 275-280.

De Rosier, D.J. de, Tilney, L.G., 1991. The role of actin and actin-binding proteins in the extension of the acrosomal process of *Limulus* sperm. In: Baccetti, B., 1991. Comparative spermatology 20 years after. Serono Symposia Publications form Raven Press, volume 75, pp 149-154.

Fischer, M.L., 1996. Biologia e ecologia de *Loxosceles intermédia* Mello Leitão, 1934 (Araneae, Sicariidae) no município de Curitiba, Paraná. Curitiba, 1996. Dissertação

(Mestrado em Zoologia) – Setor de Ciências Biológicas, Universidade Federal do Paraná.

Gibbons, I.R., 1981. Cilia and flagella of eukaryotes. The Journal of Cell Biology 9, 107s-124s.

Kato, A., Nagata, Y., Kazuo, T., 2004. δ-tubulin is a component of intercellular bridges and both the early and mature perinuclear rings during spermatogenesis. Developmental Biology 269, 196-205.

Michalik, P., Gray, M.R., Alberti, G., 2003. Ultrastructural observations of spermatozoa and spermiogenesis in *Wandella orana* Gray, 1994 (Araneae: Filistatidae) with notes on their phylogenetic implications. Tissue and Cell 35, 325-337.

Michalik, P., Dallai, R., Giusti, F., Alberti, G., 2004[a]. The ultrastructure of the peculiar synspermia of some Dysderidae (Araneae, Arachnida). Tissue and Cell 36, 477-460.

Michalik, P., Haupt, J., Alberti, G., 2004[b]. On the occurrence of coenospermia in mesothelid spiders (Araneae: Heptathelidae). Arthropod Structure & Development 33, 137-181.

Platnick, N.I., 2005. The world spider catalog, version 6.0. American Museum of Natural History, online at http://research.amnh.org/entomology/spiders/catalog/index.html.

Reger, J.F., 1970. Spermiogenesis in the spider *Pisaurina* sp.: a fine structure study. Journal of Morphology 130, 421-434.

Rosati, F., Baccetti, B., Dallai, R., 1970. The spermatozoon of arthropoda X. Araneids and the lowest myriapods. In: Comparative spermatology. Edited by Baccio Baccetti. Proceedings of the international Symposium, held in Rome and Siena, 1-5 July 1969 Accademia Nazionale Dei Lincei , Rome Academic Press, New York, London, pp 247-254.

Verderame, M., Alcorta, D., Egnor, M., Smith, K., Pollack, R., 1980. Cytoskeletal F-actin patterns quantitated with fluorescein isothiocyanate-phalloidin in normal and transformed cells. Proceedings National Academy Sciences USA 77(11), 6624-28.

FIGURES:

Figure 1. (a) Schematic representation of a ventral portion of a male *L. intermedia* abdomen showing organization of the reproductive apparatus. (b) Brown spider testicle in cross section, H&E staining; scale bar = 57 μm. (c) Second meiosis division, showing the spermatocytes in anaphase, H&E staining; scale bar = 21 μm. (d) Early spermatids connected by a cytoplasmic bridge; scale bar = 1.18 μm. (e) Mid spermatid; posterior region; tangential section; the chromatin is already in a fibrillar arrangement, but still at a low level of condensation; scale bar = 0.71 μm. (f) Cross section of mid-to-late spermatid nucleus at anterior region; the chromatin is in an advanced degree of condensation; scale bar = 0.39 μm; inset: chromatin fibrils in parallel arrangement.(g) Late spermatid in longitudinal section, showing the fully condensed chromatin; scale bar = 0.83μm. A – ampulla, AC – acrosome, arrow – microtubular manchette, arrowhead – myoid cell, * - somatic cell extension, B – cytoplasmic bridge, C – spermatocytes, CH – chromatin, D – duct, EC – ejaculatory channel, EF – epigastric furrow, empty arrow – acrosomal filament, i – implantation fossa, L – lung (showed as a point of reference), ST – spermatids, T – testicle, TL – testicular lumen, VASA – vasa deferentia.
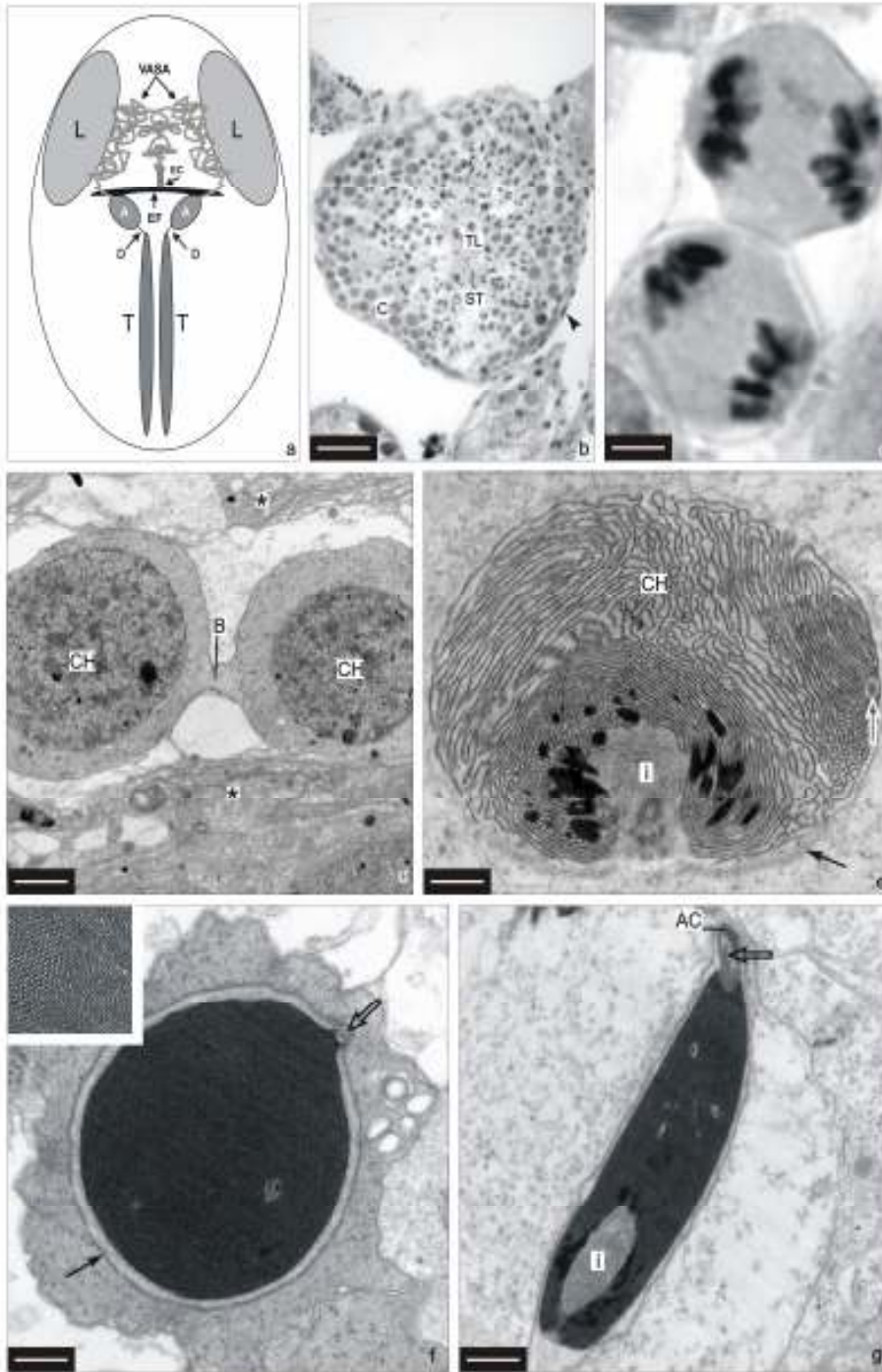
Figure 2. (a) Early spermatid with the microtubular manchette already positioned around the nucleus; scale bar = 0.31 μm. (b) Mid-to-late spermatid: note chromatin fibrils organization and microtubules positioning outside the nucleus; scale bar = 0.2 μm. (c) Late spermatids with condensed chromatin; the spermatid at the right is in longitudinal section showing the acrosomal complex, composed by the flattened acrosome and the acrosomal filament inside the subacrosomal space; the left spermatid is sectioned at the postcentriolar elongation level; in the center several sections of flagellum are seen (note the plasma membrane); scale bar = 0.32 μm. Flagellum in cross section: pattern 9 x 2 + 3 (note the plasma membrane); scale bar = 0.05 μm. (e) Posterior region of spermatid with the implantation fossa containing the flagellar basal body (circle) and tunnel formation. Notice the absence of mitochondria in the proximal portion of the flagellum; scale bar = 0.71 μm. (f) Mid spermatid with the flagellar basal body, in longitudinal section, inside the implantation fossa; scale bar = 0.24 μm. AC – acrosome, arrow – microtubular manchette, arrowhead – individualized flagellum, CH – chromatin, empty arrow – acrosomal filament, F – flagella, G – Golgi apparatus, i – implantation fossa, MT – central microtubules, NC – nuclear channel, PM – plasma membrane.
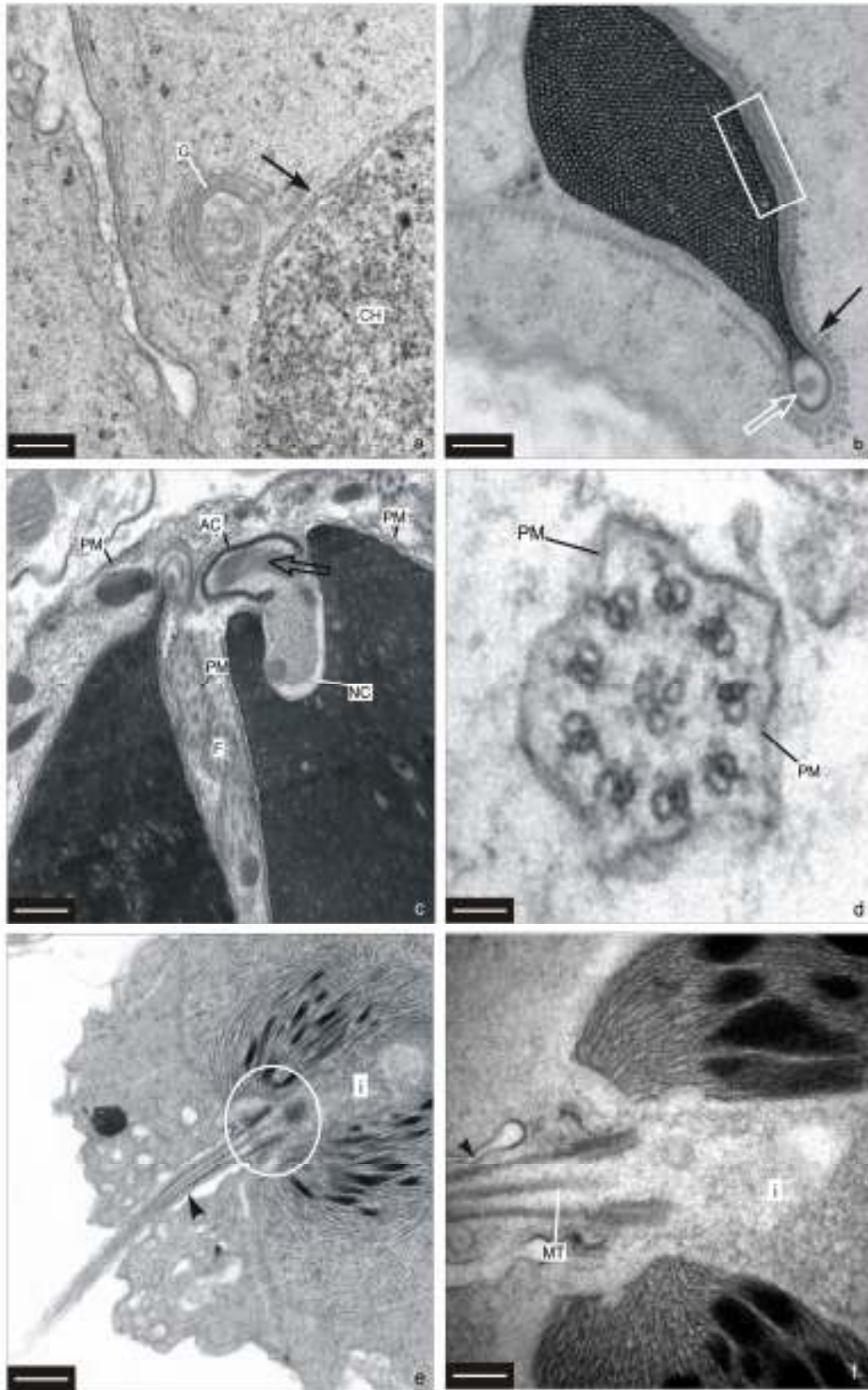
Figure 3. Formation of synspermium. (a) Tree spermatids connected by cytoplasmic bridges (detailed in the inset). Notice that section containing the bridge is at the level of the posterior portion of the cell, where the implantation fossa is located; scale bar = 1.82 μm. (b) A group of spermatids during spermiogenesis. The central spermatid is in longitudinal section; scale bar = 2.1 μm. (c) Synspermium containing late spermatids; scale bar = 1.16 μm. (d) Detail of the synspermium showed in Figure 3c. Note the plasma membrane in the nuclear posterior region, and around the flagellum; scale bar = 0.24 μm. (e) Mature synspermium with the partially individualized sperm cells and remaining cytoplasm; scale bar = 0.75 μm. (f) Mature synspermium containing 4 sperm cells. Mitochondria are seen in the remaining cytoplasm; scale bar = 0.94 μm. Arrow – inclusions at the periphery of the remaining cytoplasm, arrowhead – individualized flagellum, * - somatic cell extension, B – cytoplasmic bridge, E – postcentriolar elongation, F – flagellum, i – implantation fossa, M – mitochondria, PM – plasma membrane, RC – remaining cytoplasm.
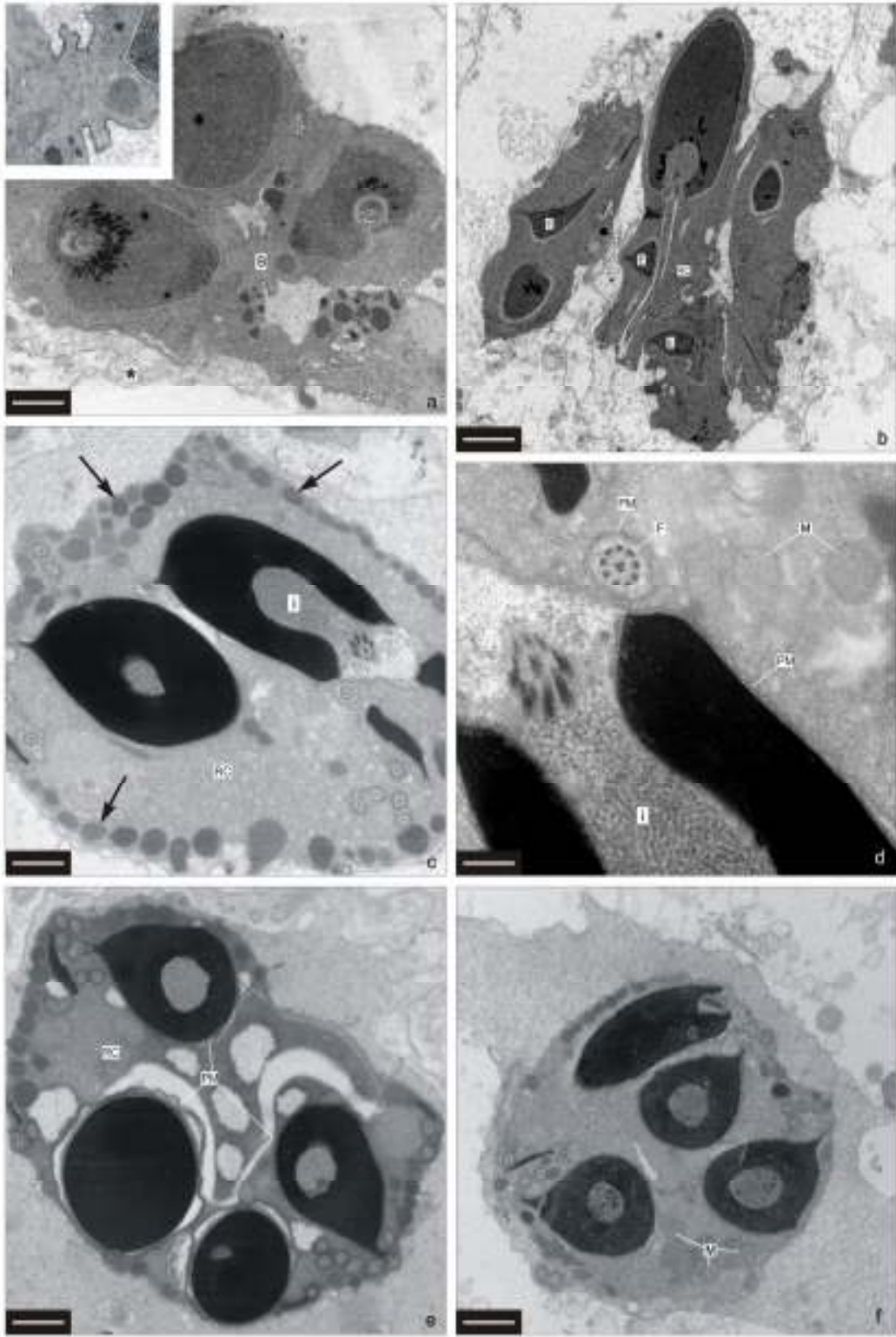
Figure 4: Schematics representing the proposed model for the arrangement of the synspermium in *L. intermedia*, based on the ultrastructural details shown in Figs. 3 and 6. (a) Sperm cells at the end of spermiogenesis, connected through cytoplasmic bridges and sharing the common remaining cytoplasm. The dotted lines indicate the points where the common plasma membrane is continuous in the round synspermium, represented flat in this figure. (b) Drawing of a section of a mature synspermium represented without the secretion around it. Four nuclei are sectioned at different levels due to the twisting. In white the extracellular space is seen around the anterior region of each cell and sections of the flagella. (c) 3D representation of a synspermium. The individualized heads and tails are represented in external view, covered by the plasma membrane. In black the remaining cytoplasm with organelles partially represented is being removed to show the individualized portions of the cells. AC – acrosome, arrow – inclusions at the periphery of the synspermium, B – cytoplasmic bridge, E – postcentriolar elongation, empty arrow – nuclear channel, F – flagellum, i – implantation fossa, M – mitochondria, N – nucleus, PM – plasma membrane, RC – remaining cytoplasm, Z – sperm cell.
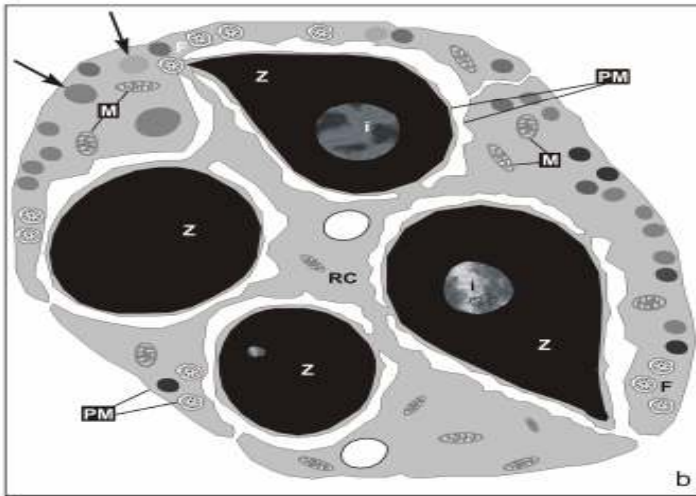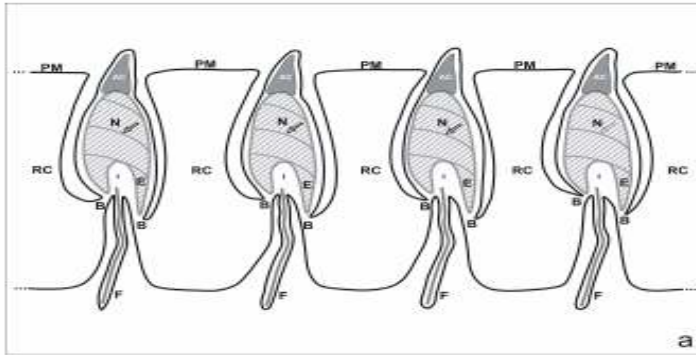
a

b

c

Figure 5. (a) Merged photomicrograph of spermatids submitted to TRITC-phalloidin treatment and DAPI staining. The reaction revealed the presence of F-actin in the acrosomal filament inside the nuclear channel and in the subacrosomal space; scale bar = 2.85 μm. (b) Central portion of *L. intermedia* testis in cross section, PAS reaction. The small testis lumen presents a PAS positive secretion. The synspermia, containing PAS positive components, are seen in the lumen; scale bar = 11.67 μm. (c) Vas deferens in cross section, PAS reaction. The secretory epithelial cells contain PAS positive granules and synspermia are seen in the lumen, involved by the PAS positive secretion; scale bar = 17.15 μm. E – postcentriolar elongation, EC – secretory epithelial cells, empty arrow – acrosomal filament inside the nuclear channel, empty arrowhead – acrosomal filament inside the subacrosomal space, N – sperm cell nucleus, S – synspermia, TL – testicular lumen.
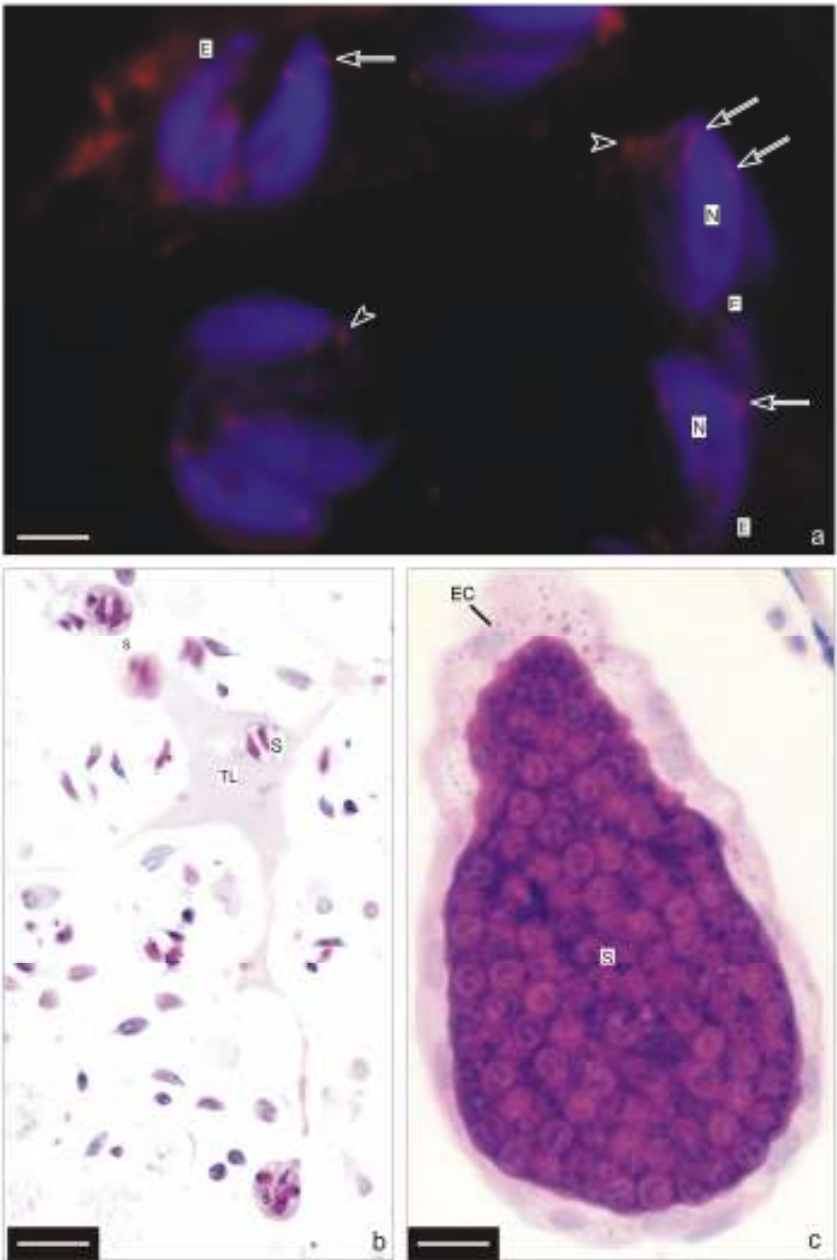
Figure 6. (a) Scanning electronmicrograph showing a mature synspermium, still inside the testis, containing four sperm cells, in the same situation as in Fig. 3d. The common plasma membrane and cytoplasm were partially removed; scale bar = 2.31 µm. (b) Scanning electronmicrograph showing a vas deferens in cross section. The secretion is uniformly distributed around the synspermia; scale bar = 10µm. (c) Transmission electronmicrograph showing mature synspermia inside the proximal vas deferens lumen. The secretion is uniformly distributes around the synspermia; scale bar = 2.2 µm. (d) Transmission electronmicrograph showing mature synspermia inside the distal vas deferens lumen. The secretion sheaths that surround the synspermia are produced by the secretory epithelial cells of the vasa; scale bar = 2.2 µm. Arrow – secretion, arrowhead – flagellum, * vasa secretion, EC – secretory epithelium, S – synspermia, SH – secretion sheath, Z – sperm cell.