

Research Article

Neutral Sterols of Cephalic Glands of Stingless Bees and Their Correlation with Sterols from Pollen

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Sterols are essential molecules in the membrane lipid composition and precursors of important sterol hormones that regulate many developmental processes. Insects are unable to synthesize sterols *de novo* and, thus, all phytophagous insects depend on an exogenous source of sterols for growth, development, and reproduction. The sterol requirements of social bees are not fully known due to the fact that there is no well-defined diet available throughout the year with regard to floral resources. Our study aimed to characterize the sterols present in pollen stored in *Melipona marginata* and *Melipona scutellaris* colonies, as well as evaluating their presence in the mandibular, hypopharyngeal, and cephalic salivary gland secretions. We analyzed the chemical composition of pollen stored in the colonies and the composition of the cephalic glands of workers in three adult functional phases (newly emerged, nurses, and foragers) by gas chromatography and mass spectrometry. The results showed that the pollen analyzed contained campesterol, stigmasterol, sitosterol, isofucosterol, lanosterol, and small amounts of cholesterol. The glands showed the same compounds found in the pollen analyzed, except lanosterol that was not found in *M. scutellaris* glands. Surprisingly, cholesterol was found in some glands with relative ratios greater than those found in pollen.

1. Introduction

Sterols are essential molecules in cell membrane, they are precursors of sterol hormones and other important regulators of developmental processes. However, insects are unable to synthesize them and have to purchase sterols from their diet [1]. The dietary needs for sterols in insects were firstly reported by Hobson [2] in the blowfly *Lucilia sericata*. Moreover, in 1959, Clark and Bloch [3] showed the inability of insects to synthesize sterols *de novo*, so that they need to purchase these compounds for auxotrophy (from the Greek “to increase” and “nutrition”). Auxotrophic individuals are unable to synthesize organic compounds necessary for its growth. These authors showed the importance of sterols as structural (cholestanol) and metabolic (cholesterol) components in insects. Further studies showed that ecdysteroids derivatives from cholesterol participate in the control of insect growth and maturation [4].

Furthermore, the necessity of sterol ingestion by insects has been demonstrated in different orders including Orthoptera, Hemiptera, Diptera, Lepidoptera, and Hymenoptera [5]. Indeed, all phytophagous insects studied to date rely on exogenous sterol for growth, reproduction and development [6, 7]. Many phytophagous insects are able to convert phytosterols in cholesterol through the dealkylation of compounds containing 28 to 29 carbon atoms, such as desmosterol, campesterol, brassicasterol, fucosterol, and sitosterol [8–10]. However, honeybees are unable to perform this conversion [11, 12]. Other phytophagous insects that do not have the mechanism of dealkylation of phytosterols include two species of Hemiptera (*Oncopeltus fasciatus*, [13]; *Dysdercus fasciatus*, [14]) and one species of Coleoptera (*Trogoderma granarium*, [15]). The ability to convert phytosterols in cholesterol was not verified in other groups of bees.

The sterol requirements of bees are not assessable because their diet and pollen sources remain unknown. The main

sterol present in the pollen of plants is 24-methylenecholesterol [16–18]. However, other studies reported that besides the 24-methylenecholesterol, the pollen presents phytosterols as desmosterol, campesterol, fucosterol, sitosterol, and even small quantities of cholesterol [17].

Steroidal compounds might be incorporated and stored in different organs of a bee. Queens and workers of honey bees have a significant amount of 24-methylenecholesterol in their body [19]. Svoboda et al. [1] showed that the 24-methylenecholesterol, isofucosterol, sitosterol, and campesterol are present in hypopharyngeal and in mandibular glands of honeybee nurses. The size of cephalic glands (mandibular, hypopharyngeal, and labial) in eusocial bees vary according to the age of individuals [20]. In *Apis mellifera*, the mandibular and the hypopharyngeal glands reach a higher peak of development in nurse bees and they are related with food supply to larvae [20]. We hypothesized that, like in *Apis mellifera*, the cephalic glands of stingless bees play a role as storage organs of phytosterols. Our study aimed to characterize the sterols present in pollen stored in *Melipona marginata* and *M. scutellaris* colonies, as well as to evaluate their presence in the mandibular, hypopharyngeal, and cephalic salivary gland secretions.

2. Material and Methods

We used two colonies of Brazilian stingless bee species: *Melipona marginata* and *Melipona scutellaris*. The colonies were kept at the meliponary of Universidade de São Paulo, Ribeirão Preto. For each species, we collected 10 newly emerged workers, 10 nursing workers, 10 foragers, and two samples (1 g) of pollen storage in pots, all on the same day. We marked the new cells produced in the comb that day for later collection of newly emerged workers. The mandibular (MAN), hypopharyngeal (HYP), and labial glands (LAB) (cephalic section) were dissected under stereomicroscope and put individually in 30 μ L of hexane. The pollen samples were put in methanol (4 mL/1 g) and the extracts were centrifuged (for removal the proteins). The aqueous phase was removed and dried under N_2 and resuspended in 400 mL of hexane. The samples were injected in a combined gas chromatography-mass spectrometry (GC-MS: SHIMADZU, model QP2010). Separation was achieved on a DB-5MS column 30 m, and the gas carrier was helium at 1.0 mL min^{-1} . The oven temperature was initially set to 50°C (held for 1 min) and increased by 10°C min^{-1} until it reached 300°C (held for 15 min). Analyses were performed in splitless mode. The mass spectra were obtained by 70 eV ionization. The data were analyzed with GCMS solutions for Windows (Shimadzu Corporation), and the chemical compounds were identified based on their mass spectra by comparison with Nist Library data and with a standard solution of different synthetic phytosterols (Sigma-Aldrich).

We analyzed the pollen as described in Silva et al. [21]. The protein fractions of pollen were kept in alcohol 70% and after 24 hours the alcohol was discarded. The samples were placed in vials containing 4 mL of absolute glacial acetic acid (24 hours) according to the acetolysis protocol described by

Erdtman [22]. Following acetolysis, the pollen was placed on slides for light microscopy with 50% glycerin. We identified pollen types by comparison with pollen grains deposited on Palinoteca of Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, using a Leica DM 4000B microscope, with an increase of up to 2.560x.

3. Results

Results of sterol analyses of pollen, hypopharyngeal glands (HYP), labial glands (LAB), and mandibular glands (MAN) of *Melipona marginata* and *Melipona scutellaris* are summarized in Tables 1 and 2. The pollen samples collected in pots of the two *Melipona* species showed the same ratios of cholesterol, campesterol, stigmasterol, sitosterol, isofucosterol, and lanosterol. In general, the gland secretions showed the same sterols as pollen, with some variations according to the age of workers and the gland type. The only exception was the absence of lanosterol in the glands of *M. scutellaris* bees, even though it is present in pollen collected in pots. In *M. marginata*, sitosterol was the main sterol present in pollen and glands (except for the mandibular glands of nurse bees) while in *M. scutellaris* campesterol and sitosterol had the highest proportions. Cholesterol in pollen samples had lower relative percentage (0.53% and 0.33%) than others sterols. There was no pattern of increasing or decreasing of the relative percentages of phytosterols present in glands in relation to the age of individuals. The sterols were obtained from Angiosperms as Arecaceae (*M. scutellaris*, 1 species), Fabaceae (*M. marginata*, 4 species; *M. scutellaris*, 3 species), Melastomataceae (*M. marginata*, 1 species) and Myrtaceae (*M. marginata*, 1 species; *M. scutellaris*, 3 species), according to pollen type (Table 3).

3.1. Newly Emerged Workers. The main sterol found in *M. marginata* glands was sitosterol, followed by cholesterol (HYP and LAB) and isofucosterol (MAN). In *M. scutellaris* glands, the main compounds were sitosterol (HYP and MAN) and campesterol (LAB) followed by isofucosterol (HYP) and cholesterol (MAN). In *M. marginata* workers, stigmasterol was found only in mandibular glands and lanosterol was found in mandibular and hypopharyngeal glands. The hypopharyngeal gland and the labial gland of newly emerged workers showed a higher proportion of cholesterol when compared with the pollen in *M. marginata* (26.62%, 47.7% and 0.53%, resp.). In *M. scutellaris*, all glands of newly emerged workers showed a higher percentage of cholesterol when compared with pollen (HYP = 11.20%, MAN = 19.96%, LAB = 20.79%, and Pollen = 0.33%).

3.2. Nurse Workers. Sitosterol was the main steroidal compound found in the glands of nurse bees, except for mandibular glands of *M. marginata* (stigmasterol) and hypopharyngeal glands in *M. scutellaris* (campesterol). Campesterol was the second major sterol found in this class of workers (HYP and MAN in *M. marginata*, MAN in *M. scutellaris*). Only the labial gland of *M. scutellaris* showed isofucosterol as the second main compound. In *M. marginata*, the glands of

TABLE 1: Relative percentages (Mean \pm standard deviation) of sterols in pollen and glands of *Melipona marginata* newly emerged workers (New), nurse bees (Nur), and foragers (For). $N = 10$ (glands) and $N = 2$ (pollen samples, 1 g per sample).

	Pollen	Hypopharyngeal			Mandibular			Labial		
		New	Nur	For	New	Nur	For	New	Nur	For
Cholesterol	0.53 ± 0.1	26.62 ± 24.2	1.31 ± 0.6	—	1.20 ± 0.4	2.60 ± 0.01	—	47.7 ± 4.9	14.79 ± 2.3	—
Campesterol	14.98 ± 4.1	14.75 ± 7.3	30.11 ± 18.8	22.16 ± 2.8	6.18 ± 2.8	29.58 ± 13.9	16.49 ± 4.1	—	13.55 ± 1.9	4.06 ± 5.2
Stigmasterol	2.01 ± 0.7	—	23.08 ± 18.7	—	3.39 ± 3.4	54.41 ± 16.8	—	—	14.07 ± 4.8	34.40 ± 44.3
Sitosterol	52.16 ± 17.9	31.38 ± 23.4	35.92 ± 24.8	65.02 ± 12.1	52.45 ± 21.8	13.37 ± 9.5	69.80 ± 29.4	52.3 ± 2.6	23.26 ± 15.4	54.12 ± 69.7
Isofucosterol	23.60 ± 15.2	14.60 ± 5.2	9.58 ± 16.3	12.82 ± 11.4	31.53 ± 10.5	0.29 ± 0.11	13.70 ± 7.4	—	11.56 ± 15.7	7.42 ± 9.6
Lanosterol	6.73 ± 6.06	12.64 ± 8.6	—	—	5.24 ± 1.2	—	—	—	22.78 ± 12.9	—

	Hypopharyngeal			Mandibular			Labial	
	Pollen	New	Nur	For	New	Nur	New	For
Cholesterol	0.33 ± 0.35	11.20 ± 1.9	2.31 ± 0.8	—	19.96 ± 28.5	21.45 ± 24.0	20.79 ± 13.8	28.10 ± 36.5
Campesterol	10.97 ± 1.58	5.06 ± 2.1	52.32 ± 31.9	52.71 ± 25.5	16.39 ± 6.7	29.19 ± 11.6	31.52 ± 33.9	9.64 ± 8.1
Stigmasterol	1.42 ± 1.54	—	—	—	—	—	—	5.50 ± 8.42
Sitosterol	53.08 ± 19.2	71.27 ± 5.6	34.61 ± 21.8	37.22 ± 37.1	48.18 ± 16.1	34.68 ± 16.9	25.90 ± 15.7	43.91 ± 28.9
Isofucosterol	27.37 ± 9.9	12.47 ± 2.7	10.76 ± 4.8	10.07 ± 13.4	15.47 ± 3.5	14.69 ± 6.4	21.79 ± 24.4	12.85 ± 7.2
Lanosterol	6.85 ± 5.9	—	—	—	—	—	—	—

TABLE 3: Pollen types found in food pots of *Melipona marginata* and *Melipona scutellaris* colonies.

Families/species	<i>M. marginata</i>	<i>M. scutellaris</i>
Arecaceae		
<i>Archontophoenix alexandrae</i> (F. Muell.) H. Wendl. and Drude	–	+
Fabaceae		
<i>Anadenanthera macrocarpa</i> (Benth.) Brenan	+	+
<i>Anadenanthera peregrina</i> (L.) Speg.	+	+
<i>Leucaena leucocephala</i> (Lam.) de Wit	–	+
<i>Mimosa</i> sp.	+	–
<i>Senna macranthera</i> (DC. ex Collad.) H. S. Irwin and Barneby	+	–
Melastomataceae		
<i>Tibouchina granulosa</i> (Desr.) Cogn.	+	–
Myrtaceae		
<i>Eucalyptus grandis</i> W. Mill ex Maiden	+	–
<i>Eucalyptus moluccana</i> Roxb.	–	+
<i>Eugenia involucrata</i> DC.	–	+
<i>Eugenia pyriformis</i> Cambess.	–	+

Presence: (+) and absence: (–).

nurse bees showed a higher proportion of stigmaterol when compared with pollen (HYP = 23.08, MAN = 54.41%, LAB = 14.07%, and Pollen = 2.01%). In *M. scutellaris*, all glands of nurse bees showed a higher relative proportion of cholesterol when compared with pollen (HYP = 2.31%, MAN = 20.43%, LAB = 14.30%, and Pollen = 0.33%).

3.3. Forager Workers. Cholesterol and lanosterol were absent in *M. marginata* forager workers. Stigmaterol was present only in labial glands of the two analyzed species. *M. scutellaris* also lacked lanosterol, and sitosterol was the main compound found in the glands, except for hypopharyngeal glands (campesterol). *M. marginata* forager workers showed great relative amounts of campesterol and cholesterol in HYP and MAN gland secretions, and *M. scutellaris* workers presented higher relative concentrations of these same compounds in MAN and LAB gland secretions. In *M. marginata*, the glands of foragers showed a higher proportion of stigmaterol when compared with pollen (LAB = 34.40% and Pollen = 2.01%). Mandibular glands and labial glands of *M. scutellaris* foragers showed a higher percentage of cholesterol when compared with pollen (MAN = 28.43%, LAB = 28.10%, and Pollen = 0.33%).

4. Discussion

This study shows that once sterols are ingested they are distributed among different glands of two stingless bee species. As phytosterols play an important role in sterol metabolism and ecdysteroid production [8, 10, 23–26], the ingestion of exogenous sterols is essential for structural and metabolic components. Sterols seem to be absorbed mainly in the midgut, particularly in phytophagous species [27, 28], moving quickly through mucosal cells [28].

The analyzed pollen and glands lacked 24-methylene-cholesterol, differently from results found for *Apis mellifera* by Svoboda et al. [1]. The pollen samples collected in pots of the two *Melipona* species showed the same ratios of sterols despite the differences in pollen origin. Patterson [29] showed that the most abundant sterols found in Angiosperms were sitosterol and stigmaterol, but many species also contain campesterol and dihydrobrassicasterol. The pollen of most plant species accumulate intermediates such as 24-methylenecholesterol and 9 β ,19-cyclopropyl sterols [30]. However, sitosterol can be the main sterol in some species, as found in *Baccharis viminea*, *Juniperus uthaensis*, *Polygonum* sp., *Hydrophyllum capitatum*, *Pinus sylvestris*, and other plants [31]. Large amounts of stigmasterol and cholesterol were found in the pollen of heather (*Calluna vulgaris*) and in cottonwood (*Populus fremontii*), respectively.

Among bees, 24-methylenecholesterol is the main sterol available in natural diet and the dominant tissue sterol, followed by other phytosterols according to the bee's host-plants [32]. We showed in our study that the main sterols of pollen were present in glands. Moreover, despite the inability of some stingless bees of dealkylation of phytosterols into cholesterol, we found large amounts of cholesterol in some glands. Indeed, the Apocrita (Hymenoptera) lost the dealkylation ability [32], and bees use several unmetabolized sterols. Thus, the accumulation of cholesterol in their glands remains unexplained.

One of the most probable hypotheses to explain the tissue accumulation of sterols is the selective transfer of sterols from the adults' endogenous pools to the offspring [1]. Svoboda et al. [1] showed that sterols of the workers could be transferred to the developing larvae through the brood food material secreted from the hypopharyngeal and mandibular glands and/or the honey stomach of the workers. Tissue sterol profiles can differ between males and females. Female's reproductive organs have higher concentration of cholesterol in *Eurycotis floridana* [33] and queen ovaries of *Apis mellifera* have higher concentrations of 24-methylenecholesterol, campesterol, and sitosterol [34]. Regarding development, sterol profile is influenced by the stage of development (egg, larva and adult) in ants, and the eggs presented the lowest diversity in types of sterol, while the queen had the highest [35].

Relative concentrations in secretions of workers of the same group have high deviations, but this can be explained by the differences in the age of the worker performing a task in colonies of social bees. Ferreira-Caliman et al. [36] showed that engagement in tasks on brood comb (nurse bees)

occurred between 5th and 12th days postemergence and the foraging activities initiated after 22 days postemergence and this task can be observed at least for 30 days.

This study is the first report on the participation of cephalic glands as storage tissues of pollen phytosterols in stingless bees. We could not assess how the accumulation of phytosterols occurs throughout life of the individual, because no bioassay using a controlled diet was performed. However, we showed that the glands of the head of stingless bees are an important storage tissues for these compounds, although many studies are still needed to understand how this transfer occurs and the role of these glands as reservoirs of sterols.

Conflict of Interests

The authors declare that there was no conflict of interests or any research financial gain concerning the results of the present study.

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