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Histochemistry and Protein Profile of the Mandibular Glands of Workers of the Ant *Atta sexdens rubropilosa* (Hymenoptera: Formicidae)

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Summary Insect mandibular glands are always associated to the mandibles; they are part of the salivary glandular system. The mandibular glands are composed by a reservoir associated to the secretory cells, with each secretory cell connected to the reservoir by means of individual canaliculi. These glands play an important role in the production of pheromones, which are compounds involved in defense, communication, and reproduction of the colony. Mandibular glands of soldiers and major and minor workers of the ant *Atta sexdens rubropilosa* were processed for different histochemical tests, total protein content, and protein electrophoretic profile determination. The histochemical tests detected the presence of lipids, DNA/RNA, polysaccharides, and proteins at different regions of the gland. The protein electrophoretic profiles showed that the total protein content as well as the number of peptides of each caste follow a progressive order in relation to the size of the individual. Thus, we suggest that the production of secretion is directly linked to the task that the individual performs in the colony.
Key words Ants, Mandibular Glands, Histochemistry, Protein, Pheromones.

The mandibular glands are structures associated with the mandible of insects, being part of the salivary gland system, while other glands are associated with mouth parts. They are commonly found in the following orders: Apterygota, Isoptera, Coleoptera and Hymenoptera (Chapman 1998).

In bees, the mandibular glands are sac-like structures found in front of the brain, differing in size and shape among the specimens considered (Cruz-Landim 1967, Graf 1970). The mandibular glands are found on each side of the head and open at the base of the mandible. Each gland consists of secretory cells a sac-like reservoir with a thin wall to associated, which some authors consider to be the proper mandibular gland (Herman *et al.* 1971).

The morphology described above occurs in the three worker castes of the ant *Atta sexdens rubropilosa*. The mandibular gland of this ant is sac-shaped and is composed by a reservoir, which ends in a well-defined excretory duct joined to the mandible, and a secretory portion comprised by secretory cells arranged in the form of acini, which are joined to the reservoir by means of extra cellular canaliculi (Pavon and Camargo-Mathias 2001).

The reservoir can be subdivided into two sections, as is the case for some meliponids, where one of them is exclusively utilized to store the secretion (Cruz 1962). In *Preponapsis pruinosa*, the secretory cell part is concentrated at the extremity of a tubular evagination of the reservoir (Mathewson 1965).

The reservoir has the function of storing the secretion which is constituted by a mixture of different compounds. It may contain compounds such as: alcohols, aldehydes, and cetones (Holldobler and Wilson 1990).

In social Hymenoptera, the mandibular glands also produce defensive secretions of the glue

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type (Wilson 1971, Wilson and Regnier 1971). These mandibular glands have been especially well studied in bees and ants, and it has been confirmed that their secretion is related to communication among the individuals in the colony.

Specific studies concerning queens of the genus *Apis* revealed that this caste possesses larger mandibular glands than the ones found in workers, and the workers' mandibular glands are larger than those of drones. In the queens, these glands produce pheromones that control the different tasks of the colony while in the workers the mandibular glands probably function in the production of saliva that will soften the cocoon at the moment of emergence (Chapman 1998).

The secretion of the mandibular glands has been studied in myrmicinae ants in general and has been found to act as an alarm pheromone (Crewe and Blum 1970). Cammaerts-Tricot (1973) showed that the mandibular gland of *Myrmica rubra* is essentially a source of attraction pheromones and that its secretion contains volatile chemical substances that influence the behavior of nearby workers. In other subfamilies studied, in addition to Myrmicinae, the mandibular glands have also been found to contain substances with an important function in colony communication, with the production of alarm pheromone (Blum 1968, Blum 1970, Blum and Brand 1973, Blum 1974, Crewe and Blum 1970, Moser 1970, Wilson 1970).

Wilson (1971) while studying ants of the subfamily Myrmicinae reported 11 types of alarm pheromones produced by these structures. Therefore, the study of the glandular functions in social insects is of great importance, since innumerable biological manifestations, such as fecundation, communication, and social integration, are directly dependent on their glandular products (Michener 1974, Wilson 1971).

The present work has the object of studying the histochemical characterization, total protein content, and electrophoretic profile of the mandibular glands of soldiers and minor and medium-sized workers of the ant *Atta sexdens rubropilosa*, in order to obtain a better understanding regarding the function of these glands in these types of individuals, in addition to verifying if differences among castes indeed exist.

Materials and methods

Soldiers and minor and medium-sized workers of the ant *Atta sexdens rubropilosa* were collected from an artificial ant hill maintained in the Center for the Study of Social Insects (CEIS)-IB-Unesp-Rio Claro, SP-Brazil.

Histochemistry

Individuals were cold anesthetized and dissected in saline solution. The mandibular glands were fixed in Bouin's solution or paraformaldehyde, depending on the technique employed. Dehydration was performed in a standard alcohol series (70, 80, 90 and 95%) at 15 min intervals. The material was embedded in histological resin and sectioned to a 5 μm thickness with the aid of a microtome. The sections were arrayed on glass slides and stained with Toluidine Blue, Nile Blue, PAS/Alcian Blue and Xylidine Ponceau depending on the specific technic employed.

Total protein content

In order to obtain the total protein content of the mandibular glands of soldiers and minor and medium-sized workers of the ant *Atta sexdens rubropilosa*, the glands were dissected in insect physiological solution and processed according to the modified Bradford method (Sedmark and Grossberg 1977).

The mandibular glands were individually transferred to an eppendorf tube and macerated with the aid of a glass rod. Each gland was then mixed with 1.5 ml of water and 1.5 ml of the Coomassie Blue reagent. After homogenizing the material, an absorbance reading was obtained for each sample in a spectrophotometer at a wavelength of 595 nm. The absorbance value obtained was used to determine the concentration of each sample with the following formula:

$$C = \frac{\Delta \times F}{V}$$

Where: C=Concentration, Δ=Absorbance, F=Correction Factor, V=Sample volume (μl)

Polyacrylamide gel electrophoresis

The polyacrylamide gradient gel (5–20%) was prepared under denaturing conditions (in the presence of sodium dodecil sulfate-SDS) for protein analysis. We followed the system of alkaline buffers (Tris-Glycine/Tris-HCl) proposed by Hames and Rickwood (1990). The gel was run in a horizontal electrophoresis chamber under a constant voltage of 150 V.

Stacking Gel: Acrylamide (30 : 0.8%), Staking Buffer (0.5 M Tris-HCl, pH 6.8), 20% SDS, 1.5% Ammonium persulfate and Temed. Running Buffer: 0.025 M Tris-base, 0.192 M Glycine, 0.1% SDS. Sample Buffer: Tris-base, 20% SDS, 80% Glycerol and Bromophenol Blue.

The samples obtained from the mandibular glands of the ant *A. s. rubropilosa* of the three different castes for total protein content determination were diluted with the sample buffer in a proportion of 1 : 1. The samples were then boiled in a water bath for 5 min and, once cooled, were transferred to the gel wells together with a high molecular weight protein standard.

After the run, the proteins were visualized by staining the gel with Coomassie blue. The gel was then distained in several changes of 7.5% acetic acid at room temperature and photographed with a Contarex Super-Xeis Ikon Camera using Kodak film, 100 ISO.

Results

Histochemistry

1. Xylidine Ponceau-Detection of Proteins: The cells of the secretory portion of the mandibular glands of the three castes of *A. s. rubropilosa* studied presented a positive reaction to the test for the detection of proteins (Fig. 1A, B, C). In the cytoplasm of the secretory cells of soldiers, we detected the presence of numerous vacuoles that reacted negatively to this test for the specific detection of proteins (Fig. 1C). This test also detected a low rate of proteins production by the secretory cells of minor and medium-sized workers, since these cells reacted weakly positive (Figs. 1A, B). This average positive reaction can be confirmed when observing the strong positive reaction obtained for this test by the muscle tissue (Figs. 1B, C).

2. Toluidine Blue-Detection of RNA/DNA: Our results showed that the nuclei of the cells of the secretory portion of the mandibular glands of soldiers and minor and medium-sized workers of *A. s. rubropilosa* were positive to this test (Figs. 1D, E, F), evidencing the presence of several nucleoli inside them (Fig. 1E). The cytoplasm of the secretory cells also showed a strong positive reaction to this test, evidencing a fine granulation that was more apparent in the minor workers (Fig. 1F). Vacuoles were also observed, probably containing a secretion produced by these cells, but that did not possess the elements detected by this histochemical test (Figs. 1E, F).

3. PAS/Alcian Blue-Detection of neutral and acidic Polysaccharides: The results obtained showed that the cells of the secretory portion of the mandibular gland of the three castes studied reacted positively to the test, thus indicating the presence of neutral polysaccharides that appeared as a fine purple granulation (Figs. 2A, C). The intracytoplasmatic canaliculi that are present in the secretory cells of minor and medium-sized worker reacted strongly positive to this test, although no granulation was observed inside them, indicating that the secretion inside the lumen of the canaliculi is more fluid and homogeneous and with a low concentration of polysaccharides (Figs. 2A, C).

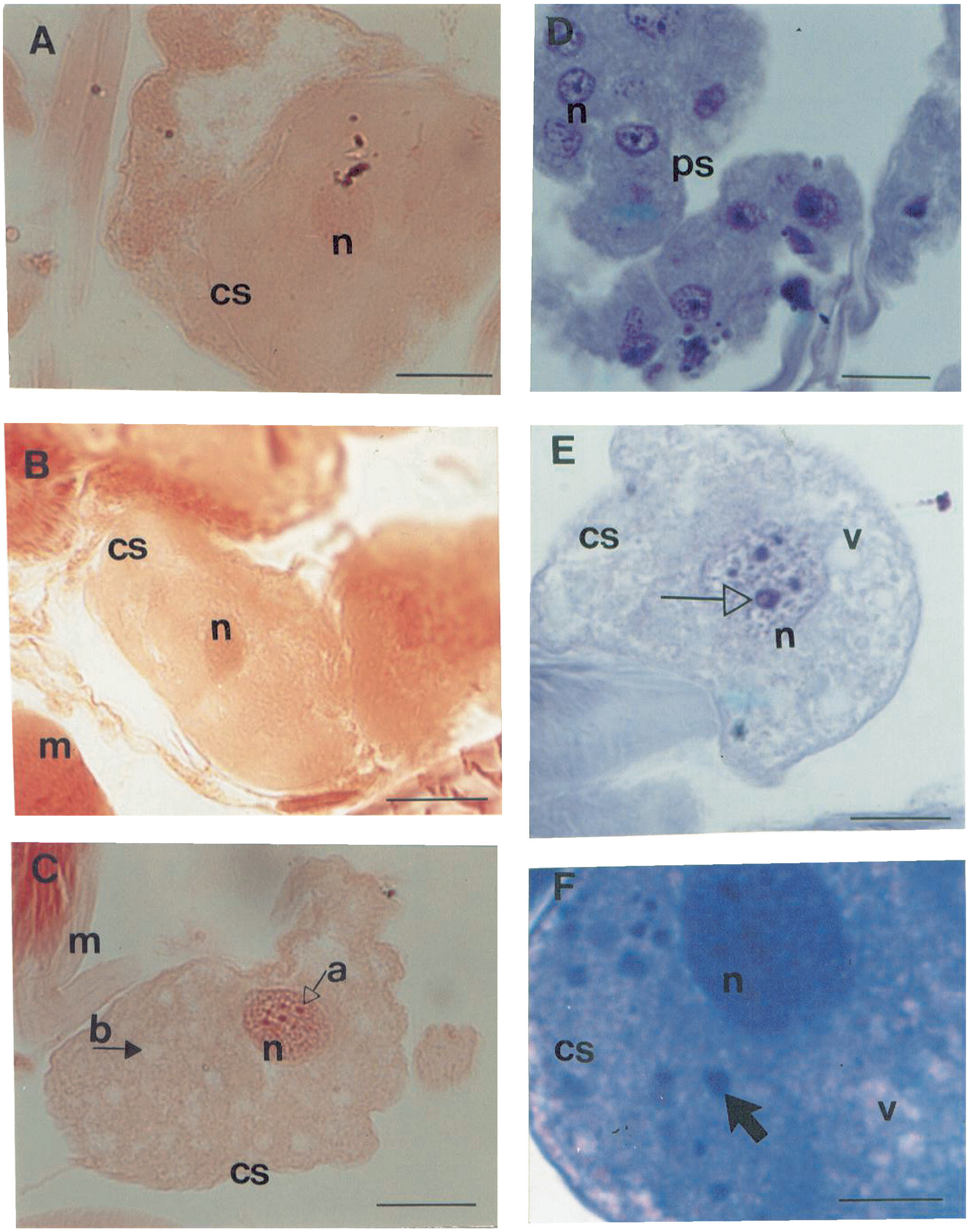

Fig. 1. Histological of the mandibular glands of workers of the ants *Atta sexdens rubropilosa* stained with Xylidine Ponceau to detect proteins presence: A) Minor-sized worker. cs=secretory cell, n=nuclei, Bar: 20 μm. B) Medium-sized worker. Bar: 20 μm. cs=secretory cell, n=nuclei, m=muscle. C) Soldier. cs=secretory cell, n=nuclei, m=muscle, arrow A=nucleoli, arrow B=vacuole. Bar: 20 μm; and Toluidine Blue to detect RNA and DNA presence: D) Soldier. General view of the secretory portion (ps), n=nuclei. Bar: 50 μm. E) Medium-sized worker. cs=secretory cell, n=nuclei, v=vacuole, arrow=nucleoli. Bar: 20 μm. F) Minor-sized worker. cs=secretory cell, n=nuclei, v=vacuole, arrow=granulation. Bar: 10 μm.

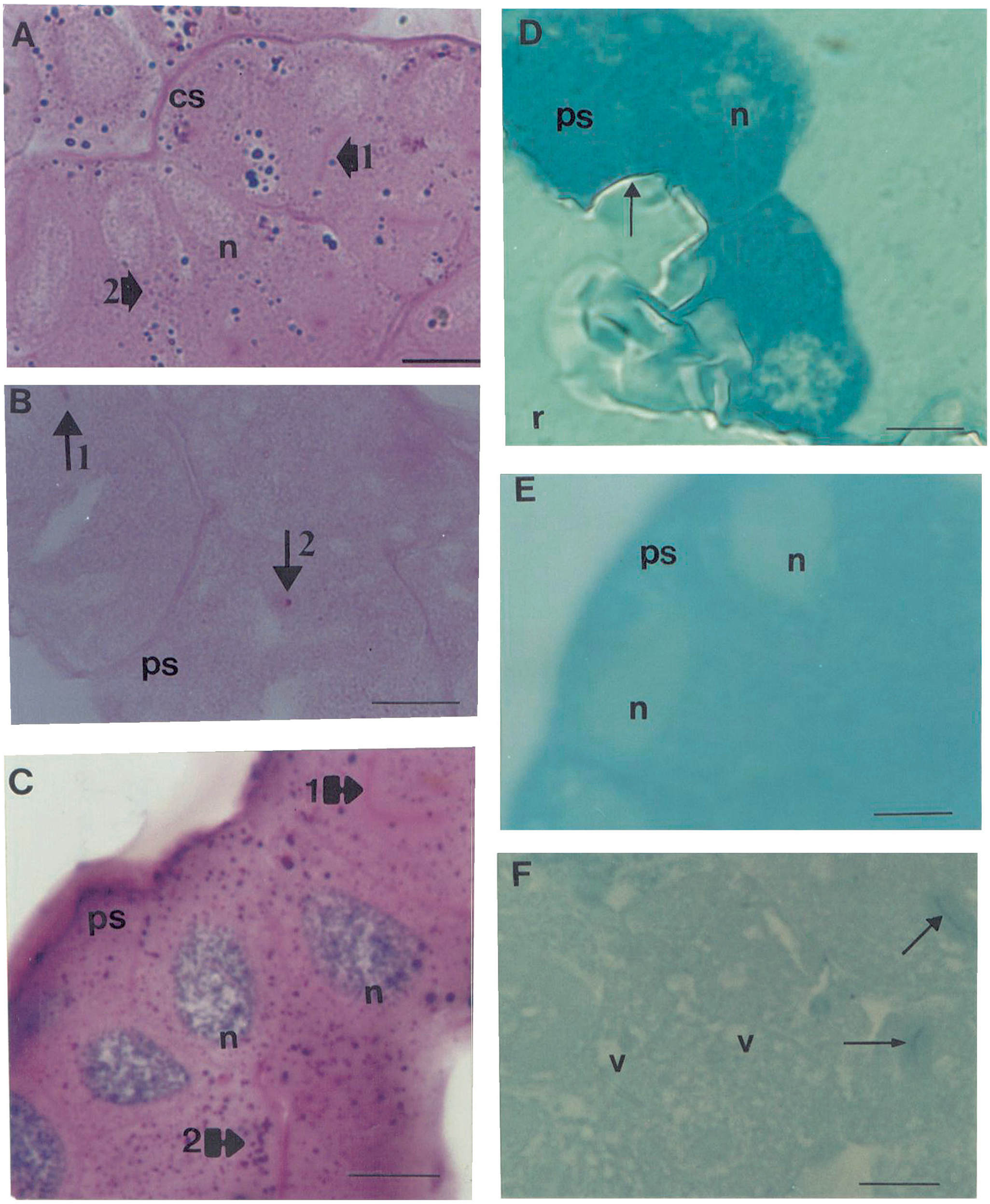

Fig. 2. Histological of the mandibular glands of workers of the ants *Atta sexdens rubropilosa* stained with PAS/Alcian Blue to detect neutral and acidic polysaccharides presence: A) Minor-sized worker. cs=secretory cell, n=nuclei, arrow 1=intracytoplasmatic canaliculi with homogeneous secretion inside the lumen, arrow 2=intracytoplasmatic granulation positive PAS. Bar: 20 μm. B) Soldier. ps=secretory portion, arrow 1=intracytoplasmatic canaliculi in longitudinal section, arrow 2=intracytoplasmatic canaliculi in transversal section. Bar: 20 μm. C) Medium-sized worker. ps=secretory portion, n=nuclei, arrow 1=polysaccharides inside the canaliculi, arrow 2=intracytoplasmatic granulation positive PAS. Bar: 20 μm; and Nile Blue to detect acidic lipids presence: D) Minor-sized worker. ps=secretory portion, n=nuclei, r=reservoir, arrow=cuticle. Bar: 20 μm. E) Medium-sized worker. ps=secretory portion, n=nuclei. Bar: 20 μm. F) Soldier. v=vacuole, arrows=intracytoplasmatic canaliculi. Bar: 20 μm.

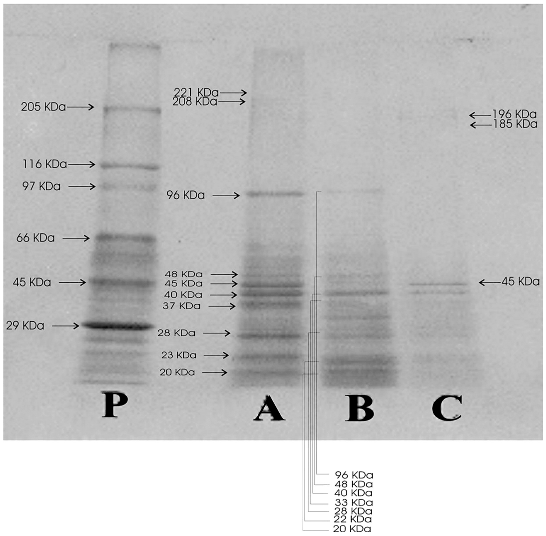

Fig. 3. Protein electrophoretic profile SDS-PAGE of the mandibular glands of soldier and minor and medium-sized workers of the ant *Atta sexdens rubropilosa*. P=standard, A=Soldier, B) Medium-sized worker, C) Minor-sized worker.

A strong positive reaction was also observed in the intracytoplasmatic canaliculi in transversal and longitudinal sections of the mandibular glands of soldiers (Fig. 2B).

4. Nile Blue-Detection of Acidic Lipids: The cells of the secretory portion of the mandibular gland of the three castes studied showed a positive reaction to this test (Figs. 2D, E, F), thus indicating the presence of lipids. The cytoplasm of these cells appeared strongly stained while the nuclei reacted negatively, which was expected since there are no lipids in this organelle (Figs. 2D, E). The secretory portion of the mandibular glands of the minor workers showed the highest concentration of lipids, followed by the medium-sized workers and, finally, by the soldiers. In the soldiers, the secretory cells of the mandibular gland showed large vacuoles that reacted negatively to the test, indicating that they contain elements of a non-lipidic nature (Fig. 2F).

In the cytoplasm of the secretory cells of soldiers, it was possible to observe a large amount of intracytoplasmatic canaliculi containing a secretion, which gave a stronger positive reaction to this test than the rest of the cytoplasm (Fig. 2F). In the gland's reservoir of the minor workers (Fig. 2D) we observed the presence of a weakly positive secretion inside it.

Table 1. The total protein contents in the mandibular glands

Castes	Contents		Mean	S.D.
Minor	0.240	0.274	0.257	0.08
Medium	0.411	0.377	0.394	0.02
Soldiers	0.908	0.959	0.934	0.06

Absolute values and means of the total protein contents (μg/μl) found in the mandibular glands of soldiers and minor and medium-sized workers of the ant *Atta sexdens rubropilosa*, including the standard error of the mean.

Total protein content

Although the histochemical tests indicated a low protein production rate by the mandibular glands of the three castes of the ant *A. s. rubropilosa* studied, the total protein content obtained for these glands (Table 1) showed that the results follow a progressive order in relation to the size of the individuals; thus, the amount of proteins found in soldiers is higher than in the medium-sized workers and this last protein content is higher than the one found in the minor workers.

Polyacrylamide gel electrophoresis

The data obtained by this method allowed the separation and determination of the number of proteins present in the mandibular gland of each caste. Fig. (3) shows the protein bands found in each sample, as well as the standards, in which the molecular weight of each band is known. Statistical estimation was made based on the migration distances by the sample and the standard. From the results of relative mobility and of the values of molecular weight (MW) of the size standards was possible to determine the values of molecular mass (in KDa) relative to the protein bands found in the mandibular glands of soldiers and minor and medium-sized workers of the ant *A. s. rubropilosa*.

Discussion

The mandibular glands of hymenopterans are part of the salivary gland system, which is constituted by at least three pairs of glands: salivary, hypopharyngeal, and mandibular. These glands secrete important substances for the process of social organization of the colony, acting directly on the various functions performed by individuals of the different castes.

The study of glandular functions in social insects is of great importance, since innumerable biological manifestations such as fecundation, communication, and social integration depend directly on the production and liberation of glandular secretions (Wilson 1971, Michener 1974). Researches related to the social organization of insects showed that some behavioral characteristics are regulated by substances that pass from one individual to another inside the community. The term "pheromone" was proposed by Karlson and Butenant (1959) for such transferable agents and the mandibular glands had been pointed out as one of the sources of these pheromones.

The morpho-histological study of the mandibular glands of soldiers and minor and medium-sized workers of the ant *A. s. rubropilosa* showed that these structures in these individuals present the same morphology found in all hymenopterans in general. Thus, these glands are described as paired structures, each located at each side of the head and associated to the mandibles. The morphology of this gland for insects in general is described as a sac-shaped structure containing a reservoir with thin walls and secretory cells that are connected to the reservoir by means of canaliculi. A well-defined excretory duct is connected to the mandible and has the function of conducing the secretion synthesized in the mandibular glands towards the exterior. This pattern described for other insects also occurs in workers of the three castes of *A. s. rubropilosa* (Pavon and Camargo-Mathias 2001).

The DNA and RNA represented by the granulation observed in the cytoplasm of the secretory cells of the mandibular glands of the three castes of *A. s. rubropilosa* could be representing the presence of DNA or several varieties of RNA, as well as organelles such as rough endoplasmic reticule and Golgi complexes because the products synthesized by these cells consist of proteins, thus requiring an efficient protein synthesis apparatus in order to perform such function. Studies of transmission electron microscopy (TEM) should confirm this supposition.

The application of the Toluidine Blue test evidenced the presence of nuclei and nucleoli inside the secretory cells and, consequently, the high concentration of nucleic acids present in these organelles. Similar results were observed by Painter (1945), who described these structures as basophilic inclusions in the hypopharyngeal glandular cells of the honeybee *Apis mellifera*. This same author correlated the number, size, and basophil level of these structures to the cellular secretory cycle, indicating that the higher the number of nucleoli, the higher the synthetic activity of the cell. In the secretory cells of the mandibular glands of *A. s. rubropilosa* of the three castes studied we found about two nucleoli per nuclei, thus indicating the high synthetic activity of these cells.

The positive reaction to PAS observed in the mandibular glands of the three castes might be due to several factors. At the intima region of the canaliculi, intra- or extra-cytoplasmatic, as well as in the cuticular lining, the positive reaction was due to the presence of chitin in the cuticle, which is also composed by polysaccharides. Complex polysaccharides might be represented in this positive reaction, such as glycoproteins or glycolipids. In the mandibular glands of the three worker castes of the ant *A. s. rubropilosa* we detected the presence of acidic polysaccharides.

The histochemical tests evidenced the presence of acidic lipids in the cytoplasm of the secretory cells of the mandibular gland of *A. s. rubropilosa* of the three castes. According to Noirot (1969) in the salivary glands of workers of the termite *Cephaloterme rectangularis*, the secretory cells of the type I would be the ones responsible for the production of lipids.

Studies conducted by Simpson (1961) showed that the mandibular glands of workers of *Apis mellifera* were responsible for producing the main lipid component of the larval meal, as well as a strongly odorous substance composed by fatty acids. The secretion produced by the mandibular gland of *A. s. rubropilosa* also possesses lipids in its composition. Therefore, we might infer that its biochemical composition is basically lipoproteic and this is a strong indication that the secretion of this gland acts as a pheromone for this species of ant.

The presence of a large amount of vacuoles in the cells of the secretory portion of the soldier's mandibular glands, which did not react to either of the histochemical tests employed, might suggest that some of those cells are already undergoing degenerative processes; thus suggesting that these glands undergo synthetic cycles of the various products and then go through the process of aging. The collection of the secretion produced by these glands is done individually, since each secretory cell is provided of an individual canaliculum. The reservoir possesses thin walls that are enveloped by a simple squamous epithelium. The cells of this epithelium probably synthesize the cuticular intima that surrounds the reservoir internally.

Studies of the larval salivary glands of *Apis mellifera* have shown that only the cells of the secretory portion of the gland are involved in the synthesis of glandular products (Silva de Morais and Cruz-Landim 1975). This same phenomenon seems to occur in the mandibular glands of workers of the three castes studied of *A. s. rubropilosa*, since the cells of the reservoir did not present the morphological characteristics of cells that synthesize and secrete substances. The squamous epithelium simply constitutes the wall of the storage sac for the final secretion, nevertheless, we do not disregard the hypothesis that these cells might be involved in the production process of the cuticle that surrounds the reservoir internally.

The electrophoretic profile of the secretion of the mandibular glands of soldiers and minor and medium-sized workers of *A. s. rubropilosa* showed that the number of polypeptides of each caste obeys to a progressive order in relation to size. This result is similar to the one obtained for total protein content, in which the mean protein concentration found in the soldiers is higher than in the medium-sized workers, and the latter is higher than the mean protein concentration found in the minor workers. Thus, it was verified that the soldiers possess a larger diversity of polypeptides in comparison to the minor and medium-sized workers, with the molecular weight of the proteins varying from 20 to 221 KDa and presenting 10 different polypeptide bands.

In the medium-sized workers, the results were similar to the ones found in the soldiers, presenting 5 polypeptide bands in common with soldiers: 96, 48, 40, 28, and 20 KDa. This similarity suggests that the mandibular glands of medium-sized workers might secrete substances similar to the ones secreted by the soldiers, at least during some of the life stages, which might be related to behavioral activities. This probable functional similarity or action of the glandular secretion on behavior was also evidenced in workers of *Scaptotrigona postica* and *Apis mellifera*, which had their hypopharyngeal glands subjected to protein electrophoresis (Costa and Cruz-Landim 1999). These authors compared the electrophoretic profile of the hypopharyngeal glands' protein extracts from adult males and workers of *S. postica* to those of workers of *A. mellifera* and detected differences and similarities in the function of the hypopharyngeal gland of *S. postica* that correlate directly to their behavior.

The minor workers presented few polypeptide bands (45, 185, and 196 KDa). This low diversity, when compared to the other castes studied, suggests that the secretion of this caste presents components that are different from medium-sized workers and soldiers.

We consider that more in-depth methodological analyses, such as two-dimensional protein electrophoresis, would be needed in order to acquire a better understanding of the protein composition of the mandibular glands of *A. s. rubropilosa*.

The differences among the protein extracts obtained from the mandibular glands of soldiers and minor and medium-sized workers of the ant *A. s. rubropilosa* suggest that these proteins are probably related to the different functions that the different castes perform inside the colony (Holldobler and Wilson 1990, Jaffe 1993). It is widely acknowledged that the soldiers are the individuals that exit the nest in order to forage, in addition to being involved with the defense of the colony. Such a situation leaves the soldier ant very vulnerable to infections and predation by other animals. Thus, a higher protein content would be expected in the mandibular gland soldiers in order to obtain a higher efficiency of this gland.

In the case of the medium-sized workers and, specially, the minor workers, which are specialized in taking care of the delicate fungal portions that feed the colony, these apparently present a lower protein content in their mandibular glands, which is coherent and directly related to the function they perform inside the colony.

The mandibular glands, through their secretions, are involved in the behavioral control and monitoring of social insects. The pheromone, or secreted element, plays the central role in the organization of the ant's society. Therefore, the morphological and histological understanding of the mandibular glands, as well as biochemical and protein analyses of their secretions, are necessary in order to obtain a better understanding of ant biology.

In ants of the species *A. s. rubropilosa*, the secretions produced by the mandibular glands have a lipoproteic content, which strengthens the hypothesis suggesting that this secretion acts as a pheromone.

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