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ULTRASTRUCTURAL, HISTOLOGICAL AND HISTOCHEMICAL CHARACTERISTICS OF THE EPITHELIAL WALL OF THE SEMINAL VESICLE OF MATURE *Scaptotrigona xanthotricha* MOURE MALES (HYMENOPTERA, APIDAE, MELIPONINI)

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ABSTRACT

The seminal vesicles of mature *Scaptotrigona xanthotricha* males were investigated using light microscopy, histochemistry and transmission electron microscopy. The globular seminal vesicles were ~450 μm in diameter and consisted of a sperm-filled lumen and a single layer of epithelium surrounded externally by a muscular sheath. The mitochondria-rich epithelial cells had many inclusions in the basal region. These inclusions were relatively large and contained membranous structures similar to myelin figures. The epithelial cells of the seminal vesicle showed none of the features characteristically associated with a secretory function, which suggested that the material in which the spermatozoa were immersed in the vesicle lumen was produced elsewhere along the ducts and/or during sexual maturation of the males. Spermatozoa were occasionally seen inside the inclusions, which suggested a possible spermiophagic activity for this epithelium.

Key words: Morphology, myelin figures, reproductive system, stingless bees

INTRODUCTION

In insects, the male reproductive system consists of a pair of testicles connected by ducts to a gonopore. In many groups, including the Hymenoptera, there are regions along the ducts, such as the seminal vesicles and the secretory accessory glands, where spermatozoa are stored until mating. The secretions of the accessory glands vary in chemical composition and function among different insect groups. In bees, the secretions consist basically of proteins, but may also include sugars and lipids [3,6-8,10,24]. These substances are added to the sperm and affect all phases of the reproductive biology of the mated female, including the protection, storage and activation of sperm, competition among sperm, inhibition of receptivity and enhancement of fecundity [2,3,6-8,10,11,13,16,19,24,28,31,35,36,38,39].

Some insects, such as stingless bees [12,20,27] and sandflies, have no accessory glands, while in others the epithelial cells of the seminal vesicle or of the ejaculatory duct have the organelles typically associated with a secretory function. The products of these epithelial cells have the same function as those of the accessory glands [9,13,16,19,37]. In other insects, the seminal vesicle apparently produces no secretion [15,32].

In only one species of stingless bees, *Melipona bicolor bicolor*, have the ducts (including the seminal vesicle) associated with the reproductive tract been studied in detail, with the conclusion that the seminal vesicle of this species showed no secretory activity [17]. To determine whether the anatomical arrangement and lack of secretory activity seen in *M. b. bicolor* are characteristic of other meliponine genera, in this study we examined the histology, ultrastructure and histochemistry of the seminal vesicle of mature *Scaptotrigona xanthotricha* males.

MATERIAL AND METHODS

Eight sexually mature males of *Scaptotrigona xanthotricha* were obtained from colonies maintained in the Central Apiary of the Federal University of Viçosa, Viçosa, MG, Brazil. The males were collected from agglomerations formed near their colony; these agglomerations are typical of this species during its reproductive period and serve to identify sexually mature males.

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Light microscopy

The seminal vesicles were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, and post-fixed in 1% OsO_4. The tissue fragments were dehydrated and embedded in Historesin®. Semi-thin sections were stained with toluidine blue and photographed. To reveal finer details of the nucleus, some of these preparations were stained for 15 min with 0.2 μg of 4,6-diamino-2-phenylindole (DAPI)/ml in phosphate-buffered saline and then examined with an epifluorescence microscope (Olympus, BX-60) equipped with a BP360-370 nm excitation filter.

Histochemistry

The seminal vesicles were fixed in 2.5% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, and embedded in Historesin®. Semi-thin sections were processed for the histochemical detection of proteins with bromophenol blue [33], polysaccharides with PAS [26] and acid phosphatase activity [4]. For the latter assay, sections were incubated for 6 h in 0.32% sodium β-glycerophosphate and 2% lead nitrate in 0.05 M acetate buffer, pH 5, followed by incubation with 1% ammonium sulfide, and then washed and counterstained with hematoxylin. To test for alkaline phosphatase activity, sections were incubated for 6 h in 0.05% veronal buffer, pH 9.2, containing 0.5% sodium β-glycerophosphate, 1% lead nitrate and 0.025% manganese chloride. The sections were subsequently treated with a 2% cobalt nitrate solution, washed, immersed in 1% ammonium sulfide and counterstained with hematoxylin. The phosphatase activity tests were adapted from Gomori´s method [4]. Control sections were processed in parallel in all of the tests.

Transmission electron microscopy (TEM)

The seminal vesicles were fixed for 3 h in 0.1 M cacodylate buffer, pH 7.2, containing 2.5% glutaraldehyde, 0.2% picric acid, 3% sucrose and 5 mM $CaCl_2$. The tissues were post-fixed in 1% OsO_4, dehydrated in acetone and embedded in Epon 812. Ultrathin sections were stained with 0.1% uranyl acetate and 0.01% lead citrate and were observed in a Zeiss Leo 906 transmission electron microscope operated at 60 kV.

RESULTS

The internal reproductive system of mature *S. xanthotricha* males consisted of a pair of degenerated testes with an extremely reduced volume and a pair of deferent ducts that emptied into the ejaculatory duct. In the deferent duct, a region proximal to the testis expanded to form the seminal vesicle that was spherical in shape and measured ~370 μm in diameter (Fig. 1A). This vesicle consisted of an external wall with a simple epithelium adhering to the basement membrane, followed by a muscular layer. The lumen contained freely swimming spermatozoa (Fig. 1A,B). The seminal vesicle did not vary in shape, size or structural organization among the males investigated.

Seminal vesicle epithelium

In mature males, the epithelium of the seminal vesicle was relatively thin and consisted of a monolayer of cubical cells (Figs. 1B and 2A) adhering to a thick basement membrane (arrow in Figs. 2C and 3A,C). These epithelial cells were polarized and the luminal surface was differentiated into microvillar-like projections (asterisk in Figs. 2A and 4B and arrows in Fig. 4C) that were frequently seen in contact with the spermatozoa (Figs. 2A,D and 4C). This epithelium also showed desmosome- and hemidesmosome-like junctions (square in Figs. 2C, 3A and 4B), and septate junctions which were more evident at the membrane interdigitations (Fig. 3D).

The apical third of the epithelial cells was especially rich in mitochondria (Fig. 2A) with an electron dense matrix and deep cristae (Figs. 3C and 4C). The middle and basal thirds contained the usual cellular organelles, including some mitochondria, the nucleus and prominent inclusions (see Figs. 2B, 3A-C and 4A,B). The nucleus, which occurred in the middle portion, varied from circular to irregular in shape, and sometimes appeared to be slightly compressed by the inclusions (Figs. 1B, 2A,C, 3C and 4B). The nuclear location and shape were clearly observed with DAPI staining (arrowheads in Fig. 1C).

The vesicular-like inclusions occupied the middle and basal portions of the cell, generally basal to the nucleus, and were clearly distinguished by light (Figs. 1A-B and 4A) and electron (Figs. 2A-D, 3A-C and 4B) microscopy. The origin of these inclusions is unclear, although the larger inclusions apparently grew by the association and fusion of smaller inclusions (asterisks in Fig. 2C). The inclusions varied in size (Fig. 2A,C), and sometimes occupied most of the cytoplasm (Fig. 2D). The content of the inclusions also varied in aspect, with light microscopy showing an opaque heterogeneous content (Figs. 1B and 4A). TEM revealed inclusions containing loose filaments that, in some cases, formed multilayered myelin-like figures (Fig. 3A-C), and frequently contained dense corpuscles and spermatozoa (arrows in Fig. 3A,B). The content of these inclusions was osmium-positive, but the histochemical tests for proteins, carbohydrates (PAS)

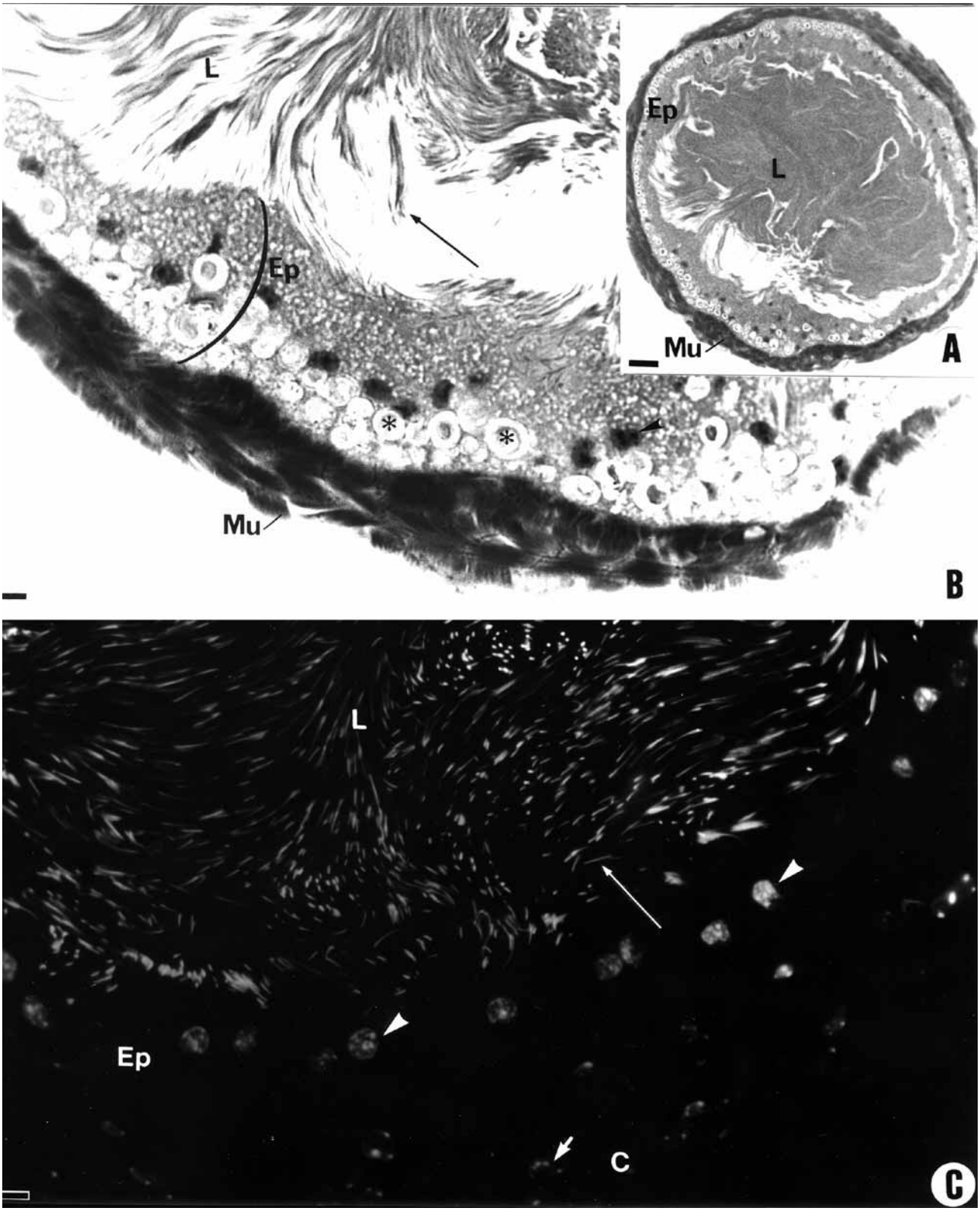

Figure 1. A-B. Light microscopy of a seminal vesicle showing the luminal region (**L**) filled with sperm (**arrow**), the epithelium (**Ep**) and the muscular sheath (**Mu**). The asterisks indicate the epithelial inclusions and the arrowhead indicates the nucleus. **C.** Fluorescence microscopy showing a DAPI-stained nucleus in the same region as panel **B**. Note the spermatic nuclei (**arrow**) in the lumen (**L**), the epithelial cell nuclei (**arrowheads**) and the nucleus (**short arrow**) of conjunctive tissue cells (**C**). **Ep** - epithelial region. Scale bars: **A -** 28 µm, **B -** 6.4 µm, **C -** 6.4 µm.

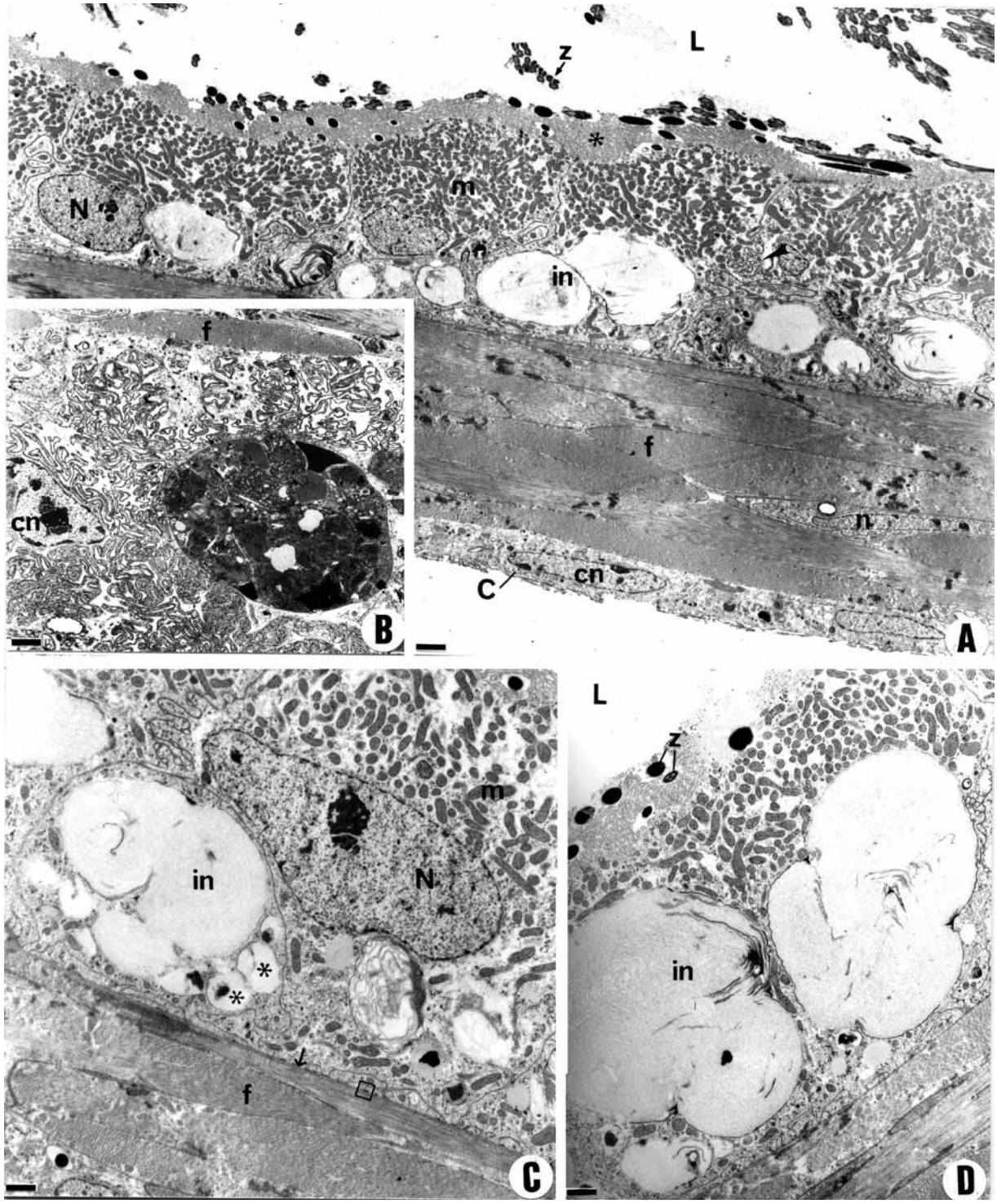

Figure 2. A. Ultrastructure of a seminal vesicle. Note the lumen (**L**) containing sperm (**z**) located close to microvillar projections (**asterisk**), the epithelial cells with their nucleus (**N**) and some inclusions (**in**), and the muscular sheath consisting of prominent muscle cells (**f**) and their nuclei (**n**). **C -** conjunctive tissue cell with a large nucleus (**cn**). **B.** Electron micrograph of the conjunctive tissue capsule showing the membrane-like structures (**arrow**) and the nucleus of a conjunctive cell (**cn**), external to a muscle cell (**f**). **C-D.** Electron micrographs of the epithelium. Panel **C** shows the fusion of smaller inclusions (**asterisks**) to form a larger one (**in**), and panel **D** shows these inclusions occupying most of the cytoplasm. The square indicates a hemi-desmosome-like junction adjacent to the basement membrane (**arrow**). **N** - nucleus, **f** - muscle fiber, **L -** lumen, **z -** spermatozoa. Scale bars: **A -** 1.3 μm, **B -** 1.56 μm, **C -** 0.68 μm, **D -** 0.80 μm.

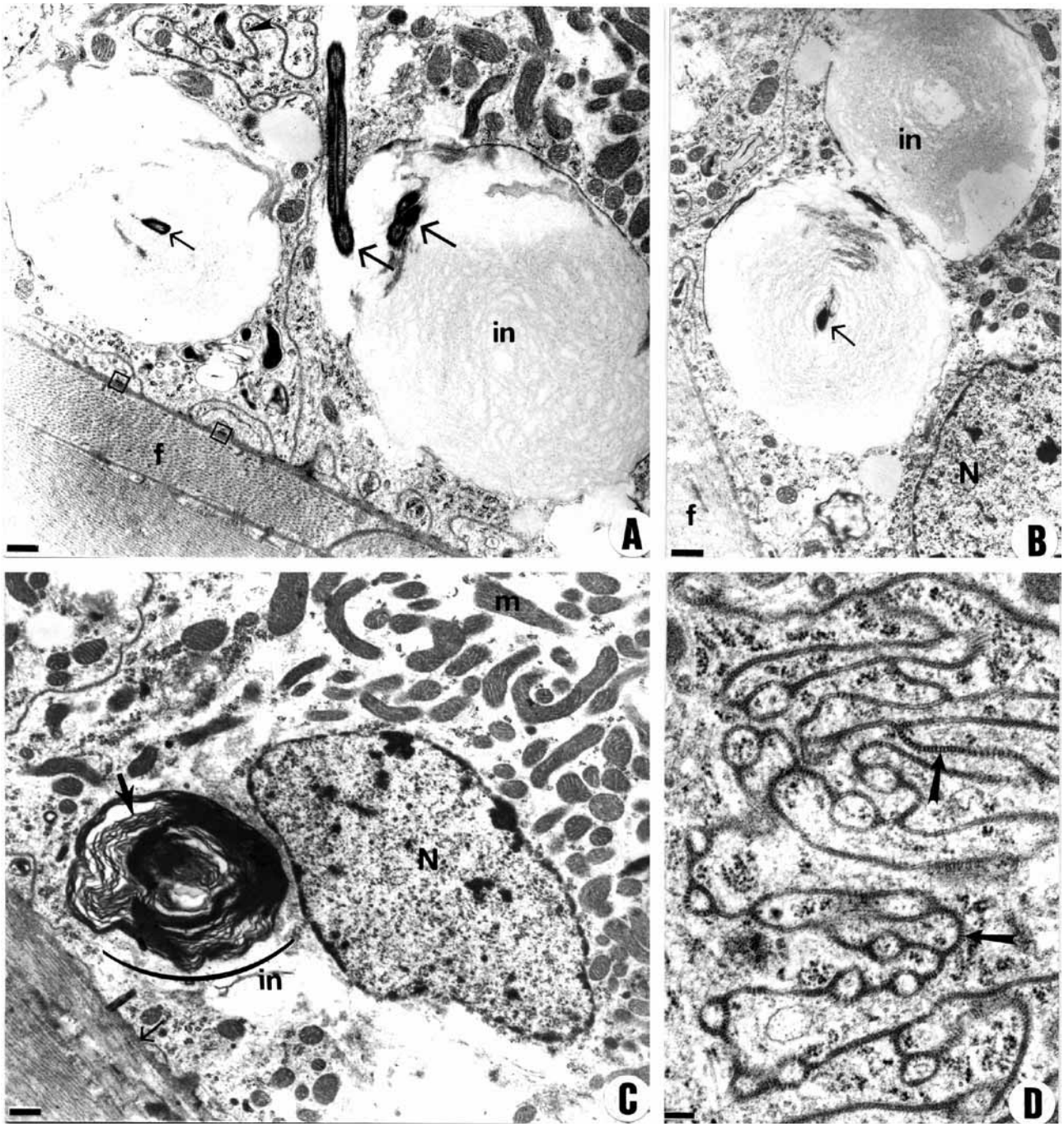

Figure 3. A-B. Ultrastructure of the epithelial inclusions (**in**) containing dense structures and altered spermatozoa (**arrows**), suggestive of spermiophagy. In (**A**), the squares indicate desmosome-like junctions attached to the basement membrane and the arrowhead indicates the septate junction. **N** – nucleus, **f -** muscle fiber. **C.** The membranous content (**arrow**) of the inclusion (**in**), similar to a myelin figure. **N -** nucleus, **m -** mitochondria, **thin arrow** - basement membrane. **D.** Ultrastructure of the septate junction (**arrow**). Scale bars: **A -** 0.35 μm, **B -** 0.50 μm, **C -** 0.4 μm, **D -** 0.17 μm.

and phosphatase activity were negative, as were also other structures associated with secretion. A positive PAS reaction was seen only in the epithelial brush border.

The muscular sheath was thick and consisted of voluminous fibers that could be observed simultaneously in their longitudinal and transverse axes (Figs. 1A,B, 2A-C and 3A). A layer of conjunctive tissue covered the muscular sheath surrounding the seminal vesicle (Fig. 2A,B).

The lumen

The luminal region was full of spermatozoa (Figs. 1A-C and 4D) immersed in an electron-dense material. Most of the spermatozoa were free swimming and were well-observed in sections in different planes (see the numerical sequence in Fig. 4D). However, some sperm bundles were occasionally seen in epithelial depressions (Fig. 4A,B). The greater thickness of the light micrographs compared to TEM clearly showed the width of these bundles (Fig. 4A) that formed deep insertions outside the epithelial cell (arrow, Fig. 4B).

DISCUSSION

The male reproductive system of *S. xanthotricha* is similar to that of other Meliponini [17,21]. According to Ferreira *et al.* [21], the male reproductive apparatus in bees (51 species representing six families) can be classified into at least four patterns, i.e. types I, II, III and IV. The type IV apparatus is present exclusively in the Meliponini, and is characterized by the absence of accessory glands and by large seminal vesicles that have a much wider diameter than the deferent ducts. This type also has four follicles (referred to as seminiferous tubules by these authors) per testis and the seminal vesicles form globular units that are encapsulated by the scrotal membrane [21]. Within the Meliponini, the reproductive apparatuses follow a basic pattern but show small, species-specific variations, such as in the dimensions of their structures. The degenerated testes found in the specimens investigated here and reported by Ferreira et al. [21] showed that these structures were sexually mature and that, as in most social insects [8,18], this species produces spermatozoa only once during sexual maturation.

In insects, the accessory glands have an important role in reproduction since their secretions have been implicated in spermatophore formation, spermatozoal maturation, activation, induction and acceleration of oviposition, control of polygamy, and mating plug formation [1,5,10,11,24,35]. Male bumble bees deposit mating plugs that effectively prevent intromission by other males and demotivate queens from additional sexual activity [3]. Similar mating plugs occur in fire ants [31] and probably in fungus-growing ants [2]. Since male sandflies lack male accessory reproductive glands, the plug in this case is presumably produced by the seminal vesicles [19]. In the Meliponini, the retention of the male genitalia by the queen during mating [30] can act as a mating plug so that in these bees accessory glands and their secretions would not be needed to guarantee monoandry. In addition, this characteristic could be considered a secondary development pattern for males to control mating.

Various kinds of secretions are produced by the epithelial cells along the reproductive tract and are mixed with the spermatozoa to form semen [22]. However, as in the *M. bicolor* [17], the epithelial cells of the seminal vesicle in mature *S. xanthotricha* males do not show the morphological features of secretory cells. This feature has been found in some insect groups in which the vesicular epithelium has an ultrastructure typical of a secretory cell, and its products support functions typical of accessory glands [9,13,16,23,28,29,36,37]. Hence, in *S. xanthotricha*, as in *Melipona bicolor* [17], the amorphous material in which spermatozoa are immersed could be produced by cells in other regions of the vas deferens, or even by the seminal vesicle during the previous phase of sexual maturation.

Myelin figures in seminal vesicles have been observed in ants (*Camponotus* spp.) [41] and *M. b. bicolor* [17], including in the degenerating testes of the latter [15]. The origin and function of these myelin figures are unclear. Some authors have suggested that they may represent a special type of secretion [17,25,34], while others consider that the presence of these structures could be the result of intracellular digestion since they are normally observed in senescent organs. Both ants and stingless bees have these figures in their seminal vesicles and they do not increase their supplement of sperm after sexual maturity. However, since ants have accessory glands, the myelin figures seen in ants and *S. xanthotricha* are not likely to be secretions and probably resulted from autophagy in elderly cells and/or spermiophagy, as reported by Dallacqua and

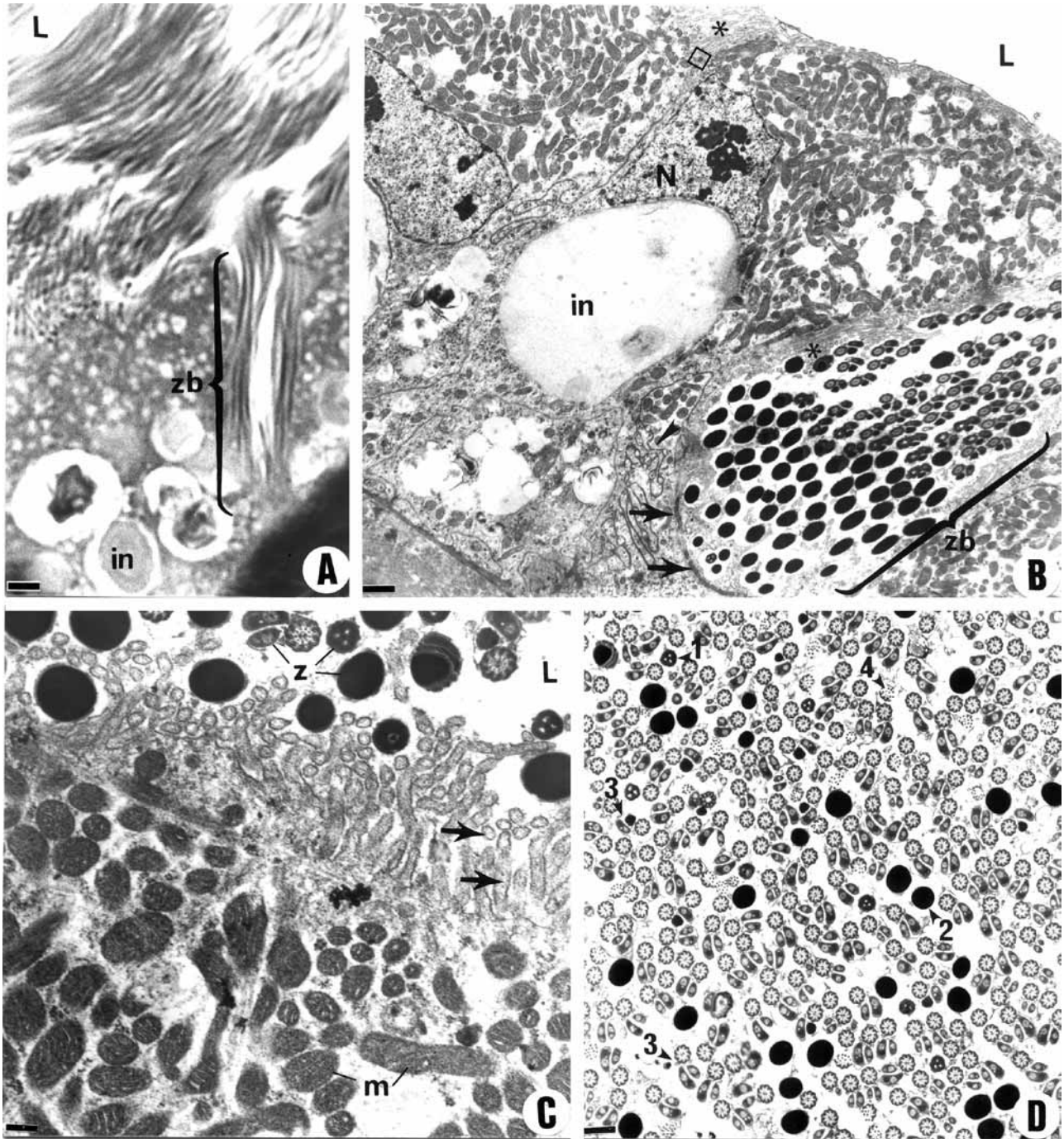

Figure 4. Light **(A)** and electron **(B)** micrographs showing large spermatic bundles (**zb**) inserted between epithelial cells. In panel **B**, note the plasma membrane of an epithelial cell (**arrows**) and some microvilli (**asterisk**) beside the spermatic bundle. The square indicates a desmosome-like junction. **L -** lumen, **in -** inclusions, **N -** nucleus. **C.** The ultrastructure of the apical region showing the abundance of mitochondria (**m**) and the microvilli sectioned transversely and longitudinally (**arrows**). **L -** lumen, **z -** sperm cells. **D.** Transverse sections showing luminal sperm cells at different levels of the spermatic vesicle. Scale bars: **A -** 2.4 μm, **B -** 0.8 μm, **C -** 0.20 μm, **D -** 0.45 μm.

Cruz-Landim [17], Viscuso *et al.* [40] and Wheeler and Krutzsch [41].

The abundance of mitochondria in the apical portion of the epithelium of the seminal vesicles indicated a high metabolic activity for these cells, especially in this region of the cell. This pattern was also found in *M. bicolor* [17], *Apis mellifera* [14] and *Camponotus* spp. [41] with the abundance of this organelle in these species being related to the regulation of the luminal pH.

In conclusion, since the seminal vesicles of mature males of two genera of stingless bees, *Scaptotrigona* and *Melipona* [17], show none of the features that are usually characteristic of a secretory function, it is possible that this condition may occur in all stingless bees. In addition, the presence of myelin figures in the seminal vesicle of these bees, as well as in ants [41], suggests that this may be characteristic of all Hymenoptera that produce sperm only up to sexual maturity.

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