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Ultrastructural detection of lipids in the cephalic salivary glands of *Apis mellifera* and *Scaptotrigona postica* (Hymenoptera: Apidae) workers

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ABSTRACT. Secretory cells of the cephalic salivary glands (CSGs) of eusocial bees produce and accumulate lipid-like secretion in the lumens of their alveoli. Correspondingly, secretory cells present typical ultrastructural features of lipid-compound producers. Previous work on bees has revealed inter-specific differences in the chemical composition of secretion, and the production mechanisms and secretory cycle of secretory cells. In this work a comparative analysis of the mechanisms of lipid storage in the CSGs of *Apis mellifera* (Linnaeus, 1758) and *Scaptotrigona postica* (Latreille, 1807) workers was carried out. The ultrastructural location of lipids was ascertained using imidazole-osmium (IO), using individuals in different stages of their life cycles. Lipid deposits were identified inside glandular cells and in the alveolar lumens in all individuals, but differences were observed between the species. The glandular cells of *A. mellifera* workers presented positive reactions to IO as droplets dispersed in the cytoplasm, as vesicles and in the channels formed by apical plasma membrane infolds. In *S. postica*, lipid compounds were detected inside the mitochondrial matrix and in smooth endoplasmic reticulum cisterns. In both species, forager workers exhibited the largest amounts of lipids stored in the alveolar lumen. The differences between the species are discussed, taking into account specific behavioral differences.

KEY WORDS. Cytochemistry, excretory, imidazole-osmium, insect, salivary system.

All insects possess exocrine glands of epidermal origin that are attached to their mouthparts. These glands are named according to the appendix to which they are connected. For instance, mandibular glands are connected to the mandible, and labial or salivary glands are connected to the labium. The salivary glands of insects generally have their secretory portion located in the thorax. In some species of bees, however, branches of the salivary glands can be found in the head. These glands, known as cephalic salivary (or labial) glands (CSGs), are fully developed and functional only in Apinae species (Cruz-Landim 1967).

In eusocial bees, the products of the exocrine glands and their functions vary not only among the classes of colonial individuals, but also among their functional life phases, showing interesting adaptations within species, and great plasticity (Katzav-Gozansky et al. 1997, Jarau et al. 2004, Santos et al. 2009). This plasticity is observed not only among different species, but also between the sexes and castes of the same species. For example, males of *Bombus pratorum* (Linnaeus, 1761) and *Bombus lapidarius* (Linnaeus, 1758) use the CSG secretion to mark flight routes and attract virgin queens (Bergman & Bergström 1997), while females (queens and workers) of *Bombus terrestris* (Linnaeus, 1758) use CSG as a reproductive status signal (Amsalem et al. 2014). The function of the CSG secretion of *A. mellifera* is not clear, but it has been hypothesized to soften wax during nest construction (Heselhaus 1922), and to lubricate mouthparts (Simpson 1960). In meliponines, according to Jarau et al. (2004, 2006, 2010), Schorkopf et al. (2007) and Stangler et al. (2009), the production of volatile components plays a role in communication through scent trails. Poiani et al. (2015), upon investigating the CSG secretion of a few species of bees, found oxygenated compounds in *S. postica*; a mixture of oxygenated compounds and hydrocarbons in *Melipona quadrifasciata* (Lepeletier, 1836), and hydrocarbons in *A. mellifera* workers (Silvana Beani Poiani, unpublished data).

A typical colony of eusocial bees is composed of males and females (queen and workers). Males are present in the colony under some special conditions, and their function is to fertilize a new queen. Female members are divided into reproductive and partially non-reproductive castes. The queen is long-lived, and is responsible for egg production (reproduction). Workers are numerous, short-lived females, and are responsible for all services necessary to maintain the colony, such as brood care, building, attending the queen, guarding the entrance of the

ZOOLOGIA An International Journal for Zoology

colony, and activities outside the nest (Snodgrass 1956, Wilson 1971). Workers of eusocial bees perform tasks according to their age, a phenomenon known as age polyethism or polyphenism. The development and functional cycles of several glands are directly related to the physiological state and life phase of the bee; in other words, the glands are activated when their secretion is necessary to perform tasks inside or outside the colony. A number of studies on the CSG of bees have investigated the chemical compounds at a specific phase of life or physiological status, for example: foragers (Jarau et al. 2004, Schorkopf et al. 2007) or mature males (Bergman & Bergström 1997). However, there have been no studies on how developed the CSGs are in different phases of the bee's life, or which changes occur on the morphology of these glands in different phases of the worker's life or physiological status.

We conducted a detailed morphological study of the CSGs of workers of *A. mellifera* and *S. postica* in different phases of their lives, in order to clarify the origin of the lipids stored in their CSGs. For that, we used a cytochemistry technique.

MATERIAL AND METHODS

Previous ultrastructural studies of bee's CSGs (Poiani & Cruz-Landim 2009) described the main features of gland cells. The cytochemical analysis presented here brings additional information about this gland.

In the ultrastructural cytochemical studies, we used CSGs from 10 newly emerged (NE) workers, 10 workers working in the brood comb area (CA), and 10 forager (FO) *A. mellifera* and *S. postica* workers. They were chosen based in the phase or task that they were performing in the colony at the moment they were collected. The specimens were collected in the apiary and meliponary maintained by the Institute of Biosciences of Universidade Estadual Paulista (UNESP) Rio Claro, SP, Brazil.

NE of both species were captured during their emergence from brood cells. CA of *S. postica* were collected while provisioning brood cells for the queen; and of *A. mellifera*, when feeding larvae. FO were collected when they returned from the field to the nest, with pollen on their legs.

Lipids were located using the imidazole-osmium (IO) method (Angermüller & Fahimi 1982). All glands were dissected in insect physiologic solution and fixed for 2 hours in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. Subsequently, the glands were washed twice in cacodylate buffer for 15 minutes per bath and in 0.1 M imidazole buffer, pH 7.5, for an additional 15 minutes. Post fixation was performed in 2% imidazole buffer containing 0.1 M osmium tetroxide, pH 7.5, for 30 minutes in the dark and at room temperature. The glands were washed twice in 0.1 M imidazole buffer, pH 7.5, next in phosphate-buffered saline (PBS), and finally in 10% ethanol, for 15 minutes per bath. The glands were dehydrated in increasing concentrations of acetone for 5 minutes each and embedded in Epon-Araldite resin. Resin polymerization was performed at 60°C for 24 hours. Thin sections were placed in copper grids and examined unstained in a Philips transmission electron microscope (TEM).

RESULTS

CSGs from NE, CA and FO workers treated with IO revealed several ultrastructural differences between the species and among the life phases.

Apis mellifera. The gland cells of *A. mellifera* NE workers exhibited smooth endoplasmic reticulum, medium electron density in the mitochondria, and nuclei with thick heterochromatin points. The alveolar space was narrow and contained small amounts of secretion. IO-highlighted droplets were scattered in the cytoplasm of the secretory cells and the secretion in the lumen (Figs. 1-2).

In CA workers, droplets were randomly dispersed in the cytoplasm (Fig. 3) as well as in heterogeneous granules positive for IO (Fig. 4). Most of the alveoli contained lumens loaded with IO-stained secretion (Figs. 3-4). The lumen of the alveoli of the CSG was lined by cuticle. In the glands of CA and FO, the gland cell apical plasma membrane was invaginated to form sub-cuticular channels, between which elongated mitochondria of medium electron density were present (Figs. 5-6).

In FO workers, IO positivity was found in the vesicles (Figs. 7-8) of the apical region and in the channels formed by the apical infolds (Fig. 6). The lumens of almost all alveoli were filled with IO-positive secretion.

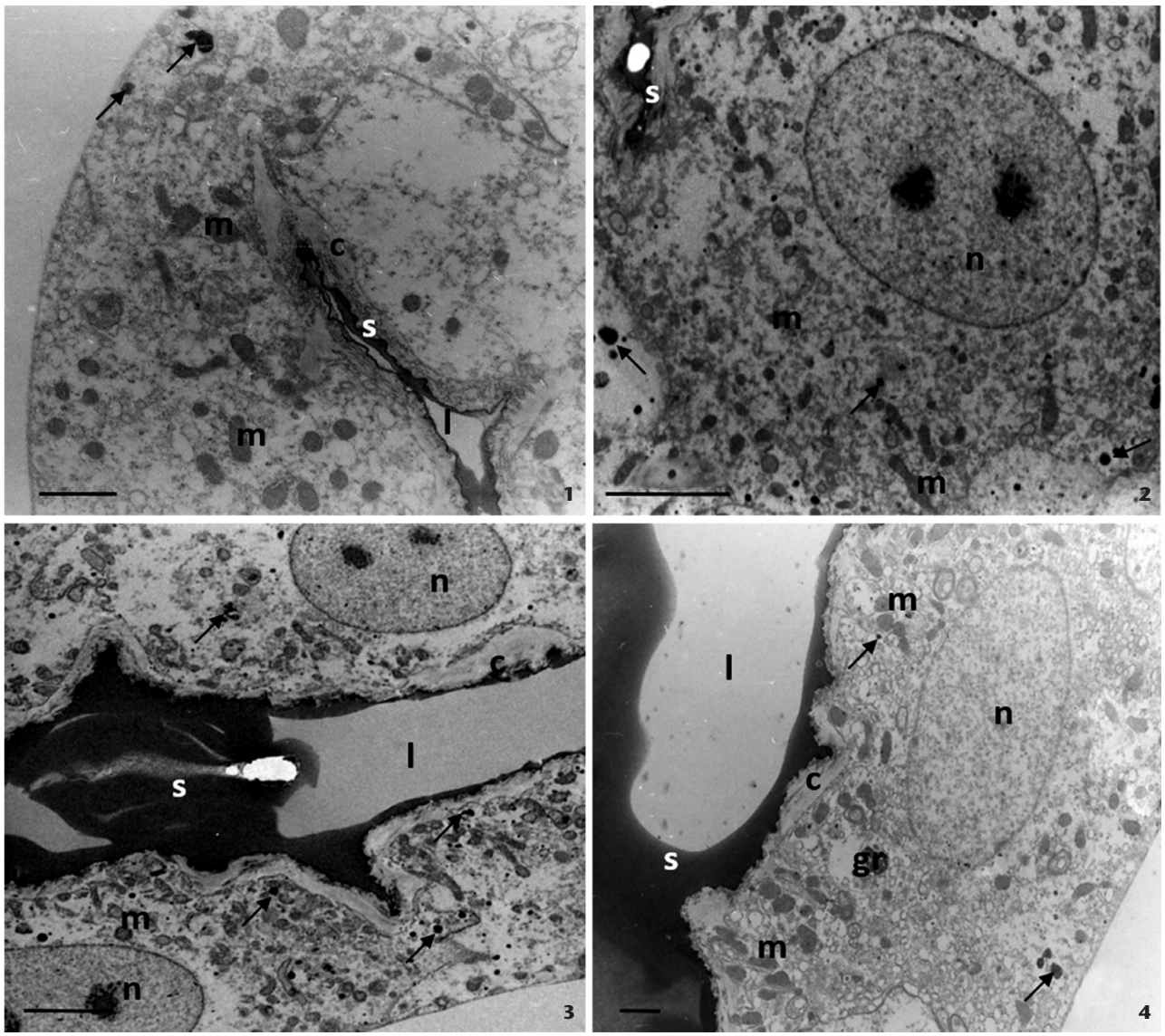
Scaptotrigona postica. Glandular cells from NE presented a low positive response to IO treatment, which appeared in the cytoplasmic vesicles and as a tiny, electron-dense lining of the alveolar luminal cuticle (Fig. 9). As noted in *A. mellifera*, the amount of IO-positive material increased in the CA glandular cells and alveolar lumen (Fig. 10), but showed a different pattern in FO cells, which exhibited few positive structures in the cells but the alveolar lumen filled with secretion.

In CA, some mitochondria appeared to react positively to IO, showing lipid content in the matrix. Additionally, the smooth endoplasmic reticulum cisterns contained positive material with the same electron density observed in the mitochondria and in the alveolar lumen (Fig. 10).

In FO workers, a few cells in the CSG were cuboidal (Fig. 11) and had large electron-dense droplets in the cytoplasm. However, the majority of cells were flat (Fig. 12) and, although some positive reaction to IO appeared in the cytoplasm of these cells, IO positivity was almost exclusively present in the alveolar lumen (Fig. 12). At this worker stage, glandular cells presented sparse organelles.

DISCUSSION

Cellular morphology displays features that provide clues about cellular function. The CSG of bees has been extensively studied and the results of these studies have documented that

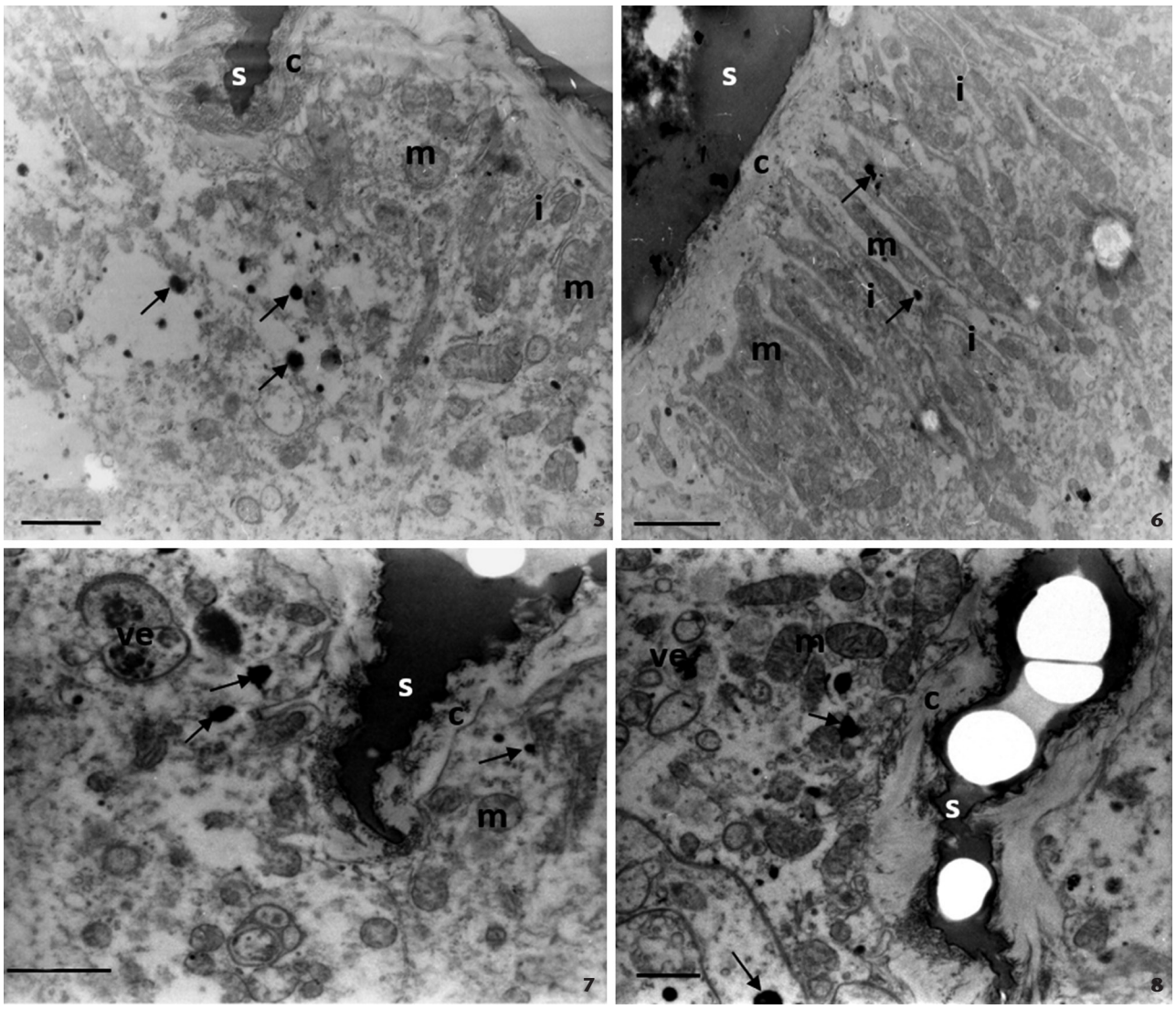


Figures 1-4. Lipids, detected using imidazole-osmium, in cephalic salivary gland (CSG) cells of *Apis mellifera* workers. (1-2) Small droplets of lipid (arrows) dispersed in the cellular cytoplasm of a newly emerged worker (NE), mitochondria (m) of medium electron density and narrow alveolar lumens (l) containing scarce IO-positive secretion (s). (3-4) Gland cells from workers working in the brood comb area (CA). Note lipid droplets dispersed in the cytoplasm (arrows) (1C), heterogeneous granules (gr) (1D) and large amounts of lipid secretion (s) in the alveolar lumen (l). (c) Cuticle, (n) nuclei. Scale bars: 1, 2, 4 = 1 µm, 3 = 3 µm.

these glands have secretory function (Snodgrass 1956, Simpson 1960, Santos et al. 2009). The morphological clues indicating cell secretory activity vary with the type of secreted product. Our cytochemistry results revealed the dynamics of producing and/or up-taking compounds by CSG cells and storing these compounds until they are used.

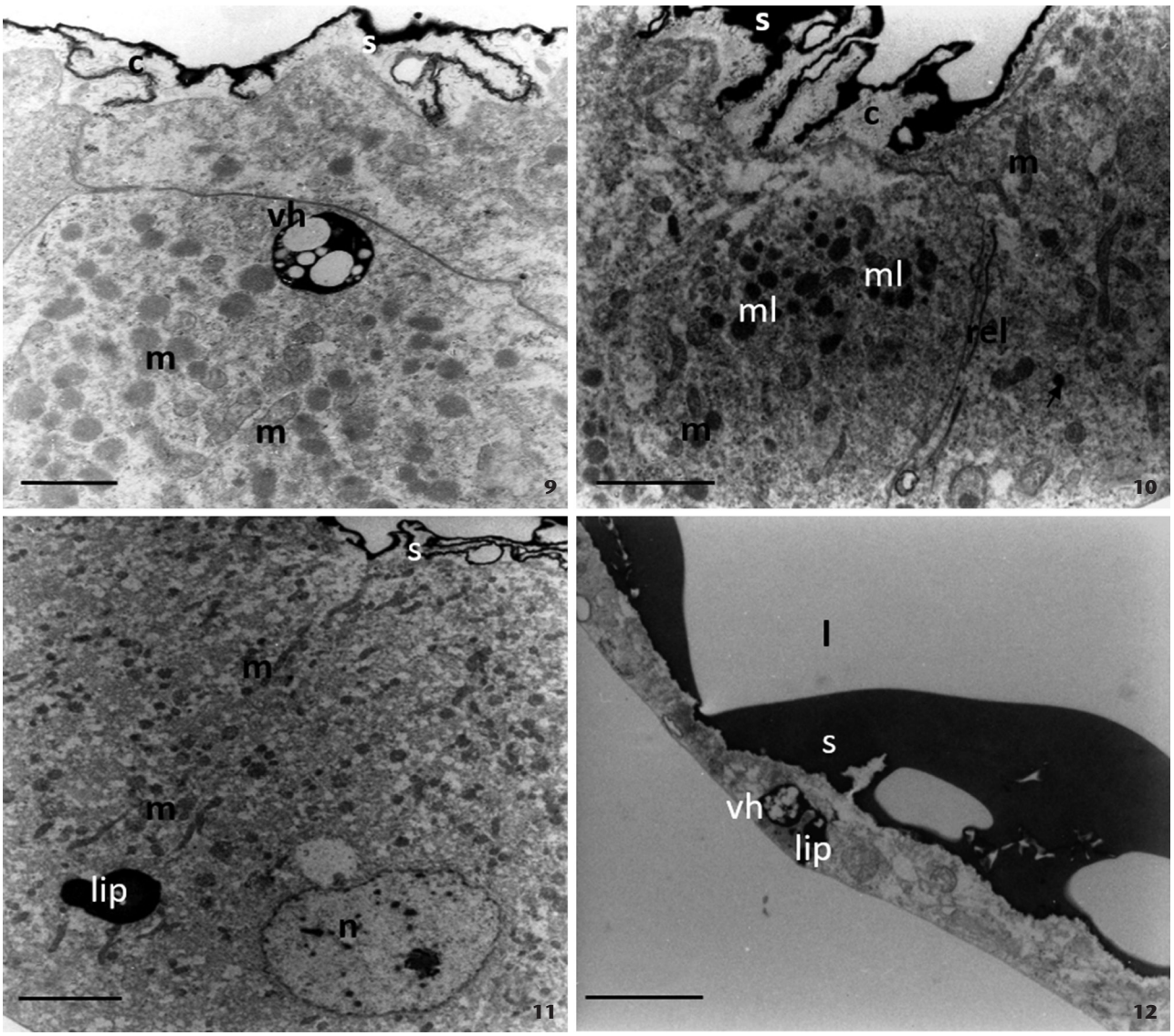
The glandular cells of workers and queens of *A. mellifera* and *S. postica* begin their secretory activity as soon as the adult emerges, and the resulting secretion is progressively accumulated in the gland's alveolar lumen as the bee ages (Poiani & Cruz-Landim, 2009, 2010a, b, c).

The results presented here have shown that the CSG of *A. mellifera* workers not only produces, but also intakes lipid compounds from hemolymph as the worker ages. This gland, then, has both secretory and excretory functions. It has been reported that some lipids present in the hemolymph might



Figures 5-8. Imidazole-osmium preparations for lipid detection in gland cells from *Apis mellifera* workers. (5-6) Gland cells from worker working in the brood comb area (CA) (5) and forager (FO) (6) showing lipid droplets dispersed in the apical region (arrow), infolds (i) of the apical membrane forming channels flanked by mitochondria (m). (6) Note the presence of lipid droplets in the apical channels (arrows). (7-8) Osmium-imidazole-positive dots spread in the cytoplasm (arrows) and vesicles (ve) of forager cellular glands. (c) Cuticle, (s) secretion. Scale bars: 5 = 3 µm, 6 = 2 µm, 7-8 = 1 µm.

be excreted by exocrine glands (Hefetz et al. 1996, Meirelles et al. 2001). The material taken by the cells may be processed in their cytoplasm before being delivered, characterizing secretory activity; or it may just pass through it, characterizing excretion. Well-developed infoldings of the basal cell membrane found in CSGs (Poiani & Cruz-Landim 2009) increase the contact surface between the cell and its environment, and might be associated with excretion. Insect Malpighian tubules and the convoluted nephron tubules of vertebrates are both excretory organs and, as such, have cells with highly developed basal membrane infolds. In some insects, for instance Collembola, which do not have Malpighian tubules, the salivary glands have an excretory role (House & Ginsborg 1985), and that function can be morphologically detected through the presence of very developed basal infolds of the gland cell membranes. The basal infolds of the cells are frequently continuous with the smooth endoplasmic reticulum. Materials uptake by the infolds are from exogenous origin and might circulate within the cellular reticular system, as seen in the Dufour glands of *B. terrestris* queens (Hefetz et al. 1996, Abdalla et al. 1999a, b) and *A. mellifera* queens (Kat-



Figures 9-12. Detection of lipid by imidazole osmium (IO) in cells from the cephalic salivary glands of *Scaptotrigona postica*. (9) Gland cell from newly emerged (NE) and (10) worker working in the brood comb area (CA) showing vesicles containing heterogeneous material (vh) in the apical region. Mitochondria (m) are abundant and variable in electron density and shape. Note the droplets (arrow), smooth endoplasmic reticulum cisterns (rel) and some mitochondria showing IO-positive lipid content inside (ml). (11-12) Glandular cells from forager (FO) workers. (11) Cubic gland cells showing large lipid droplet (lip). (12) Gland cells flattened with drop-shaped lipid vesicles (lip) and heterogeneous vesicles (vh). Note the large alveolar lumen (l) accumulating secretion (s). (c) Cuticle, (m) mitochondrion, (n) nucleus, (s) secretion. Scale bars: 9 = 1 µm, 10, 12 = 2 µm, 11 = 3 µm.

zav-Gozansky et al. 2000), and in the CSGs of *A. mellifera* (Poiani & Cruz-Landim 2009).

The IO treatment in CSG of *A. mellifera* workers has demonstrated the presence of lipids in the cytoplasm of CSG gland cells and in the alveolar lumens of NE workers, increasing continuously until the FO phase. Therefore, in this species, the gland cells continue their activity in foragers, in contrast with what happens in *S. postica*. The CSG cells present extensive infolds of the cellular apical membrane and IO-positive material could be detected within the channels formed by the infolds, as well in the smooth endoplasmic reticulum. That observation suggests that lipids absorbed from the hemolymph, through the basal plasma membrane infolds, reach the lumen by traveling through the endoplasmic reticulum until they are delivered to the lumen,

where they mix with the gland's secretion. Apical membrane infolds mean an increased surface for secretion delivery and the presence of mitochondria located along the channels that are so formed indicates an active transport of content from the inner cell to the alveolar lumen. All these features confer an excretory function to the CSG gland of *A. mellifera* workers, besides a possible secretory role revealed by proteomic analysis (Fujita et al. 2010), which revealed enzymes related to lipids metabolism.

There are older reports about the use of the CSG secretion by *A. mellifera* workers (Heselhaus 1922, Simpson 1960) and recent research has found a correspondence between the contents of the CSG and the chemical profile of the cuticle (Silvana Beani Poiani, unpublished data), suggesting that the CSG secretion may have these compounds exported to the body surface, where they act as recognition pheromones.

In contrast, the gland cells of *S. postica* do not exhibit specific morphological features that indicate excretory function. Therefore, it can be concluded that the lipids present in the alveoli lumen only originate through secretory gland activity. The CSG of *S. postica* produces lipid compounds during the NE and CA phases, storing it in the alveolar lumen until the worker becomes a forager and needs to use it. The IO treatment demonstrated the presence of small amounts of lipids in the cytoplasm and alveolar lumen of the CSG gland cells of NE workers. At the CA phase, the amount of lipids increased in the cytoplasm and alveolar lumen. These lipids were visualized as droplets in the cytoplasm and as a strongly IO-positive homogeneous material in the lumen. The presence of lipids into a network of smooth reticulum, vesicles and droplets present in the cytoplasm signals a secretory function. In FO, alveolar cells are flat, with almost no visible organelles. The alveolar lumen is full of secretion. The fact that the FO gland cells of *S. postica* were much more flattened and poor in organelles when compared to NE and CA indicates that the secretory activity is finished in FO phase, as proposed by Poiani & Cruz-Landim (2009). However, the alveoli of FO workers were still full with secretion, strongly suggesting that the secretion produced was not discharged until the forager phase. The CSG of *S. postica* synthesizes oxygenated compounds, especially esters which are phase-related (Poiani et al. 2015) and may be used in trail scents. In fact Jarau et al. (2004, 2006, 2010), Schorkopf et al. (2007) and Stangler et al. (2009) attributed scent trail activity to these glands in forager meliponines, which use it as trail pheromone. Another function was attributed to the CSG of *Plebeia emerina* (Friese, 1900) in which the glandular secretion from middle-aged nurse workers is used to soften the propolis balls dispersed in the colony as a defense against invaders (Santos et al. 2009). Elias-Santos et al. (2013) identified enzymes related to lipid synthesis in the CSG of *M. quadrifasciata anthidioides*, which is consistent with the results of the present work.

In conclusion, the morphology of the CSG and its function differ between the species studied and among the life phase of the workers. The CSG has excretory and secretory functions in *A. mellifera* workers, while in *S. postica* it has only a secretory function.

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