New characteristics of the male reproductive system in the Meliponini bee, *Friesella schrottkyi* (Hymenoptera: Apidae): histological and physiological development during sexual maturation*

Pedro BRITO\(^1\), Uyrá ZAMA\(^2\), Heidi DOLDER\(^1\), José LINO-NETO\(^3\)

\(^1\) Departamento de Anatomia, Biologia Celular e Fisiologia, Instituto de Biologia, UNICAMP, PO Box 6109, Campinas, São Paulo, CEP 13083-863, Brazil
\(^2\) Departamento de Ciências Biológicas, UFOP, Ouro Preto, Minas Gerais, CEP 35400-000, Brazil
\(^3\) Departamento de Biologia Geral, UFV, Viçosa, Minas Gerais, CEP 36570-000, Brazil

Received 24 April 2009 – Revised 28 August 2009 – Accepted 5 September 2009

**Abstract** – Despite the biological importance of Meliponini bees, there are still many doubts about their reproductive physiology and phylogeny. When compared with other bee species studied up to now, the reproductive system of *Friesella schrottkyi* (Friese) has very different characteristics during sexual maturation, which includes: (1) the release of individualized testicular spermatozoa, rather than spermatozoa in bundles, (2) spermatozoa reaching the seminal vesicles only nine days after emersion of the adult bees, (3) holocrine secretion carried out by the seminal vesicle epithelium, (4) big lipid droplets abundant in the seminal vesicle epithelium and (5) membranous inclusions found in the seminal vesicle epithelium during the period of sexual maturation. These characteristics may represent important physiological characteristics as well as evolutionary differences in relation to other Meliponini studied and may be useful in studies of this bee group, as soon as similar descriptions are obtained for other closely related species.

**Friesella schrottkyi** / seminal vesicle / morphology / holocrine secretion / Hymenoptera / reproductive system

1. **INTRODUCTION**

The Meliponini tribe (Apidae), which includes *Friesella schrottkyi* (Friese), is distributed in all tropical and subtropical regions of the Southern Hemisphere (Michener, 2000; Silveira et al., 2002). Besides the use of these species to enhance agricultural production, the interest in studying and preserving these species derives from their importance as pollinators of native vegetation. In order to protect a species, it is important to understand its reproductive behavior, as well as its systematics. Despite the importance of the stingless bees, there are many unresolved points related to their reproduction, taxonomy and phylogeny (Rasnitsyn, 1988; Dowton and Austin, 1994; Dowton et al., 1997; Ronquist et al., 1999).

Secretions from accessory glands of the male reproductive system have been associated with reproductive success in many species. Some functions of these secretions are: spermatozoon nutrition, helping spermatozoon displacement along the ducts and inducing post-copula behavior in females (Gillott, 2003). In species that form spermatophores, sperm are packed by secretory
materials from the glands before to their transfer to the female (Chen, 1984; Sturm, 2003). Therefore, it is noteworthy that Meliponini bees do not have such glands (Kerr, 1948; Ferreira et al., 2004).

Measuring almost 3 mm in length, *F. schrottkyi* is a small bee that is distributed throughout the Brazilian southeast (Silveira et al., 2002). These bees are not aggressive; their colonies are small with approximately 300 individuals and irregular combs are found inside the colonies. Males of *F. schrottkyi* are produced throughout the year from queen or workers’ unfertilized eggs (Velthuis et al., 2005).

*Friesella* is a monotypic genus (Camargo and Pedro, 2008) that is closely related to another Meliponini genus: *Plebeia* (Michener, 1990, 2000).

This study describes morphophysiological aspects of the male reproductive system of *F. schrottkyi* during sexual maturation, and aspects related to sperm release from the testes, with the intention of contributing to the study of reproductive physiology.

2. MATERIAL AND METHODS

To obtain adult males of known ages, from 0 (just emerged) to 14 days, combs were taken from colonies of *F. schrottkyi*, maintained in the Central Apiary of the Universidade Federal de Viçosa (Viçosa, MG, Brazil), and conditioned in Petri dishes. These were maintained in BOD at 28.7 °C. They were inspected daily and the males were marked with a nontoxic paint for later age identification. As each adult emerged, the individuals were transferred to a “colony” previously mounted with workers, also maintained in BOD, and fed daily with a sucrose solution and pollen taken from colonies at the apiary.

2.1. Histology

Reproductive systems were dissected in cacodylate buffer (pH 7.2), fixed in Stefanini liquid (Stefanini et al., 1967), dehydrated in an alcoholic series and embedded in historesin. Histological sections were submitted to: (1) PAS (Junqueira and Junqueira, 1983) for neutral carbohydrate detection; (2) Alcian Blue pH 2.5 and pH 0.5 (Bancroft and Stevens, 1996) for acid glycoconjugates and (3) Xyline Ponceau (Mello and Vidal, 1980) for protein detection. They were also submitted to acid and alkaline phosphatase detection (Bancroft and Stevens, 1996).

To identify the nucleus, some histological slides were stained for 15 min in 0.2 µg/mL 4,6-diamino-2-phenylindole (DAPI) in phosphate buffered saline. They were examined with an epifluorescence microscope (Olympus, BX60), equipped with a BP 360–370 nm excitation filter.

2.2. Histochemistry

Reproductive systems were dissected in cacodylate buffer (pH 7.2), fixed in Stefanini liquid (Stefanini et al., 1967), dehydrated to absolute alcohol and xylene, re-hydrated and post-fixed in osmium tetroxide. Thin sections were stained with toluidine blue.

2.3. Transmission Electron Microscopy (TEM)

Reproductive systems were fixed in a solution containing 2.5% glutaraldehyde, 0.2% picric acid, 3% sucrose and 5 mM CaCl2 in 0.1 M cacodylate buffer, pH 7.2. The material was post-fixed in 1% osmium-tetroxide in the same buffer for 2 h. Dehydration was carried out in acetone and embedding of the organs in epoxye. Ultrathin sections were stained with 2% uranyl acetate and 0.2% lead citrate and observed with the Zeiss Leo 906 transmission electron microscope, at 60 kV.

3. RESULTS

3.1. Reproductive system

The reproductive system of *F. schrottkyi* males consists of two testes, each made up of four follicles. From each follicle originates an efferent duct, and the four tubular structures with Entellan (Merck), examined and photographed with a photomicroscope, Olympus CX31.
Reproductive system of male *F. schrottkyi*

3.2. Spermatogenesis and spermatozoan release

The spermatozoa of *F. schrottkyi* have synchronized development; that is, all spermatozoa mature at the same time. When *F. schrottkyi* males emerge, their spermiogenesis is already finished, with spermatozoa already elongated and organized in cysts, their heads pointing to the efferent duct (Fig. 1B). No changes were observed in their development until approximately the ninth day, which
is marked by the formation of a glycoprotein cap which encloses the spermatozoan heads in each cyst (Fig. 1C) (Tab. I). From this stage on, the testicular cysts began to disorganize and separated spermatozoa were found in the follicles (Figs. 1D, 2A). On the 12th day, the follicles showed some degenerative figures and neither any bundles of spermatozoa nor their remains were observed (Fig. 1D). Spermatozoa were found in the seminal vesicle only after the ninth day (Fig. 2B). Rarely were spermatozoa bundles or bundle fragments observed in the seminal vesicle, since the spermatozoa are mostly separated when they reach the deferent duct (Fig. 2C).

3.3. Seminal vesicle

In *F. schrottkyi*, the seminal vesicle consists of a monolayer of prismatic epithelial cells, externally covered by a muscular layer (Figs. 3A, B). During sexual maturation, the epithelium undergoes drastic changes (Tab. I).

In newly emerged individuals, the seminal vesicle lumen is reduced (Fig. 3A), epithelial cell height was approximately 50 μm and basal membrane infoldings supplied anchorage structures (Fig. 3C). Adhesion junctions were observed in the apical portion of the epithelium (Fig. 3D). Nuclei, positioned in the basal third of the cell, showed loose chromatin and at least one strongly staining nucleolus. The chromatin was not condensed during the entire maturation period. In just-emerged adults, the epithelial cells had a few and irregular microvilli (Fig. 3D). In one day old adults, the epithelial cells present bubble-like apical projections (Fig. 3B).

From the third day on, the seminal vesicle epithelium is very irregular, with large apical projections. In general, the nuclei are included in these projections, with no evident chromatin alteration (Figs. 4A–C). These projections are irregular and present constrictions between them and their cytoplasmic bases (Fig. 4C). Beside these cells, with apical projections, are some epithelial regions with half of the original epithelial height (Fig. 4B). The apical projections are released in the vesicle lumen, originating the cell fragments found during this phase, which characterize holocrine secretion.

Figure 2. (A) Longitudinal section of the transition (arrow) between a testicular follicle (f) and its efferent duct (ef), of a 10 day-old adult, (B) DAPI of a 9 day-old adult seminal vesicle, with some luminal cell structures (open arrows), some free spermatozoa (z) and some sperm bundles (b), (C) TEM of an efferent duct of a 14 day-old adult, with free spermatozoa (z) and some sperm bundles (b).
Reproductive system of male *F. schrottkyi*

Table I. Main characteristics of the male reproductive systems of *F. schrottkyi* and their development during sexual maturation.

<table>
<thead>
<tr>
<th>AGE</th>
<th>CHARACTERISTICS</th>
<th>Testes</th>
<th>Seminal vesicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Just emerged</td>
<td>Spermiogenesis completed</td>
<td>Seminal vesicle lumen reduced, epithelium 50 µm high, basal nuclei, few membranous inclusions (&lt;1 µm diameter), lipid droplets</td>
<td></td>
</tr>
<tr>
<td>3–8 days</td>
<td>No changes</td>
<td>Seminal vesicle lumen increased, large epithelial apical projections containing nuclei, holocrine secretion, larger membranous inclusions (irregular shape), lipid droplets</td>
<td></td>
</tr>
<tr>
<td>9 days</td>
<td>Formation of the glycoprotein cap, bundle disorganization, sperm individualized inside testes</td>
<td>Sperm arrive in seminal vesicles, holocrine secretion not observed, lipid droplets</td>
<td></td>
</tr>
<tr>
<td>14 days</td>
<td>Testes degenerated</td>
<td>Seminal vesicle full of spermatozoa, epithelium 25 µm high, apical nuclei, large membranous inclusions (2 µm diameter), fused membranous inclusions, lipid droplets</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3. (A–B) Histology of adult seminal vesicles: (A) just-emerged, with reduced lumen, (B) epithelial detail of a one day-old adult with bubble-like projections (bb), (C–D) TEM of the seminal vesicle of one day old adult; (C) basal cellular portion, note anchorage structures (open arrows) in cellular infoldings, (D) apical cell portion. (b) basal layer, (e) seminal vesicle epithelium, (j) cell junctions, (lu) seminal vesicle lumen, (ms) muscular layer, (mv) microvilli, (arrow) epithelial cell nuclei, (arrowhead) dark lipid droplets.
Figure 4. (A–C) Histological sections, mounted with Entellan, of four day-old adult’s seminal vesicles. Note: (A) the lumen, full of cellular fragments, (B) a prismatic epithelium region beside a cubic epithelium region, (C) epithelial cells with apical projections, containing nuclei, (D) TEM of four day-old adult luminal cell fragments, (E–F) histological sections: (E) of a seminal vesicle stained with DAPI to identify cell fragment nuclei. Notice the area occupied by secretion (s) that flowed from the deferent duct; (F) a post vesicular deferent duct, with cell fragments embedded in the PAS positive secretion. (e) vesicle epithelium, (ep) deferent duct epithelium, (ld) lipid droplets, (ms) muscular layer, (n) nuclei, (arrows) clear lipid droplets, (open arrows) luminal nucleated cellular structures, (arrowheads) membranous inclusions.
Reproductive system of male *F. schrottkyi* 209

The cell fragments include a large volume of cytoplasm (Fig. 4D), with the same characteristics as the epithelial cell cytoplasm. Most of the luminal cell fragments have a nucleus (Figs. 4D, E), with no distinguishing morphological features in relation to the epithelial cells. These structures are seen in the seminal vesicle lumen up to an age of nine days, when the spermatozoa begin to arrive (Fig. 2B). Sometimes, cellular structures were observed in the post-vesicular deferent duct lumen, embedded in a PAS positive, fibrous secretion (Fig. 4F).

In 14 day-old adults, the seminal vesicle lumen was full of sperm and none of the cell structures, which had filled the lumen till the ninth day, were observed (Fig. 5A). The seminal vesicle epithelium is regular in height, measuring approximately 25 µm, with nuclei located in the apical third of the cell (Fig. 5B).

The acid and alkaline phosphatase tests were negative in the epithelial cells and cell fragments. No mitotic figures or stem cell nests were observed during the entire period studied.

### 3.4. Lipid droplets

In all ages, big lipid droplets were observed in the epithelial cells of the seminal vesicles. These droplets had variable sizes, some of them reaching 4 µm in diameter. They were brown when mounted with 50% sucrose (Figs. 3A, B and 5A, B), but were transparent when mounted with Entellan (Figs. 4B, C) and empty after lipid removal (with xylene). These droplets were also observed in the cell structures of the seminal vesicle lumen (Figs. 4B, C) and in the post-vesicular deferent duct (Fig. 4F). They were negative to histochemical tests for sugar and protein detection.

Transmission electron microscopy revealed that these droplets were not surrounded by membranes and consisted of homogeneous, low-density material (Figs. 4D, 6A–C and 7A).

### 3.5. Membranous inclusions

Transmission electron microscopy revealed the presence of a great quantity of membranous inclusions. These inclusions were made of heterogeneous material and were present in the epithelial cells and luminal cell structures in males of all ages (Figs. 4D, 6A–F and 7A, B). The content and diameter of these inclusions varied during maturation: in recently emerged adults they are scattered,
with less than 1 µm in diameter and their content is electron dense with some electron lucid regions (Figs. 6A, B). These inclusions are more common in adults of about four days of age, where they are bigger and sometimes irregular in shape (Fig. 6C). They consist of totally or partially layered electron dense material, which covers irregular electron lucid figures, alternating with areas of median electron density (Figs. 6D–F). In sexually mature males, the inclusions can reach about 2 µm, and contain mostly medium electron dense material, with electron lucent regions and some layered electron dense material (Figs. 7A, B). At this stage, it is easy to observe membranous inclusions that appear to have fused (Fig. 7B).

4. DISCUSSION

The male reproductive system of F. schrottkyi is similar to that observed in other Meliponini bees and corresponds to type IV described by Ferreira et al. (2004), which is chiefly characterized by the absence of accessory glands.

In F. schrottkyi, spermatozoa reach the seminal vesicle only nine days after adult emergence, which differentiates this species.
from other known Meliponini. In *Melipona mondury* (Smith) (Lima et al., 2006) and *Scaptotrigona xanthotricha* (Moure) (Araújo, 2005), spermatozoa can be observed in the seminal vesicle on the fourth day, while in *M. quadrijasciata* (Lepeletier), they occur on the seventh day (Camargo, 1984). It is important
to note that even with the longer period that *F. schrottkyi* spermatozoa take to leave testes, the sexual maturity of these bees is reached almost at the same age as *M. mondury* (Lima et al., 2006), *S. xanthotricha* (Araújo, 2005), *Apis mellifera* (Linnaeus) (Snodgrass, 1978) and *Bombus terrestris* (Linnaeus) (Tasei et al., 1998), that is around 12 to 15 days after emergence.

As a rule, Aculeata release their spermatozoa from the testes in bundles, and they are individualized only in the seminal vesicles (Cruz-Landim, 2001; Zama et al., 2001, 2004, 2005; Moreira et al., 2004). On the other hand, spermatozoa of ‘symphyta’ wasps are not observed freed from spermatodesmata (Quicke et al., 1992; Schiff et al., 2001; Lino-Neto et al., 2008). However, in *F. schrottkyi* most of the bundles are disorganized already in the testes, therefore bundles or even bundle fragments are rarely observed in the seminal vesicles. This characteristic is probably related to the long period required for spermatozoan release from the testes, which must influence the spermatozoan maturation and activation process. Early bundle disorganization in the testes may also be related to the smaller size of the *F. schrottkyi* glycoprotein cap when compared with other species, or to some difference in its composition.

There are only few reports describing insect seminal vesicle morphology (e.g. Davis, 1968; Lai-Fook, 1982a; Couche and Gillott, 1988; Viscuso et al., 1999; Fausto et al., 2000), and only a minor set of these deals with Hymenoptera. The most complete papers for this order are those concerning *Camponotus* spp. (Wheeler and Krutzsch, 1992), *Melipona bicolor* (Lepeletier) (Dallacqua and Cruz-Landim, 2003), *M. mondury* (Lima et al., 2006) and *S. xanthotricha* (Araújo et al., 2005). In *Friesella schrottkyi*, the general morphology of the seminal vesicle is similar to that of other Meliponini. However some characteristics of this species have not been observed in any other Hymenoptera.

Secretory activity has been already reported in ducts of the male reproductive system of different insects. Apocrine secretion was observed in the seminal vesicles of Phasmoidea (Viscuso et al., 2005) and Orthoptera
Holocrine secretion was reported in *Drosophila* accessory glands (Perotti, 1971), in the ejaculatory duct of *Musca domestica* (Leopold, 1970; Riemann, 1973) and in the ejaculatory simplex of one Lepidoptera (Amaldoss, 1990). In seminal vesicles, holocrine secretion was suggested for two Orthoptera species based on different epithelium heights (Viscuso et al., 1999). In a *Camponotus* ant this type of secretion was suggested by the epithelium degeneration in males collected during nuptial flight (Forbes, 1954), however, it is not clear if this observation is related only to male senescence.

Some authors have already described bees’ seminal vesicle epithelial cells as changing from cylindrical to cubic and presenting apical bubble-like projections (apocrine secretion), during sexual maturation (Cruz-Landim and Cruz-Hofling, 1969; Araújo, 2005). However, drastic cellular changes, such as long apical projections and many nucleated cell fragments in the seminal vesicle lumen, have not yet been observed elsewhere in insects.

Mature males of *M. bicolor* (Dallacqua and Cruz-Landim, 2003) and *S. xanthotricha* (Araújo et al., 2005) did not present any secretory characteristics in their vesicle epithelium. But, in *F. schrottkyi* the seminal vesicle epithelium could be described as secretory during all stages of adult life, especially between the fourth and ninth day after emergence, when cell structures were observed in the vesicle lumen, which represents holocrine secretion. We suppose that the fibrous secretion found in the post-vesicular deferent ducts may play an important role in the seminiferous secretions. Despite the eventual presence of this secretion in the seminal vesicle, this secretion is apparently produced in the post-vesicular deferent duct and can sometimes flow back into the seminal vesicle.

It is possible to say that the big inclusions observed in the vesicle epithelium are lipid droplets, since they did not respond to tests for proteins and sugar and were preserved only after osmium post-fixation. Under the transmission electron microscope they were electron-lucid and showed no surrounding membrane. The presence of large lipid droplets has never been described in the seminal vesicle epithelium of other insects.

Possibly, the membranous inclusions revealed with electron microscopy, in *F. schrottkyi* vesicle epithelium, are analogous to those observed in *S. xanthotricha* (Araújo et al., 2005), *M. bicolor* (Dallacqua and Cruz-Landim, 2003) and *Camponotus* spp. (Wheler and Krutzsch, 1992). However, in these species the inclusions were observed only in sexually mature adults. These authors proposed that the inclusions must be related to secretion, or spermiophagy. Membranous inclusions (“myelin figures”) were also reported in the apocrine secretion of ejaculatory simplex in Lepidoptera and were related to spermatophore formation (Lai-Fook, 1982b). In *F. schrottkyi*, these inclusions were observed even in immature males and undergo morphological changes during sexual maturation. Since they did not react to acid phosphatase tests, and are also present in the cell fragments (holocrine secretion), these inclusions probably are components of this holocrine secretion.

The “late” release from the testes of already separated spermatozoa, freed from the bundle arrangements, as well as the presence of holocrine secretion in seminal vesicle, with two types of inclusions (lipidic and membranous) in the vesicle epithelium during all stages, are characteristics that differentiate *F. schrottkyi* from other Hymenoptera, including closely related genera within the Meliponini. Possibly these morphophysiological differences in the *F. schrottkyi* sexual maturation of males may reflect the evolutionary history of the group. Therefore, the study of these structures in other bee genera, such as *Plebeia*, would be interesting for comparisons between genera as well as to explain the reproductive physiology and systematics of these Hymenoptera.

5. CONCLUSIONS AND FUTURE AIMS

In order to understand spermatozoon maturation and activation in *F. schrottkyi*, the long period between adult emersion and the
beginning of spermatozoan release from the testes must be investigated. For example, in this species the flow through the ducts must be intense because of the “late” beginning of spermatozoan release and this could be related to a differentiated muscular sheath of the prevesicular deferent duct. The dynamics of the spermatozoa before reaching the seminal vesicle also must be investigated.

As the seminal vesicle epithelium presents holocrine secretion and we did not observe mitosis or stem cell nests in this epithelium, further investigation must be undertaken to explain how it regenerates, or even if the remaining cytoplasmatic fragments adhering to the basal lamina are efficient for epithelium maintenance. Also the biochemical characteristics of this holocrine secretion should be investigated in the future, in order to compare this secretion with that of accessory glands of other insects.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Dr. Lucio A.O. Campos (UFV) for the invaluable support given during the development of this work as well as two anonymous referees for very helpful comments on manuscript. This research had the financial support of the Brazilian agencies CAPES, FAPEMIG and FAPESP.


Obwohl bei F. schrottkyi die Spermiien die Samenblasen erst relativ spät erreichen, ist die sexuelle Reifung zum gleichen Zeitpunkt abgeschlossen, wie bei anderen Bienen. Die Individualisierung der Spermiien bereits in den Hoden ist ein Merkmal, in dem sich diese Art von anderen Aculeaten unterscheidet (Moreira et al., 2004). Auch eine holokrine Sekretionsbildung durch das Epithel der Samenblase ist bisher bei keiner anderen stachellosen Biene beschrieben (Dallacqua and Cruz-Landim, 2003; Araújo et al., 2005). Weitere vergleichende Untersuchungen an stachellosen Bienen sind erforderlich, um diese bei F. schrottkyi erhobenen neuen Befunde vergleichend verstehen zu können.

Friesella schrottkyi / Samenblase / Morphologie / holokrine Sekretion / Hymenoptera / Reproduktionssystem

Friesella schrottkyi (Hymenoptera : Apidae): développe-ment histologique et physiologique au cours de la maturation sexuelle.
REFERENCES

Amaldoss G. (1990) Fine structure of the regional differentiation of the ductus ejaculatorius simplex (EJ1, EJ2, EJ3) along with the role of their secretions in the sperm activation and motility in two Noctuid species, Heliothis armigera (Hibbner) and Spodoptera litura (Fabricius) (Lepidoptera). II. Fine structure and function of simplex 2 (EJ2), Proc. Indian Acad. Sci. 99, 19–38.


REFERENCES


