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Morphology and protein patterns of honey bee drone accessory glands

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ABSTRACT. We used light and transmission electron microscopy to examine the morphology of the accessory glands of immature and mature adult males of *Apis mellifera* L. We also made an electrophoretic analysis of the protein content of the mature gland. The glands of the immature male actively secrete a mucous substance that can be seen in the lumen of the gland of the mature male. This secretion stains with mercury bromophenol blue and with periodic acid-Schiff reaction, which stain glyconjugates. The protein content was higher in the lumen secretion than in the gland wall extracts. The electrophoresis patterns of the wall extracts were different from those of the secretion found in the gland lumen.

Key words: Bee, *Apis mellifera*, Reproductive apparatus, Mucus gland

INTRODUCTION

The male accessory genital glands of insects may be ectodermal or mesodermal in origin, known as ectadenia and mesadenia, respectively. The number and arrangement of the accessory glands vary considerably between the different groups of insects. In some insects, as in Lepidoptera, there is regional differentiation and the production of two kinds of secretion, while in Orthoptera there are 7 to 15 pairs of glands that seem to produce a single kind of secretion (Chapman, 1998).

Each accessory gland consists of a single layer of epithelial cells, the fine structure of which depends on their stage of development and the nature of the secretion produced (Chapman, 1998). There is a muscular layer outside the epithelium, constituted of a variable number of sheets of muscular fibers. These muscles are generally innervated (Kimura et al., 1989).

The accessory glands become functional in the adult insect, and their secretion is involved in several mechanisms linked to reproduction, such as spermatophore production. The chemicals produced by the accessory glands are transferred to the female during copulation, and they frequently have a long-term effect on her reproductive behavior and physiology (Chen, 1984; Happ, 1984, 1992; Schooneveld et al., 1997; Wolfner, 1997; Gillot, 1998).

The honey bee reproductive tract includes a pair of accessory glands, the mucus glands, outgrowths of the posterior portions of the vasa deferentia (Snodgrass, 1956; Simpson, 1960; Kapil, 1962). These glands increase during the first nine days after male emergence. During, maturation the secretion changes; it acquires a slightly alkaline, viscous consistence and the property of immediately coagulating when in contact with air (Bishop, 1920). Consequently, this secretion serves as copulatory plugs in the female tract, whose primary function would be to prevent mating with other males, assuring paternity (Bishop, 1920; Bairate and Perroti, 1970). In addition, the mucous secretion serves as an energy source and aids in sperm capacitation and storage (Chen, 1984; Gillot, 1996).

According to Blum et al. (1962, 1967), most of the male reproductive gland secretion constituents in bees are proteins; but smaller molecules, including sugars and lipids, are also present. Despite the large amount of data on the postcopulatory stimulation of female reproduction by the male accessory gland in other insects, practically no attention has been paid to the mucus gland potentialities in bees, and few components have been characterized in its secretion (Allalouf et al., 1974; Baer et al., 2001). Nevertheless, postcopulatory stimulation of oogenesis and oviposition is also found in the honey bee (Koeniger, 1986; Melo et al., 2001; Patricio and Cruz-Landim, 2002).

The accessory glands appear to be present in the genital tract of most bee species (Ferreira, 1966), but in the meliponines, which are eusocial stingless bees, they are absent (Kerr, 1948; Ferreira, 1966; Dallacqua and Cruz-Landim, 2003). Honey bee drones are only capable of mating 8-10 days after emergence, which is also the time that the mucus gland takes to become filled with secretion (Bishop, 1920).

We made a morphological analysis of the mucus glands of sexually immature and mature drones. We also examined the gland wall extracts and secretion with electrophoresis.

MATERIAL AND METHODS

Mucus glands were dissected from young (two days old), sexually immature adult male

bees (*Apis mellifera*) and in sexually mature, 15-day-old adult males, maintained in colonies in Rio Claro, SP, Brazil.

Light microscopy

Three pairs of glands of each type of male were fixed for light microscopy (LM) studies in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.0. After dehydration, the glands were embedded in Leica historesin. Sections, 5 mm thick, were stained with hematoxylin and eosin, mercury bromophenol blue and submitted to acid periodic acid-Schiff (PAS) reaction.

Transmission electron microscopy

Another three pairs of glands were prepared for transmission electron microscopy (TEM) by fixing in 2% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. After washing in the buffer, the glands were post-fixed in 1% osmium tetroxide in the same buffer. The glands were dehydrated in an increasing concentration series of acetone and embedded in Epon Araldite. At the beginning of the dehydration, the glands were left overnight in 2% uranyl acetate in 30% acetone. The thin sections were stained with lead citrate.

Protein dosage and electrophoresis

For protein quantification, the electrophoresis glands were cold dissected from mature males and the glandular tissue and luminal content (secretion) separated and individually placed in individual Eppendorfs that were stored at -20°C. Two dilutions were used for the protein dosage; 80 or 100 µL of distilled water was added to the Eppendorfs containing the glandular tissue and the mucus. This material was homogenized and the vials containing the glandular tissue extracts were centrifuged at 10,000 rpm, for 10 min, at 5°C. Aliquots of the supernatants of the centrifuged extracts and of the mucus homogenates were dosed for protein content (Bradford, 1976), using bovine serum albumin as a standard.

The electrophoresis was carried out in a polyacrylamide gel, 5-20% gradient, under denaturating conditions (SDS-PAGE), according to Hames and Rickwood (1990). Samples containing 20-40 µg of proteins diluted 1:1 in the buffer were boiled in a water bath for 5 min, and after they had been cooled on ice, they were applied onto the polyacrylamide gel. A standard with known molecular weights (new England Bio Lab) was used for the molecular mass calculation (MBP β-galactosidase - 175 kDa; MPB paramyosin - 83 kDa; glutamine dehydrogenase - 62 kDa; aldolase - 47.5 kDa; triphosphate isomerase - 32.5 kDa; β-lactoglobulin - 2 kDa; lysozyme - 16.5 kDa, and aprotinin - 6.5 kDa. After electrophoresis, the gel was stained with Coomassie blue R-250 at 1%.

RESULTS

Morphology

Under LM, the mucus gland of the immature male presents an empty lumen, formed by an epithelium of very tall and slender cells (Figure 1A). The TEM shows a cell cytoplasm rich

in rough endoplasmic reticulum and already presenting secretion granules (Figure 1B). At the basal pole of the epithelial cell, the granules are spherical and contain a dense core surrounded by a clear halo. The apical pole of the cells contains the lumen of the vesicular rough endoplasmic reticulum cisternae filled with a flocculent, moderately electron-dense material, which also appears to occupy the gland lumen. Very long microvilli project into the lumen. The apical cell contacts are reinforced by junctional complexes (Figure 2).

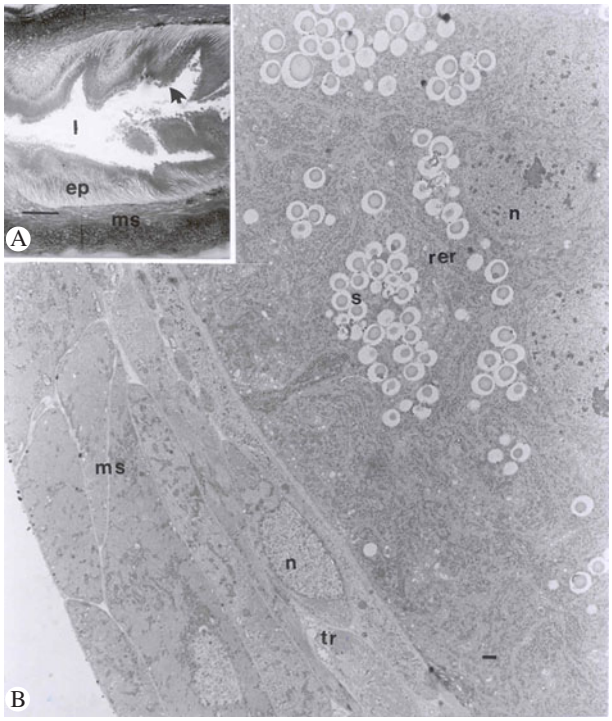

Figure 1. The immature male honey bee accessory gland. **A.** Light microscopy section of the gland showing the high epithelium (ep) and the muscular sheath (ms). The arrow points to secretion delivery. l = lumen. Bar = 100 µm. **B.** Transmission electron micrography showing the epithelial cells containing secretion (s) and the surrounding muscular sheath (ms). n = nuclei; rer = rough endoplasmic reticulum; tr = tracheoli. Bar = 1 µm.

Outside the epithelium, there is a muscular sheath, constituted of several layers of muscle fibers, among which tracheolar and nervous branches are seen (Figure 1A, B).

The LM of the gland of the mature male has very flat gland epithelium, and the lumen is filled with secretion. The secretion stained strongly with bromophenol blue and the PAS reaction (Figure 3A, B); however, the epithelial cells practically do not stain with these reactions.

The TEM also shows the flattening of the epithelium and secretion in the lumen (Figure 3C, D). In the very flat cytoplasm, only some profiles of dilated cisternae of endoplasmic reticulum, with a tubular aspect, full of secretion, are seen. The cells are covered by a dense lining of microvilli (Figure 3C).

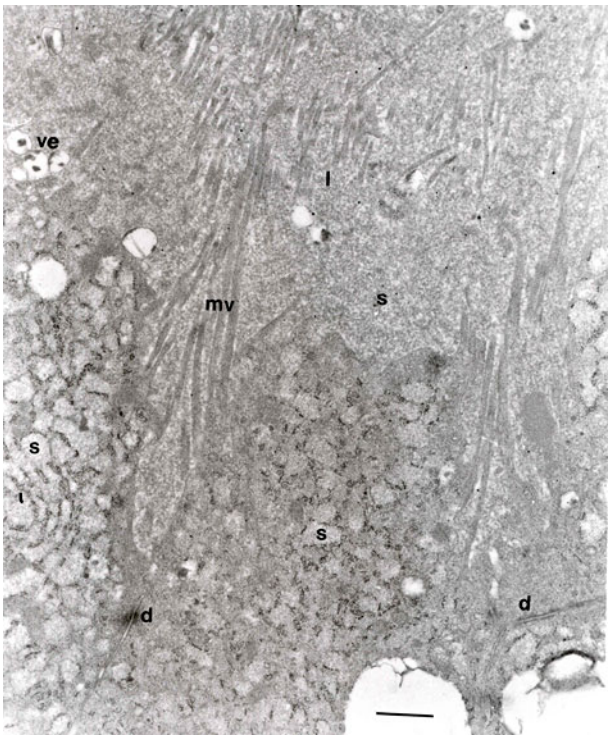

Figure 2. Transmission electron micrography of the apical region of an epithelial cell of the immature male honey bee accessory gland showing secretion (s) within the rough endoplasmic and in the lumen (l). ve = vesicles; mv = microvilli; d = desmosome-like cell junction. Bar = 1 µm.

Electrophoresis

More protein was found in the glandular tissue extracts (80-120 µm/gland pair), than in the luminal mucus (50-60 µg/gland pair).

The pattern of the water soluble proteins analyzed by SDS-PAGE showed a greater variety of protein bands in the gland wall extracts, where the bands are also more prominent than in the mucus (Figure 4).

The molecular mass of the gland wall extracts ranged from 14 to 95 kDa, while in the mucus it varied from 12 to 150 kDa. There was strong protein banding from 35 to 45 kDa, which was also, with slight differences (32 to 37 kDa), present in the gland wall extracts (Figure 4).

It was necessary to use the content from more than one pair of glands for the electrophoretic separation of the proteins. Despite the variations in the sample dilutions, the pattern of the band distribution was not affected (data not shown); only a few bands show up with great intensity in more concentrated samples.

DISCUSSION

The morphological analysis of the mucus gland of honey bee drones revealed an age-dependent developmental profile. Based on the histological and ultrastructural features of the

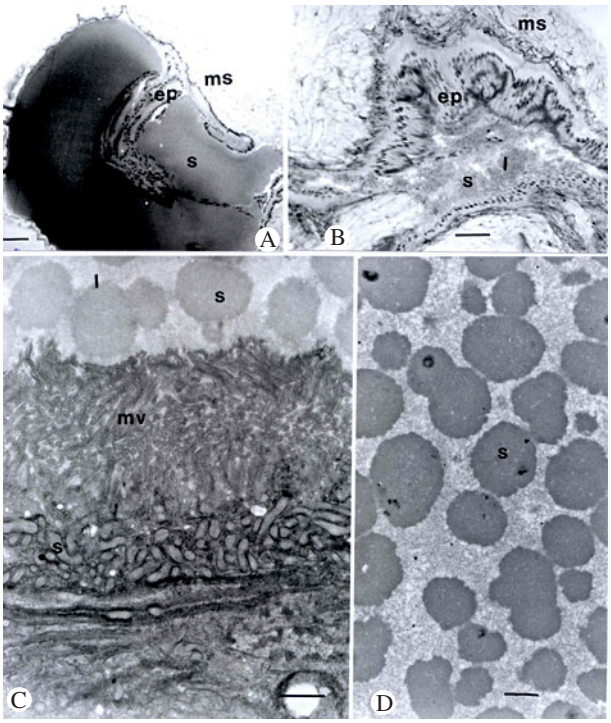

Figure 3. The mature male accessory gland. **A.** Light micrography of a cross-section of the gland showing the very low epithelium (ep) and secretion in the lumen stained by bromophenol blue. ms = muscular sheath; s = secretion. Bar = 50 µm. **B.** Light micrography of a tangential section showing the unstained epithelium (ep) and muscle layer (ms) when stained with the periodic acid-Schiff reaction. l = lumen; s = secretion. Bar = 50 µm. **C.** Transmission electron micrography of the gland wall showing the flat epithelium containing rough endoplasmic reticulum filled with secretion (s) and the lumen (l) containing secretion globules. mv = microvilli. Bar = 1 µm. **D.** The secretion (s) in the gland lumen. Bar = 1 µm.

mucus glands, the immature males already begin producing secretion in the gland cells. This secretion has a mucous aspect in the cells of the immature males and in the lumen of the sexually mature male.

The accumulation of secretion in the lumen distends the gland, promoting a flattening of the epithelium (Figure 3). Based on the ultrastructural aspect of the cells of this gland, it is no longer secreting. The secretion found in the cells has not yet been discharged into the gland lumen. Therefore, all the secretion was produced before male sexual maturation. Gland inactivity in the mature male is justified, because during mating the genitalia is lost and the male dies.

The staining of the secretion with bromophenol blue and PAS reaction is compatible with the glycoprotein nature of the secretion, which is also apparent from the ultrastructural aspect and the observed mucal consistency of the secretion.

The discrepancies between the gland extracts and the secretion may be due to the fact that cellular components, such as structural proteins and enzymes, from the epithelial and muscular cells of the lumen wall, are present in the extracts. However, the finding of mucus material not found in the gland extracts is more difficult to explain. The inclusion of substances from other parts of the reproductive tract does not appear to be possible, given the glands condition as a lateral outgrowth of the vasa diferentia. The explanation could be that secretion maturation in

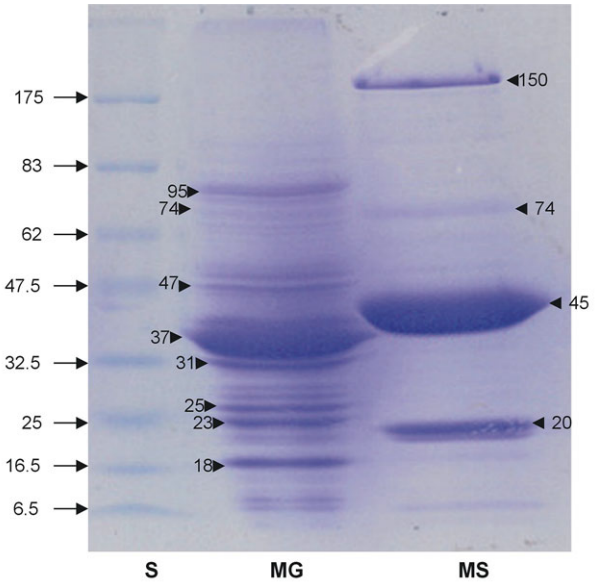

Figure 4. Electrophoresis pattern of the gland and mucus extracts. MG = mucus gland extracts; MS = mucus secretion from a mature male of *Apis mellifera*. The molecular masses are indicated by the arrows. S = standard.

the gland lumen promotes changes in its composition. The smaller number of bands in the secretion could be due to the lower complexity of the protein composition in the sexually mature males, when compared to immature males (Colonello and Hartfelder, 2003).

The mucus gland of *A. mellifera* initiates secretory activity in the newly emerged male; in the mature male, the lumen is full of secretion, while the secretory activity in the cells is low or absent. The protein profiles of the gland wall and stored mucus suggest secretion maturation in the gland lumen.

The bees are an interesting system for investigating how male-produced substances regulate reproductive biology. Copulation can produce reactions through mechanical stimulation, as seems to be the case for stingless bees (Melo et al., 2001), which do not have the male accessory glands (Kerr, 1948; Ferreira, 1966; Dallacqua and Cruz-Landim, 2003), or through active factors transmitted during mating (Koeniger, 1976, 1981). The main function of the compounds passed from insect males to females during copulation seems to be manipulation of female reproductive biology in order to assure paternity. Nevertheless, *A. mellifera* is a polyandrous specie and thus, whatever the substances introduced in the female genital tract with the spermatozoa, it will not be capable assuring sole paternity. These substances can have multiple roles in insects; in the polyandrous bees, they may have been transformed into an oocyte maturation stimulus.

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REFERENCES

Allalouf, D., Ber, A. and **Ishay, J.** (1974). Hyaluronidase in the mucus accessory glands of the drone (*Apis mellifera*). *Experientia 30*: 853-855.

Baer, B., Morgan, E.D. and **Schmid-Hempel, P.** (2001). A nonspecific fatty acid within the bumblebee mating plug prevents females from remating. *Proc. Nat. Acad. Sci. USA 98*: 3926-3928.

Bairate, A. and **Perroti, M.E.** (1970). Occurrence of a compact plug in the genital duct of *Drosophila* female after mating. *Drosophila Inf. Serv. 45*: 67-68.

Bishop, G.H. (1920). Fertilization in the honeybee. I. The male sexual organs: their histological structure and physiological functioning. *J. Exp. Zool. 31*: 225-265.

Blum, M.S., Glowska, Z. and **Tauber III, S.** (1962). Chemistry of the drone honeybee reproductive system. II. Carbohydrates in the reproductive organs and semen. *Ann. Entomol. Soc. Am. 55*: 135-139.

Blum, M.S., Baugarner, J.E. and **Tauber III, S.** (1967). Composition and possible significance of fatty acids in lipid classes of honeybee serum. *J. Insect Physiol. 13*: 1301-1308.

Bradford, M.M. (1976). A rapid and sensitive method to the quantification of microgram quantities of protein utilizing the principle of the quantification binding. *Anal. Biochem. 72*: 248-254.

Chapman, R.F. (1998). *Insects: Structure and Function*. Cambridge University Press, Cambridge, UK.

Chen, O.S. (1984). The functional morphology and biochemistry of insect male accessory glands and their secretions. *Ann. Ver. Entomol. 29*: 233-255.

Colonello, N.A. and **Hartfelder, K.** (2003). Protein content and pattern during mucus gland maturation and its control in honey bee drones. *Apidologie 34*: 257-267.

Dallacqua, R.P. and **Cruz-Landim, C.** (2003). Ultrastructure of the male reproductive tract of males of *Melipona bicolor bicolor* Lepeletier (Hymenoptera, Apinae, Meliponini). *Anat. Histol. Embryol. 32*: 276-281.

Ferreira, A. (1966). Contribuição para o estudo da evolução das abelhas (Hymenoptera, Apoidea). Aparelho reprodutor masculino. Ph.D. thesis, Faculdade de Filosofia, Ciências e Letras de Rio Claro, Rio Claro, SP, Brazil.

Gillot, C. (1998). Arthropoda - Insecta. In: *Reproductive Biology of Invertebrates* (Adiyodi, K.G. and Adiyodi, R.G., eds.). Vol. 3. Wiley, New York, pp. 319-371.

Gillot, C. (1996). Male insect accessory glands: functions and control of secretory activity. *Invertebr. Reprod. Dev. 30*: 199-205.

Hames, B.D. and **Rickwood, D.** (1990). Gel electrophoresis of proteins; a practical approach. 2nd edn. IRL Press, Oxford, UK, pp. 119-122.

Happ, G.M. (1984). Accessory gland development in mealworm beetle. In: *Molecular Entomology* (Law, J., ed.). Liss, New York, pp. 433-442.

Happ, G.M. (1992). Maturation of the male reproductive system and its endocrine regulation. *Ann. Ver. Entomol. 37*: 303-320.

Kapil, R.P. (1962). Anatomy and histology of the male reproductive system of *Apis indica* (Apidae, Hymenoptera). *Insectes Soc. 9*: 73-90.

Kerr, W.E. (1948). Estudos sobre o gênero *Melipona*. Ph.D. thesis, Escola Superior de Agricultura Luiz de Queiroz, USP, Piracicaba, SP, Brazil.

Kimura, T., Yasuyama, K. and **Yamaguchi, H.J.** (1989). Protocolinergic innervation of the accessory gland in male crickets (*Gryllus bimaculatus*) detection of proctolin and some pharmacological properties of myogenically and neurogenically evoked contractions. *J. Insect Physiol. 35*: 251-264.

Koeniger, G. (1976). Einfluss der Kopulation auf den Beginn der Eiablage bei der Bienenkönigin (*Apis mellifera* L.). *Apidologie 7*: 343-355.

Koeniger, G. (1981). In welchem Abschnitt des Paarungsverhaltens der Bienenkönigin findet die Induktion der Eiablage staat. *Apidologie 12*: 329-343.

Koeniger, G. (1986). Mating sign and multiple mating in the honeybee. *Bee World 67*: 141-150.

Melo, G.A.R., Buschini, M.L.T. and **Campos, L.A.O.** (2001). Ovarian activation in *Melipona quadrifasciata* queens triggered by mating plug stimulation (Hymenoptera, Apidae). *Apidologie 32*: 355-361.

Patricio, K. and **Cruz-Landim, C.** (2002). Mating influence in the ovary differentiation in adult queens of *Apis mellifera* L. (Hymenoptera, Apidae). *Braz. J. Biol. 62*: 641-649.

Simpson, J. (1960). Male genitalia of *Apis* species. *Nature 185*: 56.

Schooneveld, H., de Kort, C.A., Koopmanschap, A.B. and **Smid, H.M.** (1997). A peptide from the male accessory gland in *Leptinotarsa decemlineata*: purification, characterization and molecular cloning. *J. Insect Physiol. 43*: 355-362.

Snodgrass, R. (1956). *Anatomy of the Honeybee*. Comstock Publishing Associates, Ithaca, New York, pp. 334.

Wolfner, M.E. (1997). Tokens of love: functions and regulation of *Drosophila* male accessory gland products. *Insect Biochem. Mol. Biol. 27*: 179-192.

Yi, S.X. and **Gillot, C.** (1999). Purification and characterization of an oviposition-stimulating protein of the long hyaline tubules in the male migratory grasshopper, *Melanoplus sanguinipes*. *J. Insect Physiol. 45*: 143-150.