Original article

Histochemistry of the mucus gland of *Bombus morio* Swedurus, 1787 (Hymenoptera, Apidae)

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Abstract

The male mucus gland is present in all species of bees, exception for meliponines. In bees, the function of the mucus glands is not certain so far and its contribution to form the female espermatecal fluid is not assured. With the aim to contribute to the knowledge of the mucus gland in bees we carried out a histochemical study of the gland in mature, adult males of *Bombus morio*, focusing on the histochemical nature of the secretion. The males were collected around the forest fragments into UFSCar, Campus Sorocaba. The glands were fixed and analysed under routine microscopy and for histochemistry techniques: Periodic Acid Schiff (PAS), Bromophenol Blue (BB), Sudan Black (SB) and Critical Electrolyte Concentration (CEC) variant. The results showed that the mucus gland is constituted by a pair of large, thick tubular structures, which presented their distal portion more dilated and corn shaped. The glandular cells are columnar and in the apical portion present several apocrine vesicles being released into the gland lumen. The histochemistry showed that gland secretion is very complex and contains protein (BB), neutral polysaccharide (PAS), as well as lipid (SB). The luminal gland secretion presents a background of homogenous content, presenting little dark dots stained by all techniques used for this work. The investigation of the nucleolar activity (CEC) showed that the mucus gland epithelium is evolved in protein synthesis, presenting nucleoli developed and much RNA in the cytoplasm.

Keywords: bee, development, *Bombus morio*, histochemistry, mucus gland.

1 Introduction

The male reproductive apparatus of insects presents a pair of testes constituted by variable number of solid filaments or seminiferous ducts, where the spermatogenesis proceeds within germ cell cysts. From each seminiferous tube leave thin, short prolongations that join in a single efferent duct. The last one converges into sinuous, tubular structure that forms the pre-vesicular deferent duct, following an expanded region or seminal vesicle and more distally the post-vesicular deferent duct. The post-vesicular deferent duct of each testes fusion in one duct, originating the ejaculatory duct. Associated to the male reproductive apparatus of most insects there are accessory glands of mesodermic or ectodermic origin, the mesadenial or the ectadenial glands, respectively. Therefore, the glands that open into the deferent ducts are mesadenial and those opening into the ejaculatory duct are ectadenial (SNODGRASS, 1935; CHAPMAN, 1988; FERREIRA, ABDALLA, KERR et al. 2004).

The accessory glands are present in all species of bees, exception for meliponines. In meliponines the seminal vesicles seem to play the role of the mucus gland (DALLACQUA and CRUZ-LANDIM, 2003).

According to Ferreira, Abdalla, Kerr et al., (2004) classification, the testes of *Bombus* belong to the type II, which is characterized by very long post-vesicular deferent ducts that are located outside the scrotal membrane. The remaining internal genital organs, excepting the ejaculatory duct and the accessory glands, also form a globular unit encapsulated by the scrotal membrane, which is elongated with a median constriction "U" or "S" shaped. In this type of testis, the bee species present four seminiferous ducts and the mucus gland is large.

The secretion of the mucus accessory gland usually is transmitted to the insect female during copulation, through spermatophore introduction or by secretion deposition into the *bursa copulatrix*, or *genital chamber*, of the female. Their function in bees and other insects is not definitely conclusive. In some cases, after the copulation and into the female genitalia the mucus gland secretion may suffer some chemical changes, becoming a hard, viscous mass, which forms a type of barrier against multiple mates, ensure unique paternity by physical prevent the female mates with other males, originating a plug-in like into the female genitalia (BAER, MAILE, SCHMID-HEMPEL et al., 2000). Some authors attribute to the proteinaceous content of the mucus gland role in supply energy and aid in sperm capacitation into female spermatecae. Altogether with the female spermatecal gland, the mucus gland secretion could compose the spermatecal gland fluid in fecundated females (CHEN, 1984; GILLOTT, 1996).

In *Drosophila melonogaster* the mucus gland secretion has long-term effect on the female reproduction physiology (WOLFNER, 1997), by crossing the vaginal membrane to reach the female neuroendocrine axis through hemocele flow. According some authors, the mucus gland may stimulate the juvenile hormone (JH) biosynthesis by the *corpora allata* (LUNG and WOLFNER, 1999; MOSHITZKY, FLEISCHMANN, CHAIMOV et al., 1996; FAN, RAFAELI, GILEADI et al., 1999; SOLLER, BOWNES, and KUBLI, 1997). In eusocial bees the *corpora allata* is lager in queens than in workers, but its activity as modulator of the ovary development in adults using JH is not ensured (BEIG and

CRUZ-LANDIM, 1974; BEIG and BALDISSERA, 1974; CRUZ-LANDIM and HÖFLING, 1972; HARTFELDER and ENGELS, 1998; CRUZ-LANDIM, 2008).

The start to ovary development is undoubtedly the mating, but we still do not know how the manner that the mate effectively affect the physiology of queen ovary. In Africanised honeybee queens, the ovary development is continuous until they are able to mate, after that markedly degeneration process is seen on the ovaries, as well as, on the reproductive accessory Dufour gland in non-mated virgin queens (ABDALLA, 2006; BERGER and ABDALLA, 2005).

According Colonello and Hartfelder (2003), in Africanized honeybees the mucus gland changes the protein content from immature to adult drones. The authors noticed that from 2 days old adult drones there is an increase in protein content of adult males, while from 5 days old the protein content of the mucus gland decrease, stabilizing at around 8 days old. According to the authors spite of the protein content diminish, the complexity of those proteins increase during the male adult life cycle. The authors suggest that the ecdysteroids may inhibit the protein synthesis by the mature mucus gland of adult, since its titer in hemolymph decreases at 5 days old. On the other hand, in males the JH titer in hemolymph increases after 5 days old.

According Baer, Den Boer, Boomsma et al. (2009) the female spermatecal fluid of *Apis mellifera* differs between virgin and fecundated queens. The mated queens present more specific proteins than the virgin queens into the spermatecae, but the authors can not ascertain if this protein content is due the mating. Only 5 proteins from male ejaculate are also present in mated queens. According to the authors, from 122 proteins found in spermatecal fluid, only 19 is found also in seminal fluid, some of them also found in virgin queens (17 proteins). Baer, Den Boer, Boomsma et al. (2009) suggest that the same proteins produced by the mucus gland is produced by the spermatecal gland, therefore a contribution of the mucus gland either to contribute to the spermatecal fluid and/or to change the ovarian cycle of the post-fecundated female does not be concluded in *A. mellifera*.

Histochemical and morphological studies of the mucus gland in Africanised honeybees showed that the secretion is composed by proteins and neutral polysaccharides. Additionally, the secretion is released to the mucus gland lumen by apocrine vesicles (CRUZ-LANDIM and DALLACQUA, 2005). According Dallacqua and Cruz-Landim (2003) the protein content of seminal vesicles of *Melipona bicolor*, *Scaptotrigona postica* and *Apis mellifera* present low homology, being common for all of them the presence of a protein of 47.5 kDa.

There is few investigation of the mucus gland in other species of bees, exception for *Bombus terrestris*. To contribute to the knowledge of this subject it was carried out a histochemical study of the mucus gland of mature adult in *Bombus morio* with the aim to verify the gland morphology and its histochemistry nature.

2 Material and methods

Collection and Histological Preparation of the Material: The males were collected in the forest fragments into UFSCar, Campus Sorocaba, on September 2010, 23° 35' 3" S and 47° 31' 36" W. Immediately, the bees were transferred to amber glasses and left 5 minutes in 4 °C to be dissected and extracted their whole reproductive apparatus, which was fixed in 4% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.2. After fixation, the material was embedded in resin JB - 4 (Polysciences) according to the fabricant recommendations. The resin was polymerised at room temperature. Histological sections of 2 µm thick were made by a microtome Leica (RM 2255). The material was analysed in photomicroscope Leica (DM 1000).

Histochemistry Techniques (BEHMER, DE TOLOSA and DE FREITAS NETO, 1976; JUNQUEIRA and JUNQUEIRA, 1983): Histological sections of 2 µm were stained with mercury bromophenol blue for total protein, Sundan Black for lipid detection, and submitted to Periodic Acid-Schiff reaction (PAS) before treatment with amylase for neutral polysaccharides. Critical Electrolytic Concentration variant for RNA detection was also applied. The histological sections were made in histomicrotome Leica (RM2255) with 2 µm. The material was analysed in photomicroscope Leica (DM 1000). The muscle fibbers that evolve the whole mucus gland were used as control for all histochemical techniques, additionally previous observations with hematoxylin and eosin. For variant of CEC procedures, histological sections were stained with toluide blue pH 4. Some of histological preparations with the same section region were not submitted to the CEC-variant procedures (control) and others were incubated in magnesium chlorite 0.005 M according Mello and Vidal (1989). For all histological analysis more the three individuals were used.

3 Results

The mucus gland is constituted by a pair of large, thick tubular structures, which presented their distal portion more dilated. Besides all tubular epithelial wall showed secretory features. The glandular cells are columnar and in the apical portion present several apocrine vesicles being released into the gland lumen (Figure 1a).

The histochemistry showed that gland secretion is very complex and contains protein, neutral polysaccharides, as well as lipids (Figure 1b-f). The bromophenol blue (BB) for total proteins showed that both epithelial cells and luminal secretion are strongly reactive to this technique (Figure 1b). The apical apocrine vesicles also present protein content into them, but do not so strongly stained in relationship with the cell portion and the luminal secretion (Figure 1b,c). The luminal secretion is not homogeneous, when stained with BB it presents a weaker stained region lining the whole periphery of the gland and a stronger stained inner portion, bounded by the first (Figure 1b,c). Spread among the peripheral region it was observed some dark dots both stained strongly by BB as PAS (Figure 1b-d). The gland epithelium also was stained by PAS for neutral polysaccharides (Figure 1d). Conversely to the bromophenol blue technique, the glandular cells are stronger stained at the basal portion than the apical portion by the PAS (Figure 1d). The apocrine vesicles are not evident by the PAS, but same dots observed into the secretion of the luminal gland, also observed by the BB, are strongly stained by PAS (Figure 1d). The luminal secretion presented an inverted pattern from that observed with bromophenol blue, e.g., the peripheral region that lining all

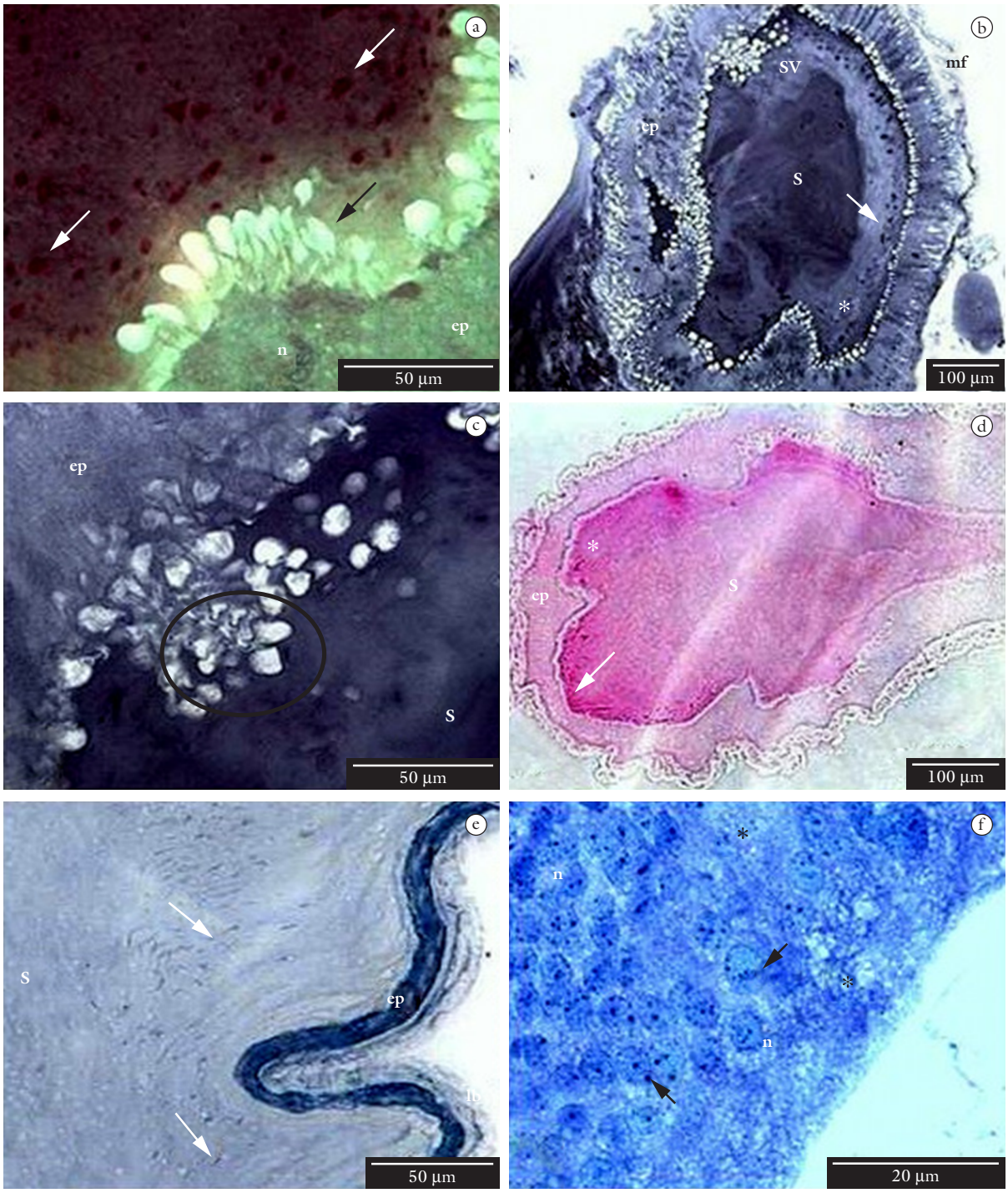

Figure 1. Micrographs of the mucus gland of *Bombus morio*. a) Detail of the mucus gland epithelium (ep) showing at the apical portion the cells apocrine vesicles (dark arrow) to the gland lumen. Notice little acidophilic dots (white arrows) and the homogenous secretion background (s), HE. b) Cross section of the mucus gland, showing the epithelium (ep) stained with bromophenol blue (BB). Notice the columnar epithelial cells (ep) and the intense apocrine secretion activity by numerous secretion vesicles (sv). Notice that the band less stained with the BB lining the apical portion of the epithelium and also present some strongly stained dots (asterisk). Upper the pale band, notice the strongly stained background of homogenous secretion in the gland lumen (s). c) Detail of the Figure b, showing the gland cells apical portion releasing apocrine vesicles (cycle), mf = muscular fibers. d) Cross section of the mucus gland stained by PAS, showing the gland epithelium (ep) stained by PAS. Notice a stronger PAS stained band lining the inner gland epithelial wall (asterisc) with strongly stained dots (white arrow). The muscular fibers (mf) were not stained due the previous amylase exposure of the material. Notice that the inner luminal secretion is less stained (s). e) Detail of gland epithelium (ep), showing the cuticle strongly stained by Sudan black, as well as, basal lamina (bl), the epithelium and the luminal secretion (s). Notice some lamellar structure more strongly stained (white arrows) and the homogenous secretion background. f) Tangential section of gland epithelium (ep) showing the orthochromatic nuclei (n) with one or more nucleoli (nu) by CEC-variant. Notice that the cytoplasm of the gland cells is intensively stained by the toluidine blue, indicating abundant presence of RNA content (asterisk).

the whole gland lumen was stronger stained by PAS than the inner region (Figure 1d). The gland epithelium was stained strongly for the Sudan black (SB) technique, showed great affinity for lipids (Figure 1b). The gland secretion also was stained by the SB (Figure 1e), presenting a homogeneous background as showed by both BB and PAS (Figure 1b-e). In this case, it additionally to the strongly stained dots, also parallel, concentric lamellae lining all the peripheral portion of the gland lumen (Figure 1e).

The investigation of the nucleolar activity (CEC-variant) showed that the gland cells present nuclei with variable number of nucleoli, most of them with evident granular and fibrillar regions (Figure 1f). Some RNA material was observed close to the nucleus envelope inner surface and the cytoplasm present abundant RNA content. In this technique the critical point is reach when the whole nucleus previously stained by toluidine blue became orthochromic, e.g., it changes its colour (from blue to green) due the substitution of toluide blue for magnesium molecules. When the nucleus became green, all structures maintained blue (without toluidine molecule exchange for magnesium) is RNA, since the competition and exchange of the toluidine for magnesium molecules starts first on the DNA molecule. For the mucus gland the critical point was reach at 2.5 minutes.

4 Discussion and conclusion

In *Bombus terrestris* it were found four principal fatty acids in the mucus gland secretion (palmitic, linoleic, oleic, and stearic acids), whose according to Baer, Maile, Schmid-Hempel et al. (2000) can form the mating plug-in the female genitalia after the semen deposition by the male first mating, avoiding other copulations. In addition, a cyclic peptide (cycloprolylproline) was also found as predominant compound of the mucus gland in males of *B. terrestris*. The peptides could play role diminishing the female receptivity to other mating (CHEN, ZOLLINGER, AIGAKI et al., 1988).

The present results showed that the mucus gland secretion of mature males of *Bombus morio* is composed by three principal general compounds, acid and neutral glycoproteins (PAS, bromophenol blue) and lipids (Sundan black). These results are in accordance with mucus gland secretion composition found by other authors in *B. terrestris* and Africanised honeybee (COLONELLO and HARTFELDER, 2003; CRUZ-LANDIM and DALLACQUA, 2005; BAER, MAILE, SCHMID-HEMPEL et al., 2000).

The presence polysaccharides by PAS indicates that the total protein (bromophenol blue) of the mucus gland secretion in *B. morio* is glycoproteins, since the material was previously exposed to amylase to avoid staining of all free polysaccharides, as glycogen per example. The secretion affinity for the Sudan black indicates the presence not only of lipids, but also could suggest the presence of some glycolipids. The mucus gland secretion of *Bombus morio*, as in *A. mellifera* (COLONELLO and HARTFELDER, 2003; CRUZ-LANDIM and DALLACQUA, 2005) may present more than one kind of protein or polypeptide, which may be conjugated with polysaccharides and lipids.

The gland secretion is not homogeneous, presenting little dots inside the gland lumen, detected for all histochemical techniques used. These dots may compose the apocrine vesicles detached from the glandular epithelium, since they are stained also by Sudan black. Besides these little dots, most of the luminal secretion presents homogeneous background, but lining all the gland lumen we observed bands stained more or less intensively, depending on the histochemistry technique used. The band formed by bromophenol blue is lesser stained, whereas the secretion right before this band is strongly stained by bromophenol blue. The band formed by the PAS technique showed just inverse pattern observed with bromophenol blue, being stronger stained in the region that lining the gland epithelium and lesser stained inner the lumen. This fact may indicate that the homogeneous portion of the secretion into mucus gland lumen in *B. morio* may suffer some kind of post- tradutional process into the gland lumen. As observed by Colonello and Hartfelder (2003) in Africanised honeybee, the number of mucus gland diminishes, but their complexity increase according the drone life cycle.

The investigation of the nucleolar activity (CEC variant) is in accordance with the results of the histochemistry techniques. The mucus gland epithelium is evolved in protein synthesis, presenting nucleoli developed and much RNA in the cytoplasm. These RNA certainly are the responsible by the protein synthesis and protein content into the glandular lumen.

From these results, more detailed studies should be done to investigate more precisely the molecular composition of the mucus gland of *B. morio*, as well as, the molecular changes that probably occur in the protein molecules into the glandular lumen. Molecular studies with this gland by SDS-PAGE are carried out.

Preliminary study of the female spermatecal gland showed that there is no correspondence of the histochemistry for polysaccharides and lipids between the spermatecal gland and the mucus gland of the same species (Marcondes, personal communication).

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