Intramandibular Glands in Different Castes of Leaf-Cutting Ant, Atta Laevigata (Fr. Smith, 1858) (Formicidae: Attini)

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KEY WORDS ant; exocrine gland; mandible

ABSTRACT Intramandibular glands have been poorly studied in polymorphic ants, where the differences between castes were unsufficiently scrutinized. Leaf-cutting ants possess one of the most complex systems of communication and labor division, which is polymorphic well as age polyethism, and makes them an ideal model for the study of intramandibular glands. This study has investigated the occurrence of intramandibular glands in female castes and subcastes of *Atta laevigata*. The mandibles of the queen, medium, and minor workers, and soldiers were submitted to histological, histochemical, ultrastructural, and morphometric analyses. The class-3 gland cells and the epidermal gland with a reservoir were found in all the castes. The queens and soldiers showed a higher number of class-3 gland cells, distributed within the mandible as well as a greater gland size in comparison to the workers. The histochemical tests, periodic acid-Schiff (PAS), mercury-bromophenol, and Nile blue, were similar for the class-3 gland cells and epidermal glands with a reservoir. However, the tests evidenced differences between the castes, with carbohydrates strongly positive in all of them, whereas neutral lipids were found in the queen and soldiers. The protein was weakly positive in the queen, whereas in the soldier, medium, and minor workers these reactions were strongly positive in the intramandibular glands. Our findings in *A. laevigata* suggest that intramandibular glands are directly involved in labor division and consequently in chemical communication between the castes. *Microsc. Res. Tech. 78:603–612, 2015*.

INTRODUCTION

Attini include the so-called leaf-cutting ants and deserve special interest, mainly because of their complex system of labor division, polymorphism, and communication (Caetano et al., 2002; Della Lucia, 2011; Della Lucia et al., 2014; Mehdiabadi and Schultz, 2009; Wilson, 1980).

In the species of the genus *Atta*, the workers polymorphism leads to a distinct labor division among subcastes, wherein, the major workers ("soldiers") are specialized in the defense of the colony, medium workers in foraging, and minor workers work within the garden fungus (Hölldobler and Wilson, 1990; Lacerda et al., 2010; Wilson, 1980).

In most species of social insects the adult workers change their activity as they age. Each species has its own pattern of temporal or age polyethism, and many of these changes follow changes in the exocrine glands (Della Lucia, 2011). The exocrine glands of insects are traditionally classified into the three main classes 1, 2, and 3, according to the classification of Noirot and Quennedey (1974, 1991). Ants are especially rich in exocrine glands. Seventy-five exocrine glands are currently known among the Formicidae (Billen, 2009), which vary in their structural, chemical, and functional complexity (Billen and Delsinne, 2013; Billen and Morgan, 1998; Caetano et al., 2002).

Unlike the mandibular glands that are the most studied, current knowledge about the intramandibular glands is restricted to the description of their occurrence and morphology in the Formicidae (Billen and Delsinne, 2013; Billen and Espadaler, 2002; Grasso et al., 2004; Martins and Serrão, 2011; Martins et al., 2013; Roux et al., 2010; Schoeters and Billen, 1994; Toledo, 1967). However, only in *Atta sexdens rubropilosa* intramandibular glands were studied aiming to compare the different castes (Amaral and Caetano, 2006). In Attini, in addition to the exocrine glands of class-3, there is a different structure characterized by the presence of an epidermal gland with a reservoir (Amaral and Caetano, 2006; Martins and Serrão, 2011).

The study of intramandibular glands in ants is important for elucidating the mechanisms involving the exocrine system and its relationship with the

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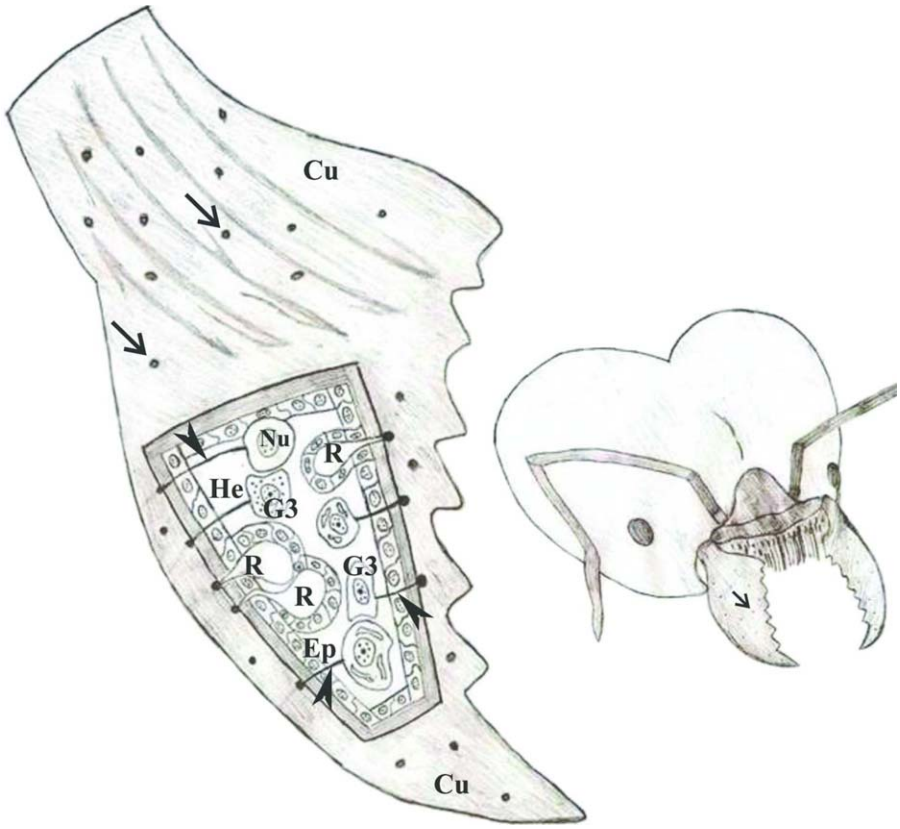

Fig. 1. Schematic drawing of *Atta laevigata* soldier showing intramandibular glands the position of mandible within the head capsule and its release site. The cut out part of the mandible shows the different types of the intramandibular glands. G3: intramandibular gland class-3 gland cells. R: epithelial glands with reservoir. Ep: epidermis with flattened cells. Arrows: Pores in the cuticle surface. Arrowheads: canaliculus. Cu: cuticle. He: hemocoel. Nu: nucleus. Not drawn with scale. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

behavior of different castes and subcastes within the colony. Thus, this study has investigated the occurrence, morphology, and histochemistry of intramandibular glands in the different castes of females of the leaf-cutting ant *Atta laevigata*, which is certainly one of the Attini species where the polymorphism is the most strongly marked.

MATERIALS AND METHODS

Ants

Three colonies of *A. laevigata* were collected in the Municipality of Teixeiras (20°39' S 42°51'W), State of Minas Gerais, Brazil, and maintained at the Leaf-cutting Ants Laboratory, Federal University of Viçosa, Viçosa, State of Minas Gerais, Brazil.

Three fertilized queens, one per colony, 12 soldiers, medium workers, and minor workers (four individuals for each colony) were separated according to their head capsule width, with the width of the minor workers being <1.5 mm, medium workers 2.0–2.5 mm, and soldiers >3.0 mm.

Histology and Histochemistry

One mandible of each pair was removed from each ant and transferred to a Zamboni's fixative solution (Stefanini et al., 1967) at 4°C for 24 h. Then the mandibles were dehydrated in a graded ethanol series (70%, 80%, 90%, 95%), at room temperature and embedded in historesin (JB4). The mandibles were sectioned longitudinally in 3 μm thin slices in motorized **Leica 2255 microtome** and stained with hematoxylin and eosin. Some slices were submitted to the following histochemical tests: Mercury-bromophenol blue for protein staining; PAS for polysaccharide and glycoconjugate; and Nile blue for lipid identification, according to Bancroft and Gamble (2007) and Pearse (1985).

Scanning Electronic Microscopy

The other mandible of the pair, after dissection was transferred to Zamboni's fixative solution for 24 h at at 4°C, dehydrated in a graded ethanol series (70%, 80%, 90%, 95%) at room temperature, transferred to hexamethyldisilazane (HMDS) for 5 min, and air dried. The pieces were gold coated (20 nm) and observed under a scanning electron microscope, LEO VP1430, in the Nucleus of Microscopy and Microanalysis Facility of the Federal University of Viçosa.

Transmission Electronic Microscopy

Three ants from each caste were dissected in sodium cacodylate to buffer 0.1*M* at pH 7.2 and the mandible was transferred to 2.5% glutaraldehyde in the sodium cacodylate buffer 0.1*M* at pH 7.2, containing 0.3*M* sucrose for 2 h. After washing the samples in the

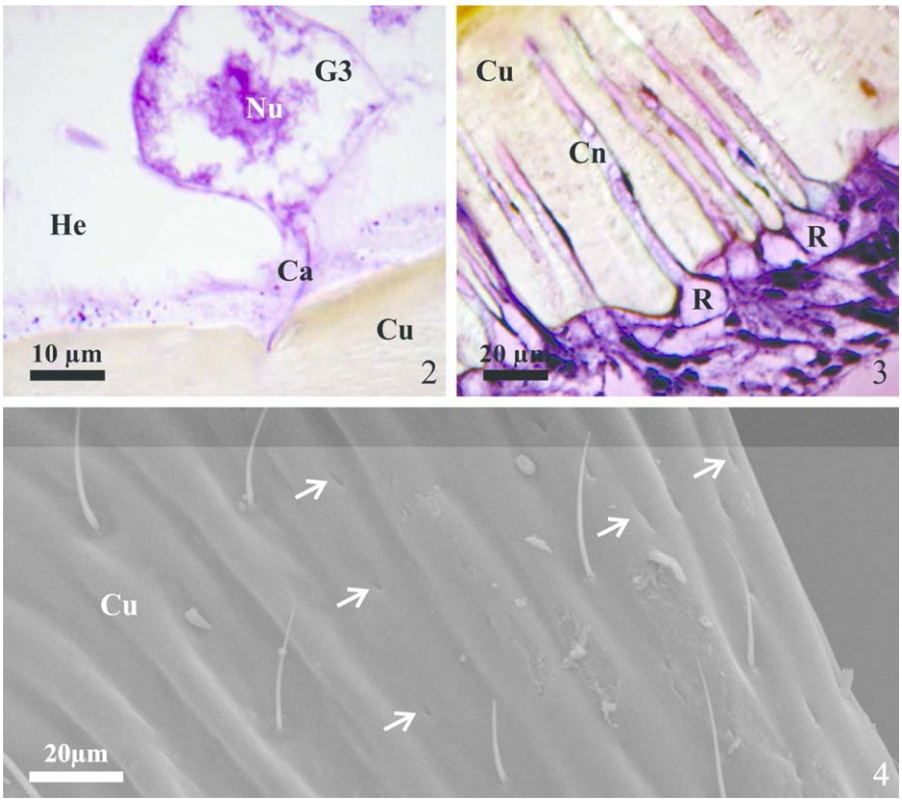

Fig. 2–4. Mandible of *Atta laevigata*. Fig. 2: Longitudinal histological sections showing intramandibular gland, class-3 gland cells (G3) queen. Hematoxylin and eosin stained. Fig. 3: Longitudinal histological sections showing epithelial glands with reservoir (R) of medium worker. Hematoxylin and eosin stained. Nu: nucleus. Cu: cuticle. Ca: canaliculus. Cn: secretory channel. He: hemocoel. Fig. 4: Scanning eletron micrograph showing the upper face of mandible of medium worker viewed SEM showing the external pores (arrows). Cu: cuticle. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

sodium cacodylate buffer, they were fixed in 2% osmium tetroxide in the same buffer for 2 h, dehydrated in 70% ethanol and embedded in LR-White resin. Ultrathin sections were stained with 1% aqueous uranyl acetate and lead citrate and examined under a Zeiss EM 109 transmission electron microscope.

Morphometry

From the histological sections, the glands were randomly selected, and measurements of the total cell area, nucleus area, and nucleus/cytoplasm ratio, using the software Image Pro-Plus, version 4.5 (Media Cybernetics). The morphometric data were obtained from 10 histological sections of the intramandibular glands of the queen ($n = 3$), soldier ($n = 4$), major workers ($n = 4$), and minor workers ($n = 4$).

Statistical Analysis

The morphometric data were submitted to generalized linear modeling (GLM), with distribution of standard errors. The statistical model resulted constituted the cell area (y) and the different castes (x). The analysis was tested by the software R (R development Core Team, 2009), followed by residue analysis, to verify the acceptability of the model used and occurrence of overdispersion.

RESULTS

In *A. laevigata*, all castes and subcastes showed intramandibular glands, characterized by a class-3 gland cells (Figs. 1 and 2) and an epidermal gland with a reservoir (Figs. 1 and 3). The class-3 gland cells were detached from the epidermis, being generally spherical and with a conducting canal that is connected to a cuticular pore in the upper face of the mandible (Figs. 1 and 2). In all castes, cuticular pores were present only on the upper face of the mandible, which were distributed in a longitudinal row along the margin of the mandibular teeth (Fig. 4).

The class-3 gland secretory cells are characterized by an end apparatus where secretory compounds are collected by duct cells (coducting canal) (Fig. 5). The plasma membrane had few invaginations 2 µm deep (Fig. 6) and the cytoplasm was rich is mitochondria (Fig. 6) and with protein (Fig. 7) and glycogen granules (Fig. 8). These secretory cells showed different morphologies, according to the caste and subcaste. In queens, soldiers, and minor workers, the secretory cells had a more vacuolated cytoplasm (Figs. 9–14) when compared with that of the medium workers (Fig. 13). Only in queens and soldiers, the cytoplasm of the class-3 gland cells had basophilic granules (Fig. 10), which are filled with protein (Fig. 7). In all the ants, the nuclei of the class-3 gland cells showed decondensed chromatin.

The histochemical analysis of the class-3 gland cells showed differences among the castes and subcastes (Table 1), except in PAS, where a strong reaction to carbohydrates occurred in all of them (Figs. 15–18). Proteins were fewer in queens (Fig. 19) than in soldiers (Fig. 20) and medium and minor workers, whereas, neutral lipids were found only in queens and soldiers (Figs. 21 and 22).

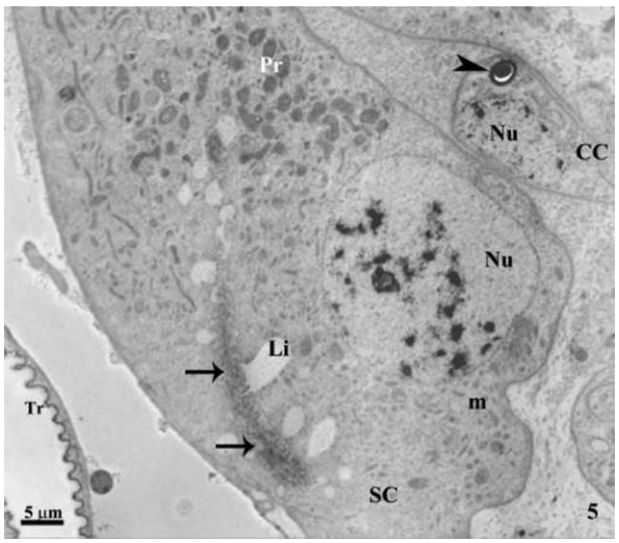

Fig. 5. Transmission electron micrograph of the intramandibular class-3 gland of *Atta laevigata* soldier showing the secretory cell (SC) with protein granules (Pr) and some lipid droplets (Li). Note the end apparatus (arrows) formed by canaliculus (arrowhead) of the conducting cell (CC). Nu: Nucleus. Tr: trachea.

The class-3 gland cells had a higher total cell area in queens (241.04 μm^2) and soldiers (272.76 μm^2) as compared to medium and minor workers, with 138.64 and 108.56 μm^2, respectively. The nucleus/cytoplasm ratio had higher values in medium workers ($F = 34.60$, $P < 0.0001$) (Table 2).

The number of class-3 gland cells varied between castes, with fewer cells in the medium and minor workers, followed by an increase in the queens with an agglomerate of gland cells within the mandible cavity. In soldiers the gland cells almost filled the mandible cavity completely (Figs. 11 and 12).

The epidermal glands with a reservoir were formed by the hypertrophy of the epidermal cells containing a reservoir within the mandible (Fig. 3). The epidermis lining the mandible, which corresponded to the non-secretory portion, consisted of flattened cells in all castes (Fig. 23), with the nuclei containing condensed chromatin.

The hypertrophied epidermal cells showed the cytoplasm rich in lipid droplets (Fig. 24) and few protein granules (Fig. 25). Cytoplasm organelles were restricted to some mitochondria and profile of rough endoplasmic reticulum (Fig. 26).

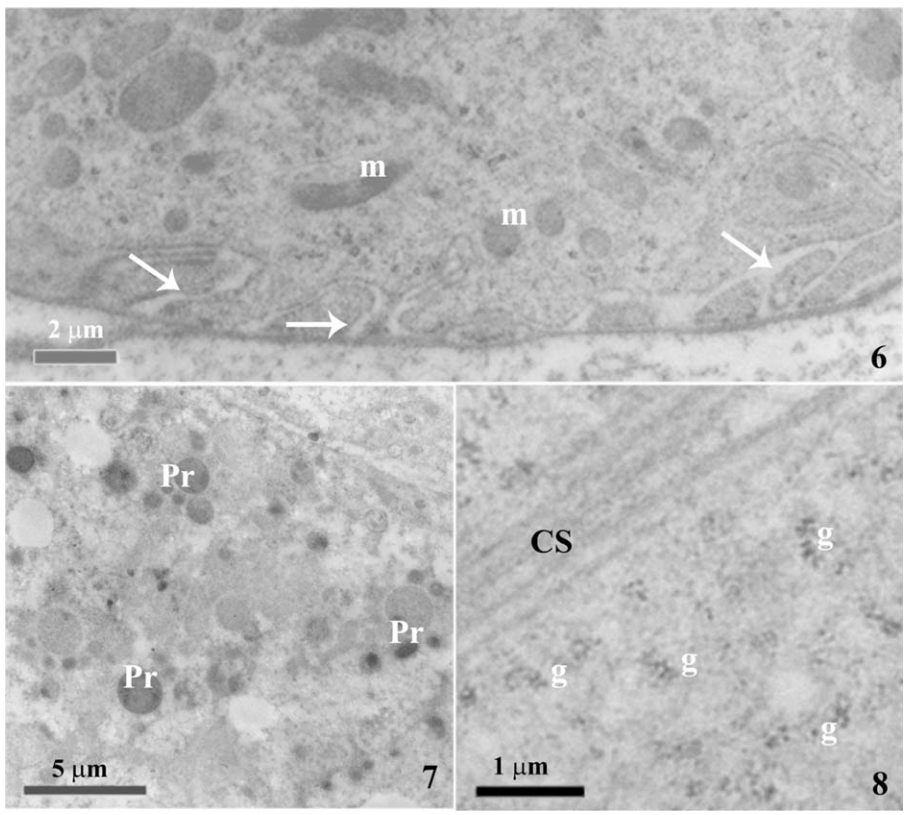

Fig. 6–8. Transmission electron micrographs of the secretory cell of intramandibular class-3 gland of *Atta laevigata* queen. Fig 6. Cell surface showing short plasma membrane infoldings (arrows) and cytoplasm with mitochondria (m). Fig. 7. Perinuclear cytoplasm showing protein granules with different sizes and electrondensities (Pr). Fig. 8. Two adjacente cells with closely surface (CS) showing glycogen granules (g) in the cytoplasm.

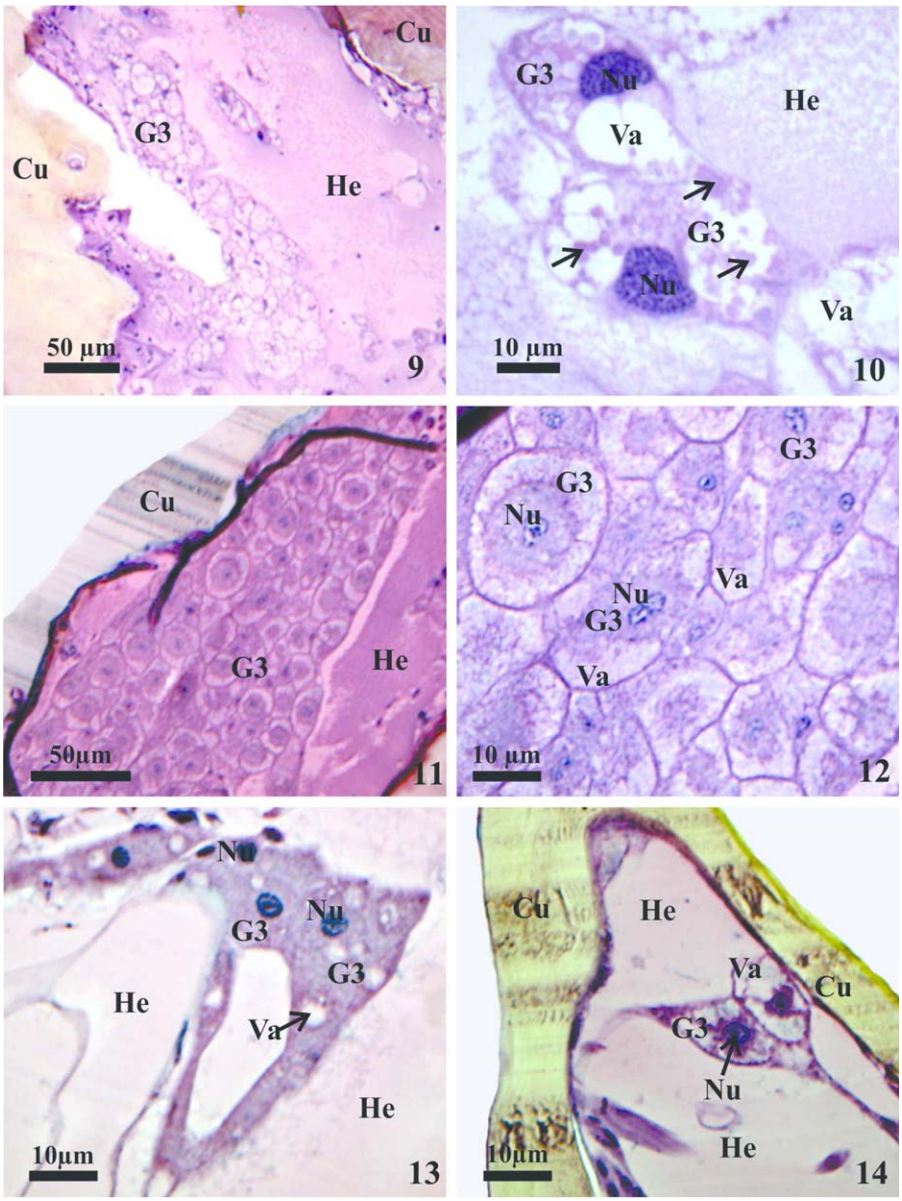

Fig. 9–14. Longitudinal histological sections of the mandible of *Atta laevigata*. Hematoxylin and eosin stained. Figs. 9 and 10: Queen. Arrow: granules, Va: vacuole. Figs. 11 and 12: soldier. Cu: cuticle, G3: intramandibular gland class-3 gland cells, He: hemocoel, Nu: nuclei, Va: vacuole, arrow: granules. Fig. 13: medium worker. Fig. 14: minor worker. Cu: cuticle, G3: intramandibular gland, class-3 gland cells, He: hemocoel, Nu: nuclei, Va: vacuole. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Histochemical analysis of the reservoirs of epidermal glands also showed differences between castes and subcastes (Table 1). These glands had a strong reaction to carbohydrates in all castes and a positive reaction to neutral lipids in queens and soldiers. Positive reaction for proteins was weaker in queens and medium and minor workers.

DISCUSSION

The occurrence of intramandibular glands of class-3 in *A. laevigata* corroborates that the presence of these glands is a common feature in social Hymenoptera, as they have been reported in bees (Costa-Leonardo, 1978; Cruz-Landim and Abdalla, 2002, 2011; Romani et al., 2002, 2003; Smith et al., 1993; Wossler et al., 2000), wasps (Romani et al., 2005; Santos et al., 2015) and ants (Billen, 2008; Billen and Delsinne, 2013; Billen and Espadaler, 2002; Grasso et al., 2004; Marques-Silva et al., 2006; Martins and Serrão, 2011; Martins et al., 2013; Roux et al., 2010; Schoeters and Billen, 1994; Toledo, 1967).

The upper face of the mandible of all castes of *A. laevigata* has some pores, which represents the oppening of class-3 gland cells. The class-3 gland cells are those in which the cellular conducting canals open into pores in the cuticle (Noirot and Quennedey, 1974, 1991), which already occurs in the mandible (Billen and Espadaler, 2002; Martins and Serrão, 2011; Santos

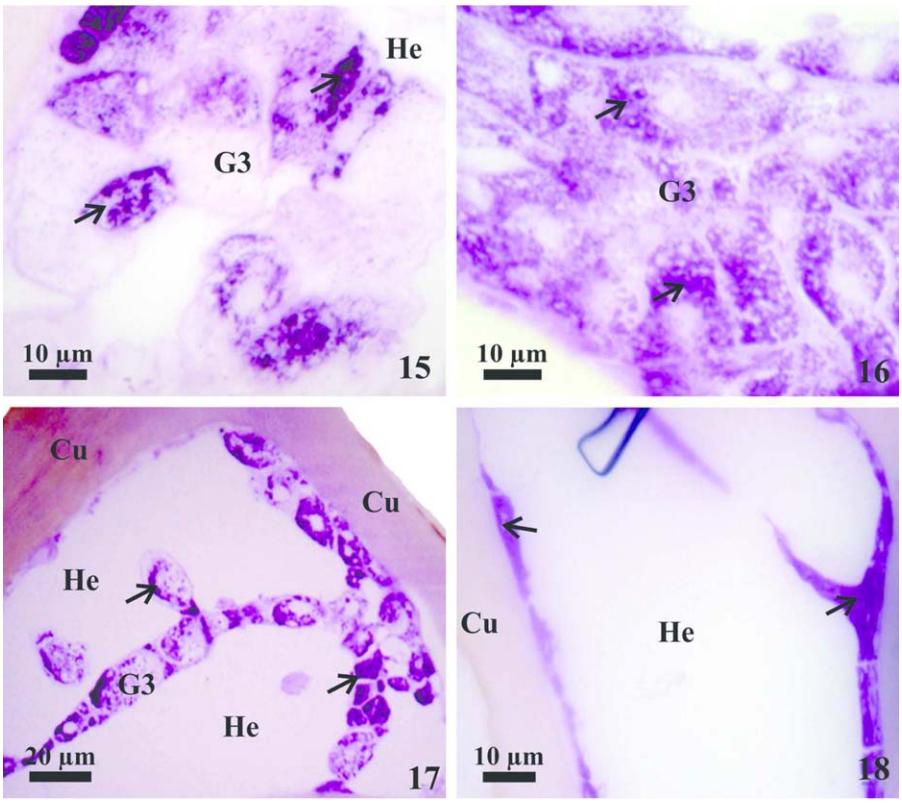

Fig. 15–18. Longitudinal histological sections of the mandibles with histochemical tests in class-3 gland cells in different castes of *Atta laevigata*. The tests were strongly positive for glycoconjugates (PAS) in all castes (arrow). Fig.15: Queen. Fig. 16: Soldier. Fig. 17: Medium worker. Fig. 18: Minor worker. Cu: cuticle, G3: intramandibular gland, class-3 gland cells, He: hemocoel. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

et al., 2009, 2015). The occurrence of pores only onto upper face of mandible of *A. laevigata* suggest that the products of class-3 intramandibular glands are released in the surface exposed to the environment other than in the surface closely to ant mouth. However, the significance of this finding remains unclear. Contrary, Roux et al. (2010) showed the presence of pores on both the under and upper faces of the mandibles in the ant *Oecophylla longinoda*.

In the castes and subcastes of *A. laevigata*, the chemical compound in the cytoplasm of the class-3 gland cells, with proteins, carbohydrates, and neutral lipids, suggests the high secretory activity of these cells. The presence of neutral lipids in the queen and soldiers only may be related to the specialization of these castes for reproduction and defense, respectively, in a similar way as several insects produce defensive secretions, pheromones and hormones, which contain lipidic compounds (Gilby, 1965).

In eusocial insects, the occurrence of exocrine glands varies with caste, and the products of these glands may be important in communication, social integration, mating, defense, food collection and storage, sexual attraction, and trail marking (Cruz-Landim and Abdalla, 2002).

In *A. laevigata*, the epithelium of the mandible, with flattened cells, does not characterize a glandular epithelium, unlike that observed in Ponerini ants, where the epidermis is characterized as a class-1 gland, with cubic or columnar epidermal cells (Martins and Serrão, 2011). Although class-1 gland cells are lacking in *A. laevigata*, we found epidermal glands with a reservoir, as reported for *A. sexdens rubropilosa* (Amaral and Caetano, 2006). The occurrence of this gland type in Attini is a potential apomorphy for the genus *Atta* (Martins and Serrão, 2011). In Ponerinae, the female dimorphism is slightly marked and the queens and workers are similar, with incipient chemical communication (Peeters and Crewe, 1984; Wilson, 1971). Ponerini workers are quite active and carnivorous; the colonies are relatively less populated, with little cooperation between workers, features supposedly similar to their ancestors (Kusnezov, 1955; Peeters and Crewe, 1984). On the other hand, in Attini, the labor division between workers is also related to the evolution of social life, as well as to worker caste polymorphism, nest structure, and age polyethism, which is greatly due to the sofistication of chemical communication and reliable signals (Jaffé, 1984; Wilson, 1971).

The functions of the intramandibular glands require further investigation, but the presence of carbohydrates, proteins, and lipid storages in both types of intramandibular glands in *A. laevigata*, suggests that these glands play a role in the production of compounds involved in chemical communication. Martins and Serrão (2011) stated that the intramandibular glands of Attini and Ponerine produce different substances with distinct functions according to the

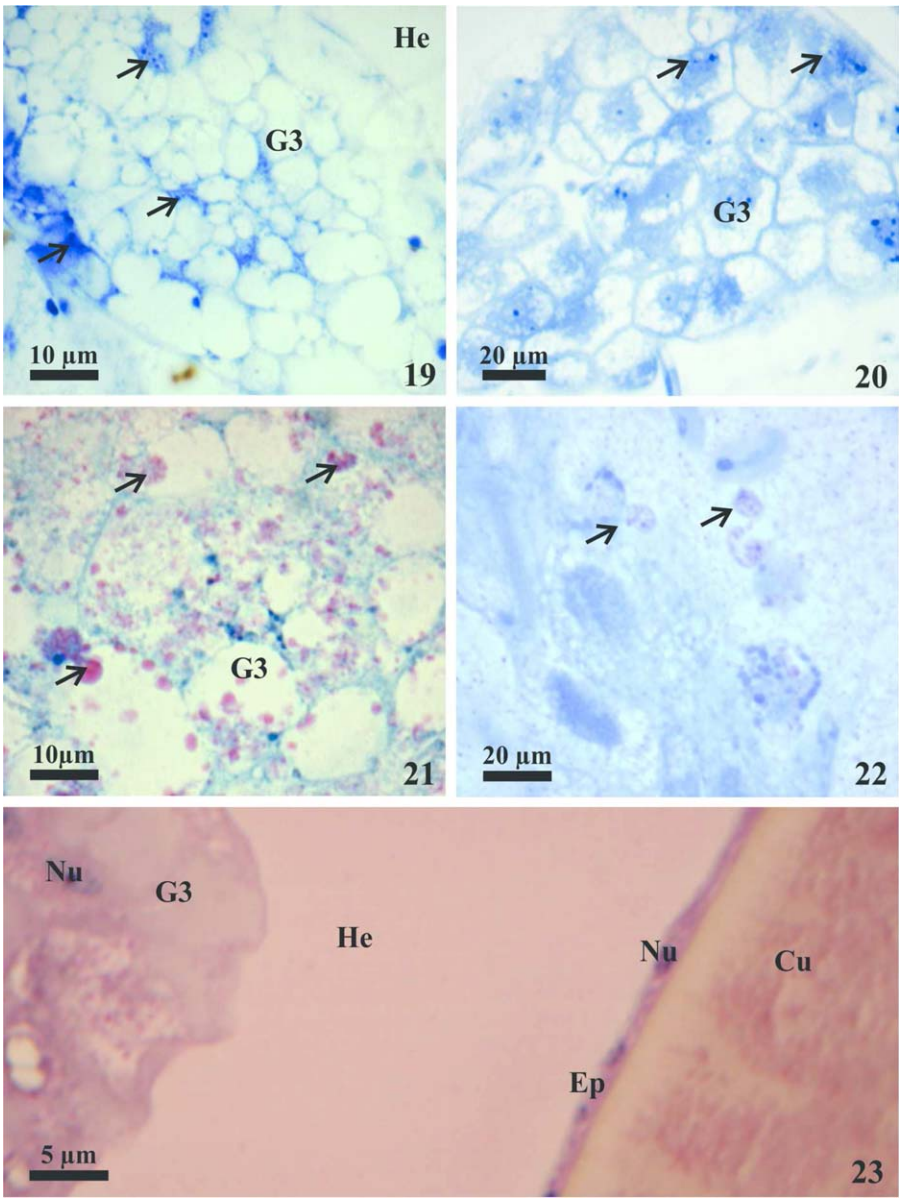

Fig. 19–23. Longitudinal histological sections of the mandible with histochemical and Hematoxylin and eosin tests in class-3 gland cells in different castes of *Atta laevigata*. Fig. 19: Histochemical tests. Positive tests for protein (mercury-bromophenol) in queen (arrows). Fig. 20: Histochemical tests. Positive tests for protein (mercury-bromophenol) in soldier (arrows). Fig. 21: Histochemical tests. Positive reaction for neutral lipid (Nile blue) (arrow) in queen (arrows). Fig. 22: Histochemical tests. Positive reaction for neutral lipid (Nile blue) in soldier (arrows). G3: class-3 gland cells, He: hemocoel. Cu: cuticle. Fig. 23: Epidermis with flattened cells (Ep) and class-3 gland cells (G3). Hematoxylin and eosin stained. Cu: cuticle. He: hemocoel. Nu: nucleus. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

chemical compound (glandular substances) and gland types. Furthermore the class-3 gland cells produce pheromones compounds as well as enzymes (Amaral and Caetano, 2006; Quennedey, 1998). Schoeters and Billen (1994) have pointed out that the class-3 gland cells releases all secretions of the intramandibular glands. In *A. sexdens rubropilosa* the mandibular glands in the three castes show lipid droplets possibly corresponding to pheromones being realeased (Pavon and Camargo-Mathias, 2005).

In *A. laevigata*, only queens and soldiers possess cytoplasmic granules, neutral lipids, and a great number of class 3 intramandibular glands, suggesting that these glands may have different functions according to the task that each caste plays in the colony, and may act in intraspecific communication.

The size of the cells in class-3 intramandibular glands is higher in queens and soldiers, but is similar in medium and minor workers. Morphometrical data consider that the cytoplasm increase its size according storage of secretion. Contrary, active and inactive nuclei have the same size. Thus, glands with low nucleus/cytoplasm ratio may be storing higher amounts of secretion.Together, these data suggest that

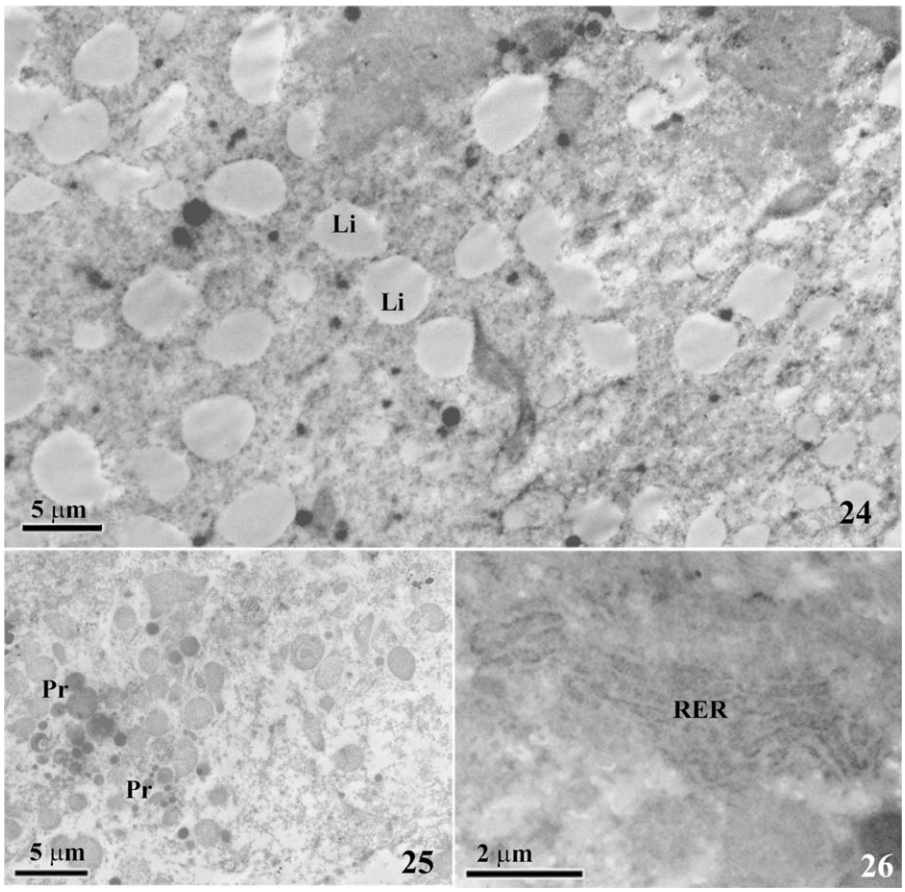

Fig. 24–26. Transmission electron micrograph of the intramandibular epidermal gland with reservoir of *Atta laevigata* soldier. Fig. 24: Cytoplasm showing many lipid droplets (Li). Fig. 25: Protein granules of different sizes (Pr). Fig. 26: Detail of rough endoplasmic reticulum (RER).

TABLE 1. Results of histochemical tests of the intramandibulares glands in different castes of Atta laevigata

	Class-3 gland cell			Epithelial glands whit reservoir		
Caste	PAS	Mercury bromophenol	Nile blue	PAS	Mercury bromophenol	Nile blue
Queen	+++	+	+++	+++	+	++
Soldier	+++	++	++	+++	nd	+
Medium Worker	+++	++	–	+++	++	–
Minor Worker	+++	++	–	+++	++	–

– No reaction, + weak positive reaction, ++ positive reaction, +++ strong positive reaction, nd-not determined.

TABLE 2. Measurements (mean ± standard deviation) of the secretory cells of the intramandibular gland in different castes of Atta laevigata

Castes	Total cell area[a] (μm^2)	Nucleus area[b] (μm^2)	Cytoplasmic area[b] (μm^2)	Nucleus/cytoplasm ratio[a]
Queen	241.04 ± 59.11a	20.58 ± 8.09	220.46 ± 52.37	0.09 ± 0.02b
Soldiers	272.76 ± 101.59a	17.72 ± 8.40	255.04 ± 96.70	0.07 ± 0.03b
Medium	138.64 ± 38.82b	14.93 ± 3.85	123.70 ± 37.47	0.13 ± 0.04a
Minor	108.56 ± 34.53b	8.99 ± 4.20	99.57 ± 31.20	0.09 ± 0.02b

[a]Data followed by different letters in the column differs among castes.
[b]Data used for calculation of nucleus/cytoplasm ratio and not submitted to statistical test.

soldiers are more similar to queens than to workers. This suggests that, contrary to the established belief in ant literature, soldiers represent an additional caste, with an origin independent to the workers (Baroni-Urbani and Passera, 1996), where the soldier caste is closer to the queens than to the workers, with different physiology, morphology, and behavior (Baroni-Urbani, 1998). In workers, medium ones shows lower activity of class-3 intramandibular glands assessed by higher nucleus/cytoplasm ratio, but the significance of this

finding should be studied in the future in association with behavioral features of these subcaste.

Different intramandibular glands in different castes suggest specific functions for each caste that remain unclear. However, the morphological features of these glands in the queens and soldiers of *A. laevigata* suggest that these glands may play a role in the release of sex pheromones or are involved some how in reproduction in queens and an alarm or defensive pheromones in soldiers, while in minor and medium workers, other functions may be attributed to these glands secretions. Production of pheromone for intramandibular glands occurs in the ponerine ant *Neoponera villosa* (Martins et al., 2015). Therefore, these differences may be associated with caste profile. The composition of pheromones varies between castes in social insects (Cruz-Landim et al., 2011; Hernández et al., 1999; Hughes et al., 2001; Nascimento et al. 1993).

In conclusion, the intramandibular glands in *A. laevigata* are the class-3 gland cells and epidermal cells with a reservoir, and their morphological, histochemical, and morphometric features support the hypothesis that these glands may play an important role in labor division in the castes and subcastes.

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