Synthesis and chemical composition of mucus gland secretions in Apis cerana indica

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Original article

Synthesis and chemical composition of mucus gland secretions in *Apis cerana indica**

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Abstract – The columnar epithelial cells of the mucus gland begin to synthesize secretory material in the late pupal stage, and this material gradually accumulates in the lumen, beginning soon after emergence of the adult drones. Histochemical tests demonstrated secretory activity in the epithelial cells and revealed the biochemical nature of the secretions as a mixture of proteins, carbohydrates and lipids. Total proteins, lipids and carbohydrates were detected in concentrations of 333.2 ± 13.883, 208.60 ± 11.69 and 44.82 ± 2.94 μg/mg, respectively, showing that proteins form the major constituents of the mucus gland secretory material. SDS-PAGE of mucus gland secretory material revealed about 15 proteins of molecular weight ranging from 2.5 to 151.2 kDa. Three proteins of 45, 43 and 37 kDa were stained intensely and can be considered as the major class of mucus proteins.

mucus gland / protein profile / *Apis cerana indica*

1. INTRODUCTION

In honeybees, the mucus glands represent the primary male accessory glands. The glands of adult drones secrete a protein-rich viscous fluid soon after emergence. During mating, the secretion has multiple functions, such as aiding in sperm transfer, providing a glue that keeps the drone's copulatory organs attached to the queen, and forming the major part of the mating sign (Snodgrass, 1956; Woyke, 1956; Woyke and Ruttner, 1958; Blum et al., 1962, 1967; Koeniger et al., 1989, 1996; Wyatt and Davey, 1996; Colonello and Hartfelder, 2003, 2005; Cruz-Landim and Dallacqua, 2005; Tozetto et al., 2007).

Most of the information on the structure, development and functions of mucus glands in honey bees is confined to *Apis mellifera.* In India, *Apis cerana indica* F. is widely domesticated and it is a dominant hive-bee of the apiculture industry. To our knowledge, however, only meager information on the reproductive physiology of *A. cerana indica* is available. The present histological, histochemical and biochemical study was, therefore, undertaken to investigate the structure of the mucus glands and to obtain information on the synthesis and chemical composition of the secretory material (mucus) in this species of the honey bee.

2. MATERIAL AND METHODS

Bees were collected from a hive established on the premises of the Department of Zoology, RTM Nagpur University, Nagpur (India).

2.1. Histological and histochemical methods

The mucus glands of the drone honeybees were dissected in insect Ringer solution and immediately fixed in Bouin's or Carnoy's fixative for 18–24 h,

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dehydrated in ethanol, cleared in xylene and embedded in paraffin wax at 58–60 °C. Sections were cut at 4–6 μm thickness. The Bouin fixed sections were stained with either Ehrlich's haematoxylin eosin (HE) or Heidenhain's iron haematoxylin-orange G (Fe-H) histological techniques. Carnoy fixed sections were stained with the Feulgen reaction (FR), toluidine blue (TB), Hg – bromophenol blue (Hg-BPB) and periodic acid Schiff's reagent (PAS) for demonstration of DNA, RNA, proteins, and mucopolysaccharides, respectively. Baker's calcium formal fixed (12 h) material was frozen immediately and 10 μm thick sections were cut on a cryostat at –20 °C. These were stained with Sudan black B (SBB) for lipids (Tembhare, 2008).

2.2. Biochemical methods

Mucus glands were dissected from newly emerged, 6- and 12 day-old drones in ice-cold Ringer solution and the fat body, trachea and muscles were carefully removed. The glands were washed in ice-cold Ringer, weighed to 0.001 mg accuracy and homogenized for 5 min at 0 °C in ice-cold phosphate buffered saline (pH 7.0) using a pestle mortar. The supernatant obtained after centrifugation at 12 000 *g* was used for estimation of total proteins, lipids and carbohydrates with the methods of Lowry et al. (1951), Frings and Dunn (1970) and Dubois et al. (1956), respectively.

2.3. SDS-PAGE

Proteins were separated electrophoretically in SDS polyacrylamide gels (Laemmli, 1970) consisting of a 3% stacking gel (pH 6.8) and a 10% separating gel (pH 8.8) containing 1% SDS. Mucus glands of newly emerged (0 day old) and 6 day-old adult drones were dissected, homogenized and centrifuged as mentioned above and the supernatant was used as the sample. 50 μL of clear supernatant were mixed with 50 μL (1:1) of sample buffer (Laemmli, 1970). The samples were heat treated for 5 min in a water bath (60 °C). The mixture was cooled on ice and 20–40 μL were applied to the gel. A wide-range molecular weight (mass weight) marker protein mix (Sigma, USA) was used to estimate molecular mass. The gel was stained with Coomassie brilliant blue for 2 h and destained with a mixture of methanol-acetic acid-distilled water until the bands on the gel became clear.

2.4. Cell measurements

The diameter of cells and their nuclei were measured using a lanometer (PZO< Poland). 25 readings were taken for each cell and nucleus from 8–10 sections to calculate means and standard errors.

3. RESULTS

3.1. Histology

The mucus glands (MG) of *A. cerana indica* drones are milky white, large bi-lobed, peanut-shaped, sac-like structures with a wide lumen. Each gland is divided by a well-defined narrow constriction into a narrow distal and a large proximal region. The proximal regions of the glands are fused forming a common sac opening into the lateral ejaculatory ducts. The lateral ejaculatory ducts are rather short and open into the median ejaculatory duct (Fig. 1).

The wall of the MG consists of a thin inner epithelial layer and a thick outer muscle coat. It is externally covered by a peritoneal sheath. The epithelial cells are large and columnar in shape and are arranged in a single tier. They contain spherical centrally located nuclei and granular cytoplasmic inclusion in their perikarya. In the distal region of the MGs, the muscle coat is composed of an inner layer of circular muscles and outer layer of longitudinal muscles, while in the proximal region, the muscle coat is composed of inner and outer layers of longitudinal muscles and a middle layer of circular muscle (Fig. 2).

3.2. Histomorphological changes

The MGs show a gradual increase in weight, length and diameter during pupal–adult development (Fig. 3). The nuclei of the epithelial cells gradually increase in diameter from 6.98 ± 0.35 μm in late pupa to 10.50 ± 0.48 μm in adult drones (Fig. 4). In the late pupal stage, the epithelial cells are packed with dense cytoplasmic inclusions. In the newly emerged drones, release of secretory material from the epithelial cells into the lumen is evident. A large amount of

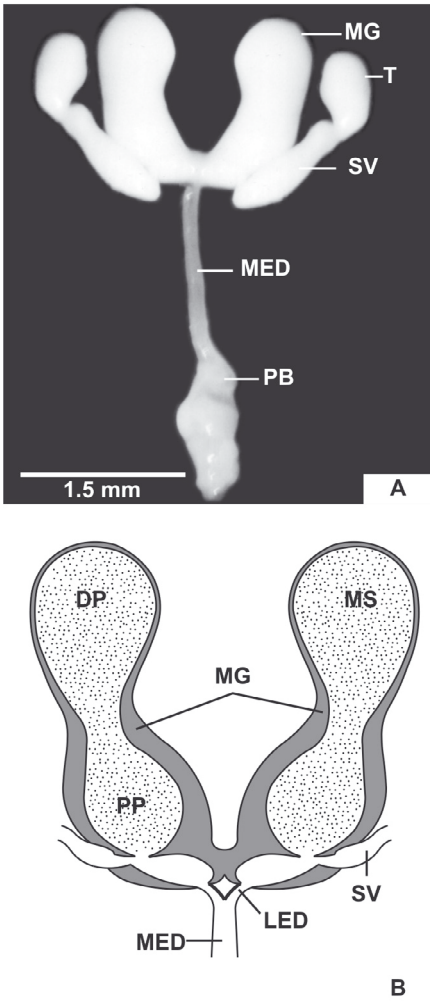

Figure 1. Reproductive system of *Apis cerana indica* drones, A. in situ preparation, B. Diagram showing opening of mucus glands and seminal vesicle into the lateral ejaculatory ducts. MG, mucus gland; T, testis; SV, seminal vesicle; LED, lateral ejaculatory duct; MED, median ejaculatory duct; PB, penis bulb; DP, distal region; PP, proximal region; MS, mucus secretion.

secretory material (mucus) is accumulated in the lumen of MGs of 6–12 day-old mature drones (Figs. 2C, D).

3.3. Histochemical results

The Feulgen, toluidine blue and Hg-bromophenol blue tests on MGs of newly emerged to 3-day old drones revealed the presence of DNA and RNA in the nuclei and protein-positive stained material in the cytoplasm of the epithelial cells, suggesting intense protein synthesis. PAS and Sudan black B tests revealed carbohydrate and lipid-positive stained material (Fig. 5) in the epithelial cells and lumen (Tab. I) demonstrating the mixed composition of the secretory material (mucus).

3.4. Protein, carbohydrate and lipid content

The total concentration of protein, carbohydrate and lipid in MG extracts of newly-emerged, 6- and 12-day old drones were analyzed (Tab. II).

The total concentration of protein and carbohydrate increased considerably after adult emergence reaching maximum levels at maturity in 6 day old drones, followed by slight a reduction until day 12. The lipid concentration increased continuously from newly emerged to 12 day-old adults. The increasing protein-lipid content could be important for the formation of a highly viscous mating sign formation left by the drone in the female genitalia.

3.5. SDS-PAGE of MGs

The electrophoretic separation of MG proteins of newly emerged, 3- and 6-day old adults showed a complex protein profile consisting of about 15 protein bands ranging from 2.5 to 151.2 kDa (Fig. 6). Among these, three proteins of 45, 43 and 37 kDa molecular mass were stained intensely representing the major class of mucus proteins distinctly.

4. DISCUSSION

The structural organization of the MG of *A. cerana indica* drones is similar to that of

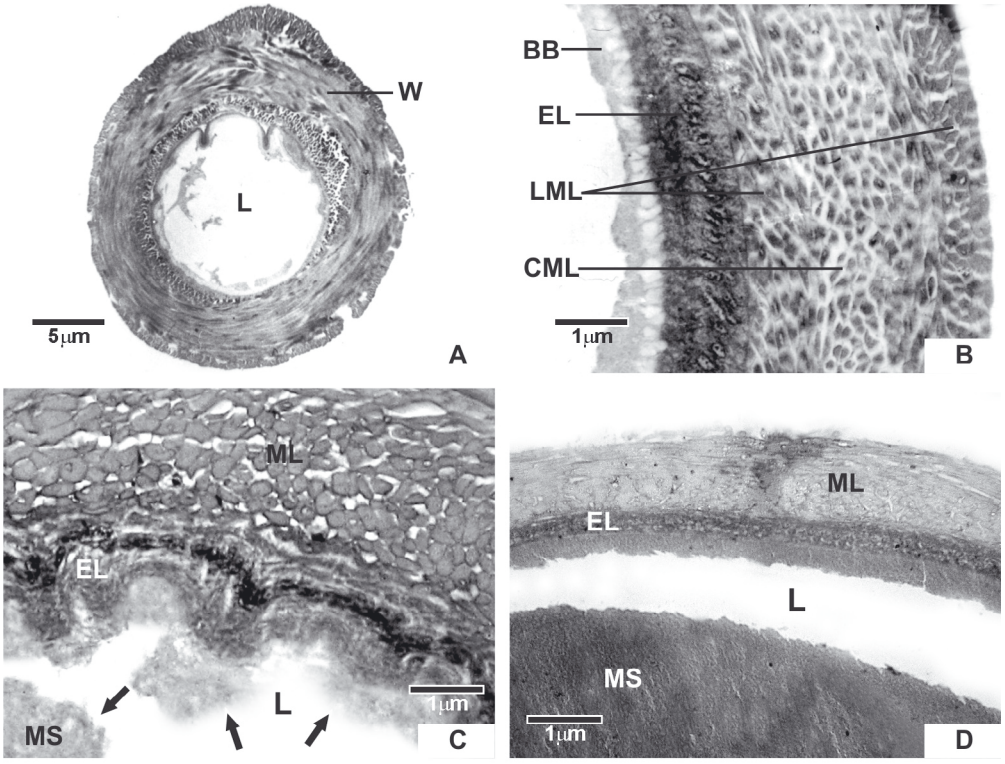

Figure 2. Histology of the mucus gland, A. Cross section of the proximal region of MGs showing a thick wall (W) and a large lumen (L), B. The wall of the MG consists of outer and inner longitudinal muscle layers (LML), a middle circular muscle layer (CML), and an epithelial layer (EL) with a brush border (BB), C. Release of mucus secretion (MS) into the lumen (L) of the MG (→) in late pupae, D. Accumulation of mucus secretion (MS) in the lumen of the MG of a 6-day old drone. EL, epithelial layer; ML, muscle layer.

A. mellifera, representing the typical mesodermal male accessory gland (Snodgrass, 1956; Woyke, 1958; Simpson, 1960; Kapil, 1962; Moors et al., 2005). Full development of the MG by the end of the pupal stage in *A. cerana indica* is also a characteristic feature of *A. mellifera* (Tozetto et al., 2007). Similarly, the time course of secretory activity in the epithelial cells and gradual accumulation of secretory material (mucus) in the lumen of the MGs resembles that seen in *A. mellifera* (Mindt, 1962; Colonello and Hartfelder, 2003; Moors et al., 2005). Bishop (1920) noticed that the honey bee drones only become capable of mating at 8–10 days after emergence, which is also the time that the mucus gland takes to become fully filled with secretions.

Histochemical reactions demonstrated Hg-BPB positive proteins, PAS positive carbohydrates (muco-polysaccharides) and sudanophilic lipids in the epithelial cells of MGs of *A. cerana indica,* similar to results obtained for *A. mellifera* (Blum et al., 1962, 1967; Ivanova, 2000; Ivanova et al., 2000; Colonello and Hartfelder, 2003; Cruz-Landim and Dallacqua, 2005) and *Bombus terrestris* (Baer et al. 2000, 2001). The biochemical analysis also showed that proteins are the major constituents of the secretions, while carbohydrates and lipids make smaller contributions to the MG secretory material of *A. cerana indica* and thus supporting the observations of Colonello and Hartfelder (2003) for *A. mellifera*. It is now well established that

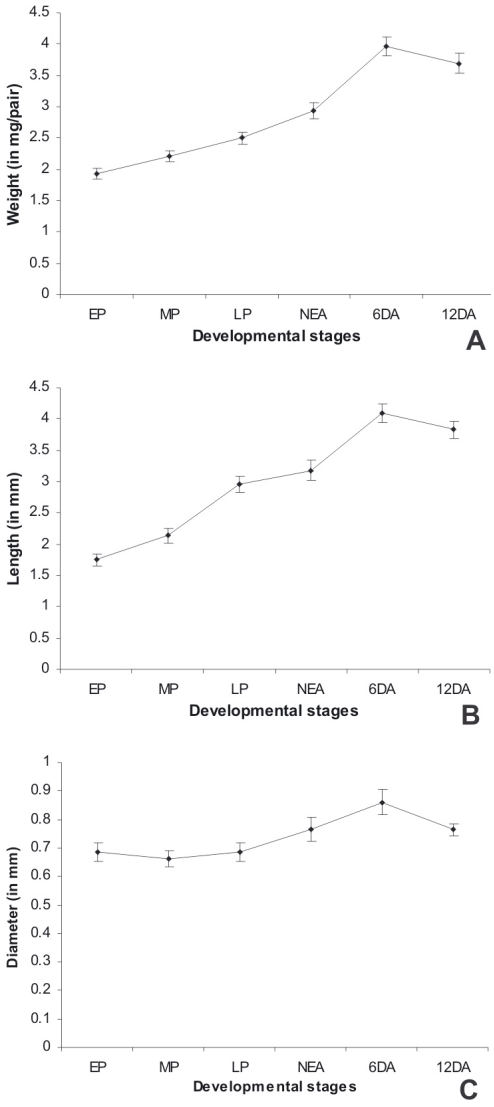

Figure 3. Changes in weight, length and diameter of the MGs during pupal-adult development, A. Weight, B. Length, C. Diameter. EP, early pupa; MP, mid pupa; LP, late pupa; NEA, newly emerged adult; 6DA, 6-day old adult; 12DA, 12-day old adult.

the male accessory glands of various insect species secrete predominantly proteins, along with some muco-polysaccharides, glycogen and lipids (Chen, 1984; Happ, 1984; Gillott, 1988, 2003; Leather and Hardie, 1995).

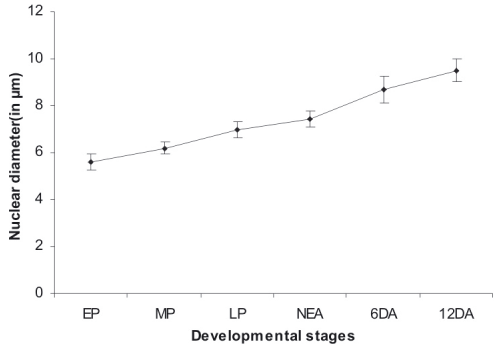

Figure 4. Nuclear diameter of epithelial cells of MG during pupal-adult development.

SDS-PAGE revealed about 16–20 proteins in *A. mellifera* mucus (Ivanova, 2000; Colonello and Hartfelder, 2003; Cruz-Landim and Dallacqua, 2005) while the present study showed the presence of about 15 protein bands in the gland extracts of newly emerged and 6-day old drones of *A. cerana indica*. In *A. mellifera* the molecular mass range of these proteins is 174 to 25 kDa (Colonello and Hartfelder, 2003) , whereas we found proteins ranging from 151.2 to 2.5 kDa molecular mass in *A. cerana indica*. Similarly, Colonello and Hartfelder (2003) also report a group of three proteins of 43–47.5 kDa appearing persistently in the mucus of mature drones of *A. mellifera* and considered them as the major mucus proteins. In *A. cerana indica,* a group of three proteins ranging from 37–45 kDa molecular mass can similarly be considered as a class of major mucus proteins.

The presence of a large number of proteins suggests a multifunctional role of the mucus (Gillott, 1988, 1996, 2003) such as a mating plug in the female genitalia after copulation to avoid polyandry (Baer et al., 2000, 2001; Sauter et al., 2001; Strassmann, 2001; Moors et al., 2005), as contributing to the mating sign (Koeniger, 1984, 1986a, b, 1991; Koeniger et al., 1996) which could have adhesive function fixing the drone to the queen while copulating freely in air and also to firmly retain the detached part of the drone's endophallus i.e. the cervix filled with sperm in the queen's vagina (Koeniger, 1984). This may represent a stimulant for oocyte

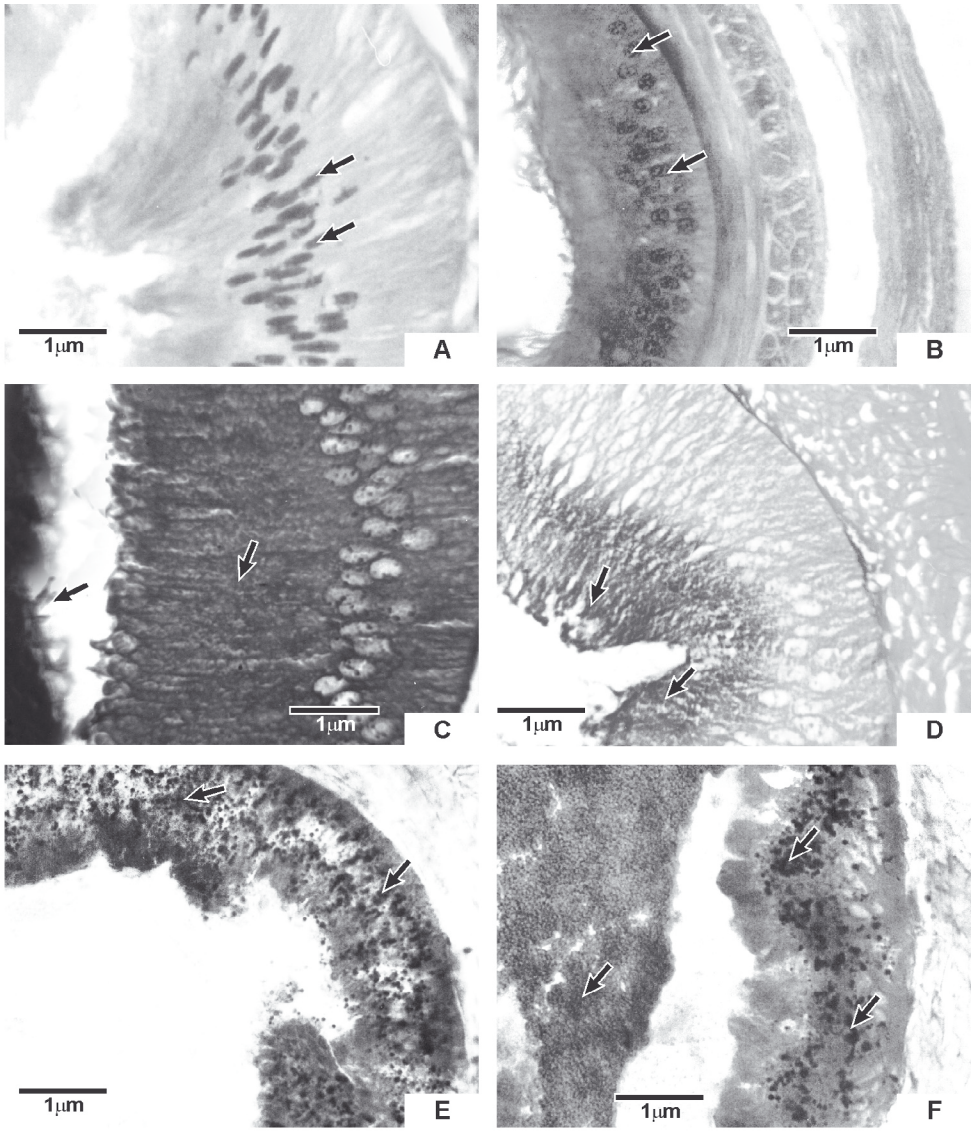

Figure 5. Histochemical reactions showing the presence of nucleic acids, protein, carbohydrates and lipids in mucus gland, A. Section stained with FR showing DNA in nuclei of epithelial cells (arrow), B. Section stained with TB showing RNA in nuclei of epithelial cells (arrow), C. Section stained with Hg-BPB showing protein secretion in epithelial cells and lumen (arrow), D. Section stained with PAS showing carbohydrate secretion in epithelial cells and lumen (arrow), E. Section stained with SBB showing lipid secretion in epithelial cells in newly emerged drone (arrow), F. Section stained with SBB showing lipid secretion in epithelial cells and lumen in 3-day old drone (arrow).

Table I. Histochemical results on MG.

	Test	Substance	Presence in mucus gland		
			Nucleus	Cell	Lumen
1.	Feulgen reaction (FR)	DNA	++	—	—
2.	Toluidine blue (TB)	RNA	++	++	—
3.	Mercuric bromophenol blue (Hg-BPB)	Protein	—	+++	+++
4.	Periodic acid-Schiff (PAS)	Carbohydrate	—	++	++
5.	Sudan black-B (SBB)	Lipid	—	+	++

+ (positive), ++ (moderate), +++ (intense), — (no reaction).

Table II. Major components of mucus glands extracts.

Age of drones Day	Total concentration of		
	Protein (μg/mg)	Carbohydrate (μg/mg)	Lipid (μg/mg)
0 (NEA)	89.68 ± 7.91	26.46 ± 0.54	85.57 ± 3.51
6	333.2 ± 13.88	44.82 ± 2.94	135.8 ± 6.83
12	273.7 ± 9.29	32.25 ± 1.90	208.6 ± 11.69

Abbr.: MG- mucus gland, NEA –newly emerged adult, ± standard error.

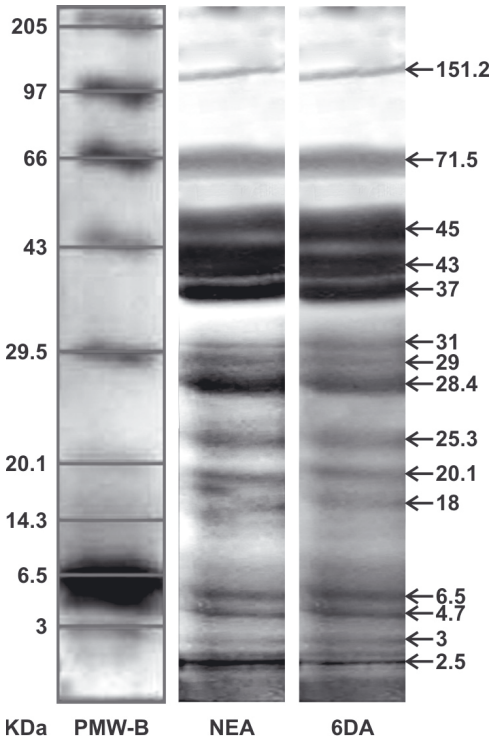

Figure 6. SDS-PAGE of MG extracts of newly emerged (NEA) and 6-day old drones (6DA).

maturation (Melo et al., 2001; Patricio and Cruz-Landim, 2002; Cruz-Landim and Dallacqua, 2005), an energy source (Colonello and Hartfelder, 2003) or be of importance for sperm capacitation and storage, similar to processes shown in other insects (Chen, 1984; Gillott, 1996).

Synthèse et composition chimique des sécrétions de la glande à mucus chez *Apis cerana indica*.

KW : glande à mucus / profil protéinique / mâle / *Apis cerana indica*

Zusammenfassung – Synthese und chemische Zusammensetzung des Mucusdrüsensektets von *Apis cerana indica* F. Obwohl *Apis cerana indica* in Indien die weitverbreitetste und am häufigsten in der Bienenhaltung anzutreffende Biene ist, sind unsere Kenntnisse über ihre Fortpflanzungsphysiologie nur beschränkt. Ziel der vorliegenden Studie war es demzufolge, die Struktur der Mucusdrüse und die Synthese und Zusammensetzung ihres Sektets mittels histologischer, histochemischer und biochemischer Methoden zu untersuchen.
Die Mucusdrüsen (MD) von *A. cerana indica* sind milchigweiße, sackartige Strukturen von erdnussartiger zweiteiliger Form und einem großen Lumen. Jede Drüse wird durch eine Verengung in ein schmales distales und ein weites proximales

Teilstück unterteilt. Die proximalen Teilstücke der beiden Drüsen bilden einen sackförmigen Ausgang in den jeweiligen medianen Abschnitt der lateralen Samenleiter. Die beiden lateralen Samenleiter sind kurz und laufen in einem medianen gemeinsamen Samenleiter zusammen (Abb. 1).

Die Wand der MD besteht aus einem inneren Epithel und einer äußeren Muskellage, die von einem Mesenthelium umkleidet ist. Die Epithelzellen sind große, Drüsenzellen von säulenartigem Format, die eine einzige Zellage bilden. Die runden Zellkerne liegen zentral und die Perikaryen weisen granuläre Zytoplasmaeinschlüsse auf. Im distalen Abschnitt der MD besteht die Muskellage aus einer inneren Ringmuskel- und einer äußeren Längsmuskelschicht, während die Muskulatur im proximalen Abschnitt von einer inneren und äusseren Längsmuskel- und einer mittleren Ringmuskellage gebildet wird (Abb. 2).

Während der Entwicklung von der Puppe zur adulten Bienen nimmt die MD an Gewicht, Länge und Durchmesser zu (Abb. 3). Eine Größenzunahme war auch für die Kerne der Epithelzellen zu sehen, von 6,98 ± 0,35 μm bei späten Puppen auf 10,50 ± 0,48 μm bei adulten Drohnen (Abb. 4).

In den Epithelzellen der MD war ab dem späten Puppenstadium die Synthese von sekretorischem Material zu sehen, das bereits kurz nach dem Schlüpfen der adulten Drohnen im Lumen der Drüsen akkumulierte. Histochemische Tests gaben Aufschluss über die sekretorische Aktivität und die biochemische Zusammensetzung des Sekrets als seine Mischung aus Proteinen, Kohlenhydraten und Lipiden (Abb. 5 und Tab. I). Bei einem Gesamtproteingehalt von 333,2 ± 13,8 μg/mg stellen Proteine die Hauptkomponente des Sekrets dar, das zudem 208,6 ± 11,7 μg/mg Lipide und 44,8 ± 2,9 μg/mg Kohlenhydrate enthält (Tab. II). In SDS-Polyacrylamidgelen ließen sich elektrophoretisch 15 Proteinbanden auftrennen, mit Molekülmassen von 2,5 bis 151,2 kDa, wobei drei Proteinbanden von 45, 43 und 37 kDa besonders hervortraten und dementsprechend die Hauptproteine des Mucusdrüsensekrets darstellen (Abb. 6).

Das komplexe Proteinmuster lässt auf eine Multifunktionalität des Drüsensekrets schließen, das eine Rolle spielen könnte sowohl im Spermatransfer, der Spermienkapazitierung, der Spermienlagerung und Energieproduktion, wie auch in der Oocytenreifung und anderen bei Bienen beschriebenen postkopulatorischen Aktivitäten.

Mucusdrüse / Proteinprofil / *Apis cerana indica*

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