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## RESEARCH ARTICLE - BEES

### Structural Changes in the Male Reproductive Tract of the Stingless Bee *Scaptotrigona xanthotricha* Moure, 1950 (Meliponini, Apidae) During Sexual Maturation

VA ARAÚJO<sup>1</sup>, JE SERRÃO<sup>2</sup>, Y ANTONINI<sup>3</sup>, LG DIAS<sup>4</sup>, J LINO-NETO<sup>2</sup>

1 - Instituto de Biodiversidade e Sustentabilidade (NUPEM), Universidade Federal do Rio de Janeiro, Macaé-RJ, Brazil

2 - Departamento de Biologia Celular, Universidade Federal de Viçosa, Viçosa-MG, Brazil

3 - Departamento de Biodiversidade, Evolução e Meio Ambiente, Instituto de Ciências Exatas e Biológicas, Universidade Federal de Ouro Preto, Ouro Preto-MG, Brazil

4 - Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas y Naturales, Grupo de investigación Bionat, Universidad de Caldas, Caldas, Colombia

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#### Corresponding author

Vinícius Albano Araújo

 <https://orcid.org/0000-0001-9387-7378>

Instituto de Biodiversidade e Sustentabilidade (NUPEM)

Universidade Federal do Rio de Janeiro

Av. São José Barreto, 764 - São José do Barreto, CEP 27965-045 - Macaé-RJ, Brasil.

E-Mail: [vialbano@gmail.com](mailto:vialbano@gmail.com)

[vialbano@nupem.ufrj.br](mailto:vialbano@nupem.ufrj.br)

#### Abstract

Stingless bees are social insects widely distributed in the Neotropical region but their populations have declined due to loss of habitats and the increase use of pesticides in agriculture. Thus, the knowledge of the biology of these insects, including reproductive biology is important for their conservation and management. This study describes the morphological changes in the male reproductive tract during the sexual maturation in the stingless bee *Scaptotrigona xanthotricha* (Moure, 1950). The gross morphology and histology of the reproductive tract was investigated from pupae to 22 days old adults. The male reproductive organs in *S. xanthotricha* are a pair of testes, each with four follicles, pair of *vasa deferentia* with enlarged seminal vesicle region that open in an ejaculatory duct. In the male reproductive tract of this bee there are no accessory glands. From brown-eyed pupae to newly-emerged adults, the epithelia of the seminal vesicle and post-vesicular *vasa deferentia* have prismatic cells, which release secretion to the organs lumen. In 5-days old adults the testes undergo degeneration, the seminal vesicle is filled with secretion and spermatozoa, and its epithelium has cubic cells rich in inclusions in the basal region suggesting that males reach the sexual maturation. Structural modifications in the reproductive tract during sexual maturation are discussed concerning the reproductive biology of Meliponini.

#### Introduction

In recent decades, efforts to increase knowledge about bees have been performed in response to the rapid decline and collapse of their populations (Freitas et al., 2009; Potts et al., 2016; Nielsen et al., 2017; Castilhos et al., 2019). The most probable causes for this worldwide phenomenon are the use of pesticides in the agriculture, infections by pathogens, and habitat fragmentation (Cortopassi-Laurino et al., 2006; Del Sarto et al., 2014; Goulson et al., 2015; Guimarães-Cestaro et al., 2020).

Stingless bees (Meliponini *sensu* Michener, 2007) are the most numerous group of social bees widely distributed

in tropics (Michener, 2007, 2013; Cortopassi-Laurino & Nogueira-Neto, 2016). In the Neotropics there are more than 400 species into 32 genera (Camargo, 2013), which performing fundamental ecosystem services as pollinators (Michener, 2007; Freitas et al., 2009) pollinating 40-90% of native plants (Brosi et al., 2009) as well as some crops (Slaa et al., 2006). The morphology of the male reproductive tract has been described in some species of Meliponini (Cruz-Landim, 2001; Dallacqua & Cruz-Landim, 2003; Ferreira et al., 2004; Araújo et al., 2005; Lima et al., 2006; Lino-Neto et al., 2008; Brito et al., 2010; Ferreira et al., 2019). Although those studies have contributed in some extension to understand the reproduction



in these insects, they are restricted to few species and gaps about the reproductive biology and evolutionary history of stingless bees needs to be filled.

An intriguing finding in the male reproductive tract of stingless bees is the absence of accessory glands (Kerr, 1948; Ferreira et al., 2004, 2019). In insects, the accessory glands associated with the male reproductive tract produce compounds that are involved in functions such as spermatozoa maturation and activation, sperm and spermatophore formation, activation of oviposition in females, polygamy control including the formation of the mating plug (Chen, 1984; Gillot, 2003; Boosmam et al., 2005; Colonello & Hartfelder, 2005; Fuessl et al., 2014). Because these glands are absent in stingless bees their function has been suggested to be replaced by secretory cells in other reproductive tract regions, mainly in the *vas deferens* and seminal vesicle (Dallacqua & Cruz-Landim, 2003; Araújo et al., 2005; Brito et al., 2010; Ferreira et al., 2019). In these organs, the epithelial cells seem to produce components of the semen fluid (Dallacqua & Cruz-Landim, 2003; Araújo et al., 2005; Brito et al., 2010).

In social bees the spermatogenesis begins in the pupal phase and is completed in adult males with different ages according to the species (Happ, 1992; Cruz-Landim & Dallacqua, 2002; Dallacqua & Cruz-Landim, 2003; Lino-Neto et al., 2008). Males emerge sexually immature and continue to undergo structural changes in the reproductive tract until complete formation and migration of spermatozoa to the seminal vesicles, when they then are sexually mature to mate and fertilize the eggs (Araújo et al., 2005; Lima et al., 2006; Brito et al., 2010; Ferreira et al., 2019).

Among 33 genera of Meliponini endemic of the Neotropical region, *Scaptotrigona* has 21 species with nine in Brazil (Pedro, 2014). *Scaptotrigona xanthotricha* (Moure, 1950) is widely distributed in the Atlantic rainforest where they build nests in hollows of trunks (Kerr, 2001). Together with other bee species, *S. xanthotricha* is responsible for 90% of pollination of Atlantic forest native trees (Kerr, 2001; Custodio et al., 2017) and has economic importance due to the honey and as pollinators of crops (Slaa et al., 2006; Nunes-Silva et al., 2013; Witter et al., 2015), such as cucumber, strawberries, carrots and watermelon (Dos Santos et al., 2008; Roselino et al., 2009; Nascimento et al., 2012).

Considering ecological and economic importance as well as the need to expand the knowledge about the reproductive biology of stingless bees, this study describes the morphological changes in the male reproductive tract of *S. xanthotricha* during sexual maturation.

## Materials and Methods

### Insects

Brood combs were obtained from colonies of *S. xanthotricha* kept in the Central Apiary of the Federal University of Viçosa, Minas Gerais state, Brazil. The pupae were removed by opening the brood cells with aid of tweezers

and males were identified by the presence of gonostylus. The age of pupae was classified according to the pattern of compound eyes color and body pigmentation, assigned into five different classes: 1) pink-eyed pupae; 2) brown-eyed pupae; 3) black-eyed and non-pigmented body pupae; 4) black-eyed pigmented body and non-pigmented wing pupae, and 5) black-eyed pigmented body and wing pupae. To obtain adults, brood combs were placed in Petri dishes at  $28 \pm 2$  °C, and newly-emerged adult males were marked on the thorax with non-toxic ink to control age of each individual. These males were caged in wooden box together with workers of different ages (in order to feed young males), resin, and brood combs containing young larvae (to be handled by workers) at  $28 \pm 2$  °C. Bees were fed on pollen grains stored in the colonies and 50% sucrose aqueous solution *ad libitum*.

### Light Microscopy

Seven males every pupal stages and adults 0-22 days old were cryo-anesthetized at -5 °C for 2 min the reproductive tracts were dissected in 0.1 M sodium cacodylate buffer pH 7.2, and transferred to 2.5% glutaraldehyde in the same buffer for 4 h. Then, samples were washed in the same buffer and post-fixed in 1% osmium tetroxide for 2 h following dehydration in a graded ethanol series (50, 70, 90 and 95%). The pieces were embedded in historesin (Leica Historesin) and 2 µm thick sections obtained with a glass knife in a rotary microtome Leica RM 2255 were stained with 1% toluidine blue-sodium borate and analyzed with light microscope. For anatomical analysis, freshly fixed reproductive tracts were photographed with an Olympus BX-60 microscope.

Some histological sections were submitted to the PAS histochemical test (Junqueira & Junqueira, 1983) to detect polysaccharides and glycoconjugates. Positive control was performed with *Bombus terrestris* midgut (Carneiro et al., 2018).

## Results

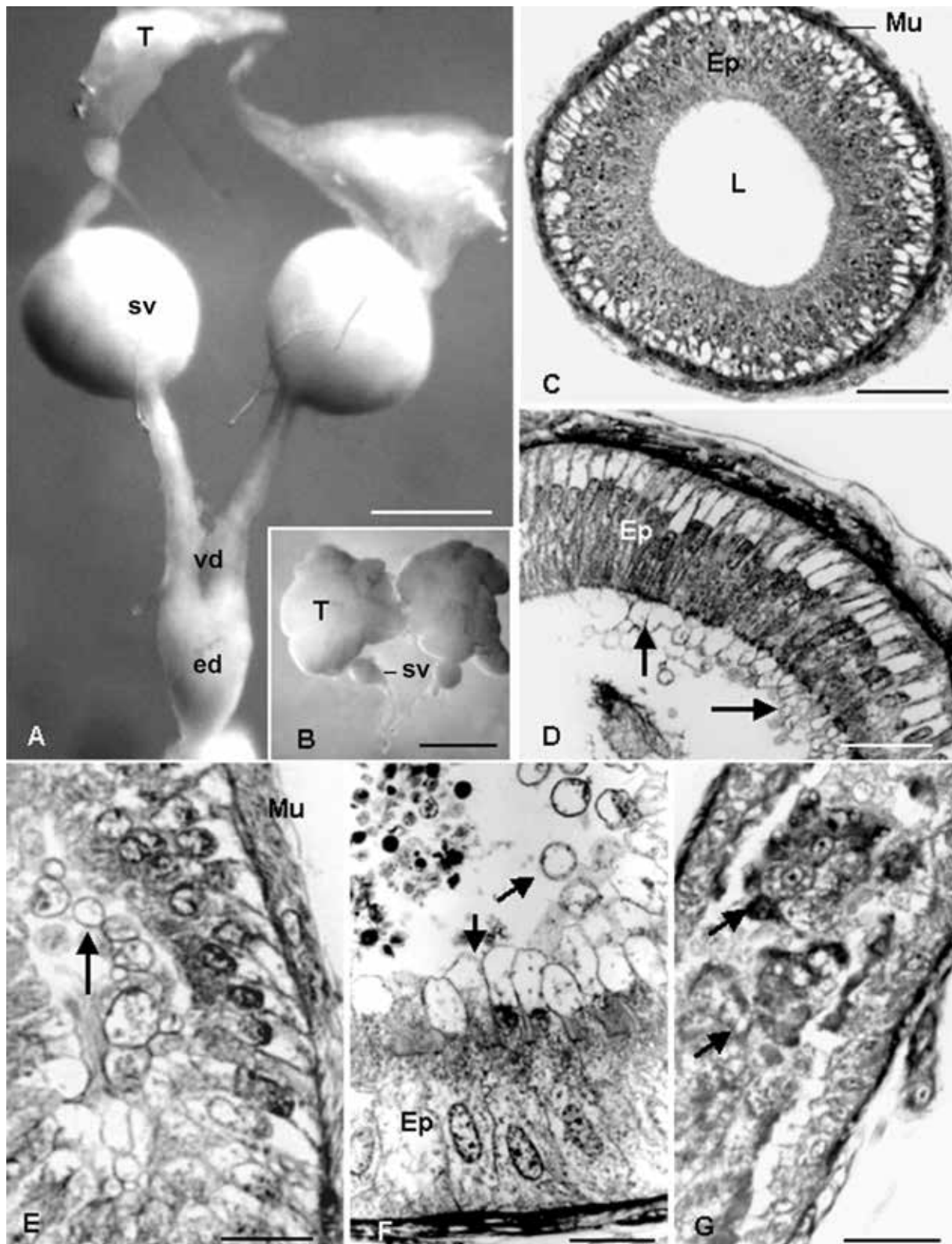
The male reproductive tract of *S. xanthotricha* had a pair of testes, a pair of *vasa deferentia* and seminal vesicles opening in an ejaculatory duct. Each testis had four follicles opening into efferent ducts that fused to form a *vas deferens*. The proximal region of *vas deferens*, closely to the testis, had an enlarged region forming the seminal vesicle, followed for a long post-vesicular region opening in a short ejaculatory duct (Fig 1A).

During post-embryonic development and adult lifespan there were morphological changes in male reproductive tracts. From pupae to 4-day-old adult, the testes were voluminous and seminal vesicles slightly dilated (Fig 1B). In 5-day-old adults, the testes begun to degenerated, which was complete in the 10<sup>th</sup> day (Fig 1A).

In the young pink-eyed pupae the *vasa deferentia* and seminal vesicles had empties lumen, and their single layered epithelia had prismatic cells (65 µm high) with well-developed median nuclei rich in decondensed chromatin (Fig 1C).

From brown- to black-eyed pigmented body and non-pigmented wings pupae, the epithelia of the *vasa deferentia* and seminal vesicles were rich in basal large vesicle-like

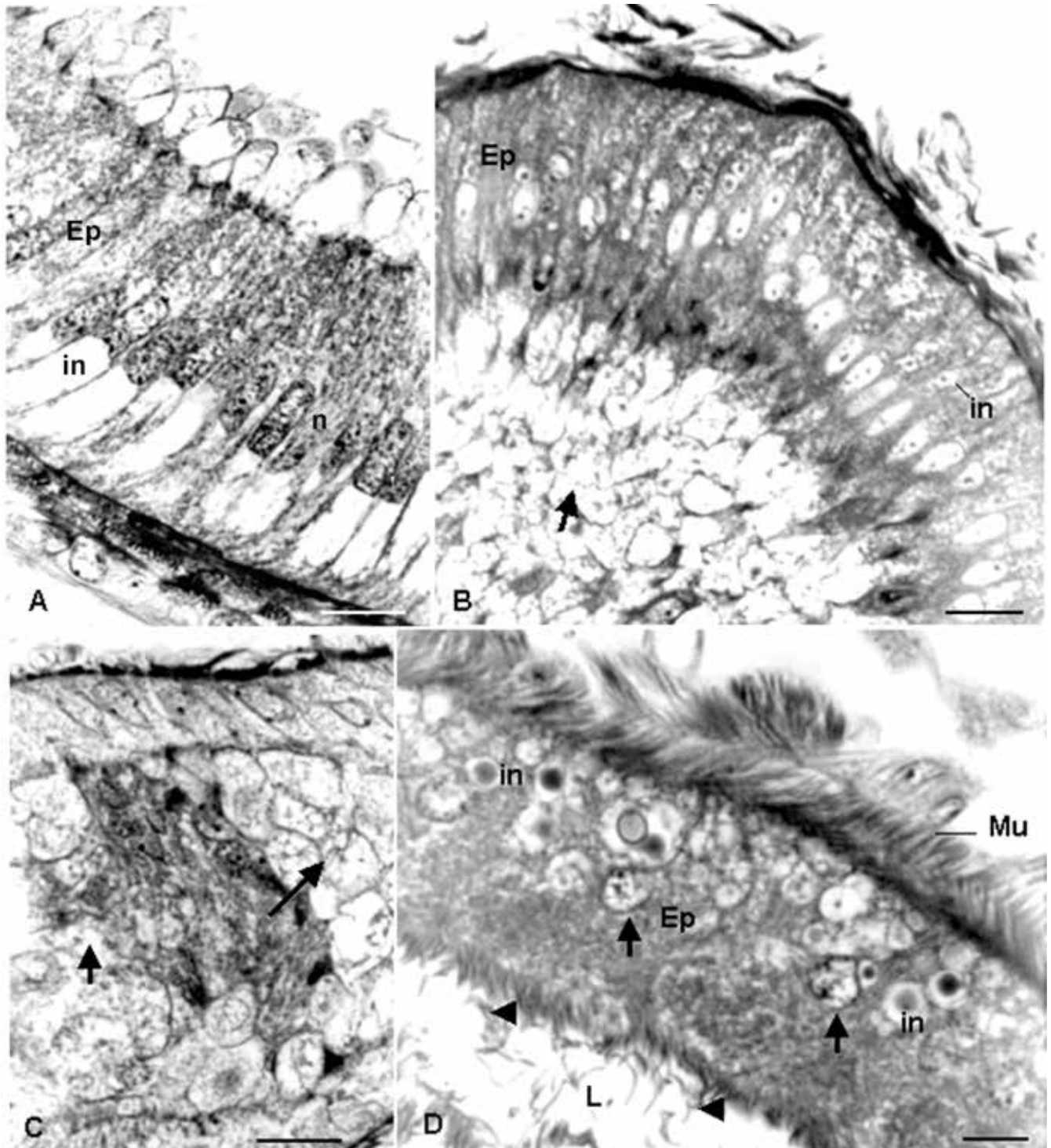
structures and apical granules which were released into the lumen, resulting in increased secretion accumulation according to the age (Fig 1D, G).



**Fig 1.** The male reproductive tract of *Scaptotrigona xanthotricha*. A. Sexually mature male 10-day-old showing degenerated testes (T), enlarged seminal vesicles (sv), *vasa deferentia* (vd) and the ejaculatory duct (ed). B. Black-eyed pupae showing well-developed testes (T) and dilated seminal vesicles (sv). C-D. Cross section of a seminal vesicle in pink-eyed pupae (C) and brown-eyed pupae (D) showing empty lumen (L), simple prismatic epithelium (Ep), vesicles (arrow), and a muscle layer (Mu). E-F. Cross section of *vasa deferentia* (E) and seminal vesicles (F) in black-eyed and non-pigmented body pupae showing the secretion in the lumen (arrows). G. Longitudinal section of a *vas deferens* in black-eyed and pigmented body pupae, showing the different secretions in the lumen (arrows). Bars: A-B = 0,7mm; C-G = 50µm;

From black-eyed pigmented body and wings pupae to 2-day-old adult, the *vasa deferentia* and seminal vesicles epithelia had prismatic cells with median and well-developed nuclei (Fig 2A). In both organs, the epithelial apical region was dilated, rich in vesicles with granular content, which protruded and detached into the lumen (Fig 2A).

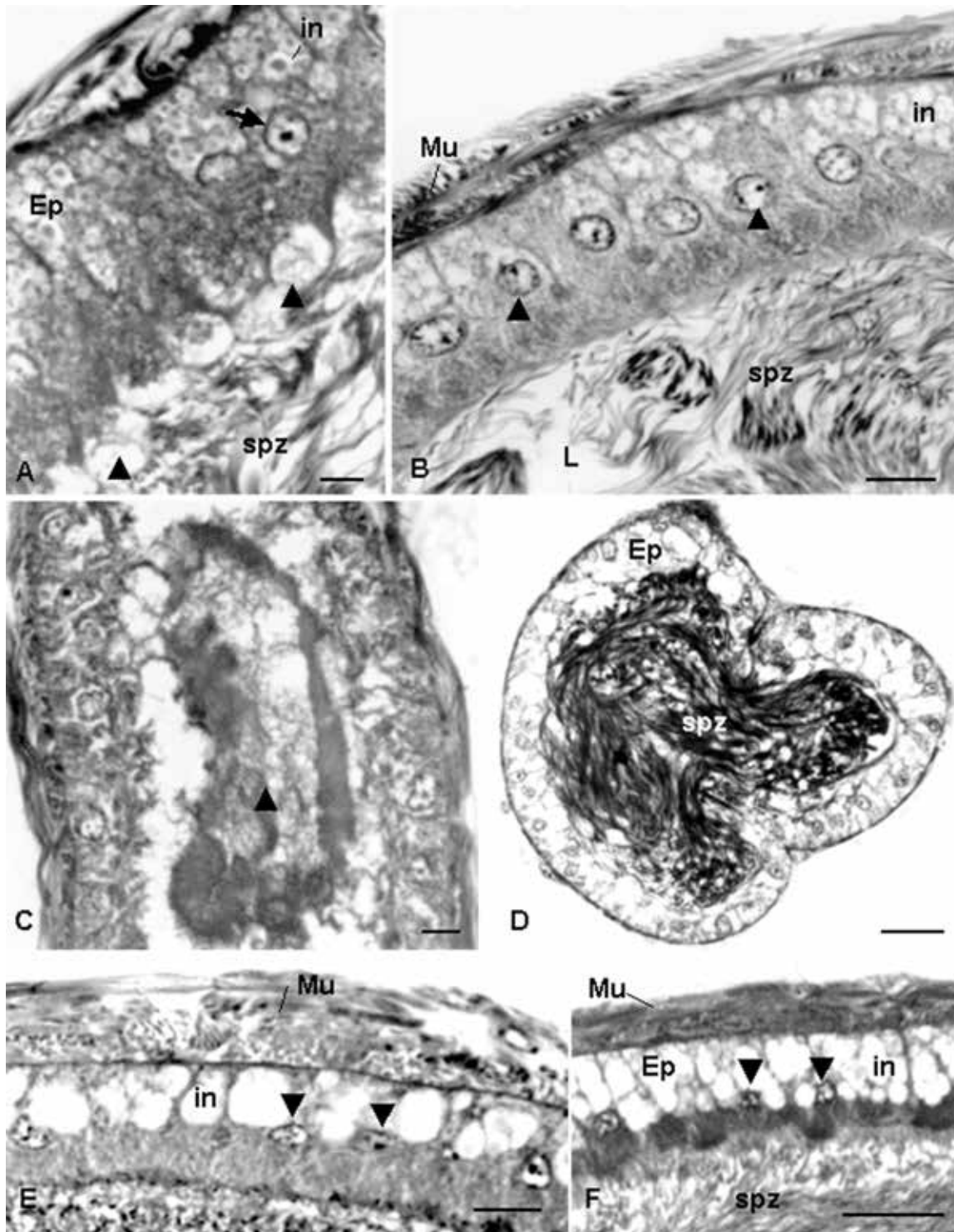
Three- and 4-day-old adults, showed the epithelia of the *vasa deferentia* and seminal vesicles with tall cells with vesicle-like structures in the basal region and nuclei smaller (5  $\mu\text{m}$  diameter) (Fig 2B) than those from previous developmental stages (8  $\mu\text{m}$  diameter). The lumens of these ducts were filled with vesicles of granular content (Fig 2B, C).



**Fig 2.** Light micrographs in transverse sections of the seminal vesicles and post-vesicular *vasa deferentia* of *Scaptotrigona xanthotricha*. A. Black-eyed and pigmented body pupae showing the epithelium of the seminal vesicle (Ep) with prismatic cells rich in apical vesicles, median nuclei (nu) and vesicles inclusions in the base (in). B-C. 3-day-old adult male showing vesicular inclusions (in) at the base of the epithelial cells (Ep) and secretions in the lumen (arrow) of the *vasa deferentia*. D. 5-day-old adult male showing cubic epithelium (Ep), with basal cell nuclei (arrow) and sperm at the apical edge of the epithelium (arrow head). Note the vesicular inclusions (in) at the base of the epithelium that is lined a muscle layer (Mu). Bars: A-C = 20 $\mu\text{m}$ ; D = 10 $\mu\text{m}$ .

Five-day-old adult revealed the seminal vesicles filled with spermatozoa with cubic epithelium (32  $\mu\text{m}$  high) showing basal nucleus (Fig 2D) and the apical region rich in vesicles with granular content (Fig 3A). In the 6- and 7-day-old adults, the seminal vesicles were filled with spermatozoa (Fig 3B),

the cubic epithelium had median nucleus and the basal region was rich in vesicles (Fig 3B). In these males, the post-vesicular *vas deferens* was filled with secretion (Fig 3C), whereas in the pre-vesicular region there was some spermatozoa in the lumen (Fig 3D).



**Fig 3.** Light micrographs of transverse sections of the seminal vesicles and post-vesicular *vasa deferentia* of *Scaptotrigona xanthotricha*. A. Seminal vesicle of a 5-day-old adult showing lumen with spermatozoa (spz), and vesicles (arrowhead) at the apex and base (in) of the epithelial cells (Ep). Nucleus (arrow). B. Seminal vesicle with cubic epithelium showing sperm (spz) in the lumen (L), and cells with spherical nucleus (arrowhead) and vesicular inclusions in the basal region. C-D. *Vasa deferentia* with secretions (arrowhead) and sperm (spz) in the lumen. E-F. Seminal vesicle of 10-day-old males showing sperm (spz) in the lumen, and many vesicular inclusions (in) in epithelial cells (Ep). Nucleus (arrowheads). External muscle (Mu). Bars: A-B = 10 $\mu\text{m}$ ; C = 15 $\mu\text{m}$ ; D-F = 30 $\mu\text{m}$ .

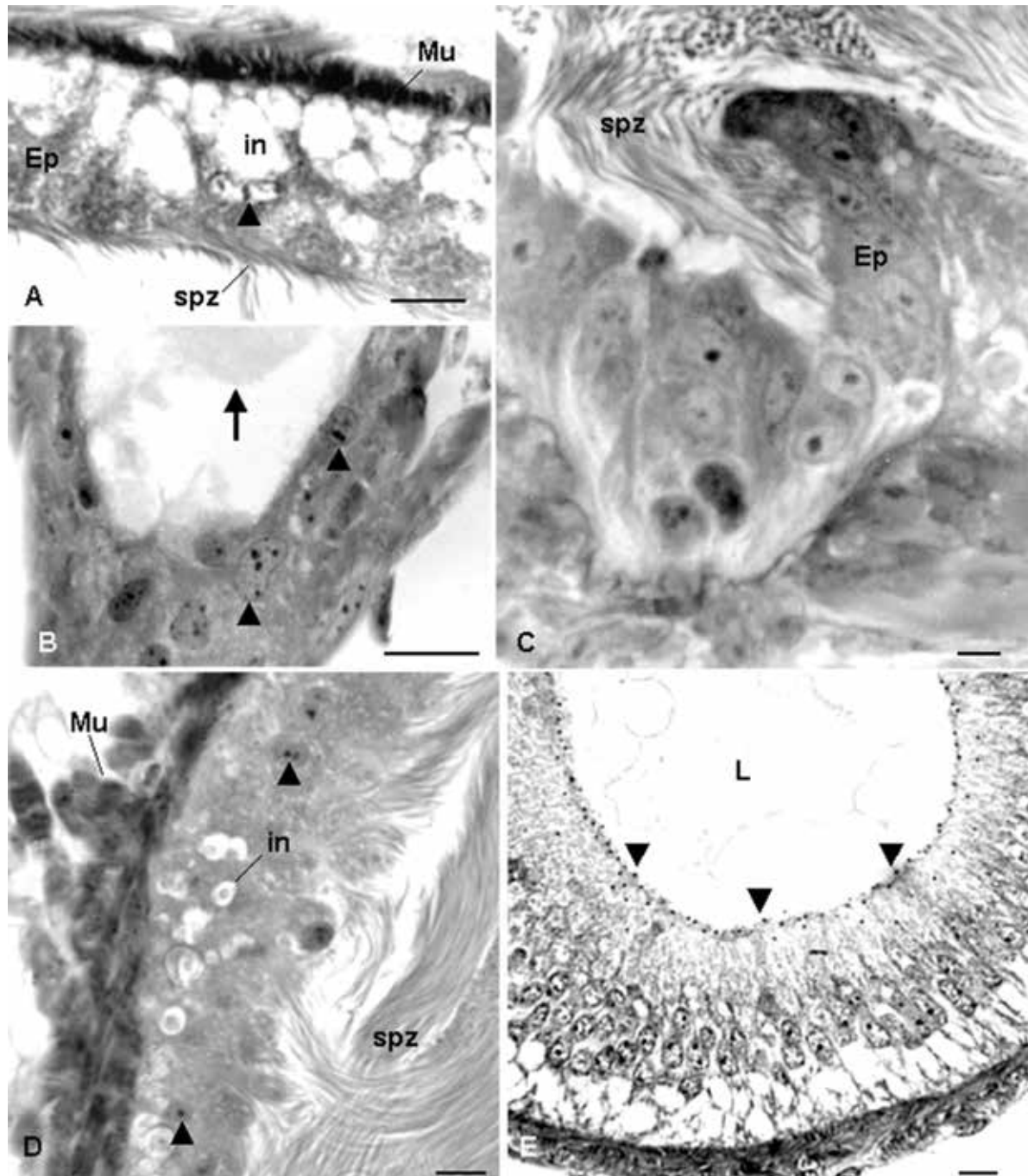


Males from 8- to 10-day-old aged showed the nucleus of epithelial cells of the seminal vesicle with irregular shape and, sometimes, compressed by numerous and large vesicles (Fig 3E, F). Many spermatozoa were associated with the apical surface of the epithelium. The post-vesicular *vas deferens* showed a similar pattern with cubic epithelium and nucleus compressed by vesicles (Fig 4A).

After the 20<sup>th</sup> day of adulthood, there was an increase in size and quantity of vesicles in the epithelium of the seminal

vesicle (Fig 4A, D). In addition, some spermatozoa bundles were associated with deep folds of the epithelium (Fig 4C). The post-vesicular *vas deferens* showed luminal secretion in male 22-day-old (Fig 4B) and the epithelium with nuclei showing an increase in the amount of condensed chromatin (Fig 4B, D).

During the development and sexual maturation, the vesicles in the apical region of the epithelium in the seminal vesicle and *vas deferens* had some PAS-positive granules (Fig 4E).



**Fig 4.** Light micrographs of transverse sections of the seminal vesicles and post-vesicular *vasa deferentia* of *Scaptotrigona xanthotricha*. A. Seminal vesicle in 10-day-old male showing sperm (spz) in the lumen, muscular cells lining externally (Mu), and the cubic epithelium (Ep) with cell nuclei (arrowhead) associated with vesicle-like inclusions (in). B-D. 22-day-old males showing (B) *vas deferens* secretion (arrow) in the lumen and nuclei with some clumps of condensed chromatin (arrowhead); (C) spermatozoa (spz) associated with deep epithelial fold (ep); and (D) basal region of the epithelium with many vesicle-like inclusions (in) and the nuclei with clumps of condensed chromatin (arrowhead). E- Representative epithelium of seminal vesicle in black-eyed pupae showing PAS-positive reaction (arrowheads). Bars: A and D = 20 $\mu$ m; B = 30 $\mu$ m; C and E = 10 $\mu$ m.

## Discussion

The male reproductive tract of *S. xanthotricha* is similar to that described for other Meliponini with a pair of testes, each with four encapsulated follicles, a pair of *vasa deferentia* and seminal vesicles opening in an ejaculatory duct without accessory glands (Ferreira et al., 2004; Brito et al., 2010).

In *S. xanthotricha*, from young pink-eyed pupae to 5-day-old adults the testes are well-developed and the enlarged seminal vesicles empty, suggesting that the spermatogenesis is in progress such as reported for other bees (Dumser, 1980; Heinze & Holldobler, 1993; Cruz-Landim & Dallacqua, 2002; Boomsma et al., 2005; Lima et al., 2006; Brito et al., 2010). However, in the stingless bees *Melipona mondury* (Lima et al., 2006) and *M. quadrifasciata* (Ferreira et al., 2019), the lumen of seminal vesicle in the pupae is filled with amorphous content.

When the seminal vesicles are filled with spermatozoa, the testes undergo degeneration and males can be considered sexually mature (Cruz-Landim & Dallacqua, 2002; Araújo et al., 2005; Brito et al., 2010). Thus, our results indicate that *S. xanthotricha* males reach sexual maturity from the 5<sup>th</sup> to the 10<sup>th</sup> day of adulthood because testes undergo degeneration and seminal vesicles are filled with sperm. In this way, they no longer produce spermatozoa after sexual maturation likely in other eusocial Hymenoptera (Boomsma et al., 2005).

In *S. xanthotricha*, the spermatozoa begin to migrate from testes to the seminal vesicles in the 4<sup>th</sup> day of adulthood and testes are almost totally degenerate in 10-day-old male. The sexual maturation in bees varies according to the species with spermatozoa stored in the seminal vesicles of *M. mondury* on the 4<sup>th</sup> day of adult life (Lima et al., 2006), 7<sup>th</sup> day in *M. quadrifasciata* (Camargo, 1984), 9<sup>th</sup> day in *Friesella schrottkyi* (Brito et al., 2010), and 12<sup>th</sup> day in *Apis mellifera* (Snodgrass, 1978) and *Bombus terrestris* (Tasei et al., 1998).

The absence of male accessory glands in the reproductive tract of *S. xanthotricha* and all studied Meliponini is intriguing because these glands play important reproductive functions in other bees, such as the production of the mating plug, mating signals, changes in the female behavior, and maintenance of spermatozoa viability (Colonello & Hartfelder, 2005; Den Boer & Boomsma, 2009).

In sexually mature males of *S. xanthotricha* it was observed the storage of secretions in the seminal vesicles (Araújo et al., 2005), but results here obtained show that during sexual maturation, substances are produced and stored by seminal vesicles and the post-vesicular *vasa deferentia*. In some stingless bees, secretion rich in glycoproteins are produced by the epithelium of the seminal vesicles in pupae and adults, although these compounds occur in small amounts in the lumen (Dallacqua & Cruz-Landim, 2003; Araújo et al., 2005; Lima et al., 2006; Ferreira et al., 2019). Therefore, it is plausible to suggest that in *S. xanthotricha*, the *vas deferens*, including the region of the seminal vesicle, might have different functions during sexual maturation, such as

synthesis of glycoproteins in early stages of post-embryonic development and storage after sexual maturation.

The vesicles in the epithelial cells of *vasa deferentia* and seminal vesicles in sexually mature *S. xanthotricha* males have been reported as myelin figures under high resolution (Araújo et al., 2005) and may indicate a function of spermophagy throughout the epithelium, as found in *M. bicolor* (Dallacqua & Cruz Landim, 2003) and in the ant *Camponotus* spp. (Wheeler, 1992). However, myelin figures have also been suggested to be structures of storage and secretion, mainly of lipids with specialized functions in the extracellular environment (Schmitz & Muller, 1991). These vesicles occur during sexual maturation of *S. xanthotricha*, such as in *F. schrottkyi* (Brito et al., 2010), which may release components of the sperm, including lipids to be transferred to female during mating.

This work describes the relationship between age and spermatogenesis, testicular degeneration and sexual maturation in *S. xanthotricha*. These male bees reach sexual maturity between the 5<sup>th</sup> and 10<sup>th</sup> day of adulthood with morphological changes in the testes, epithelia of the *vasa deferentia* and seminal vesicles throughout the sexual maturation process. Overall, these data contribute to a better understanding of reproductive biology contributing to the management and conservation of this important stingless bee.

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## Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Vinícius Albano Araújo, José Eduardo Serrão, Yasmine Antonini, Lucimar Gomes Dias, José Lino-Neto. The first draft of the manuscript was written by Vinícius Albano Araújo and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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