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Chemical identity of recently emerged workers, males, and queens in the stingless bee *Melipona marginata*

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Abstract – Epicuticular hydrocarbons are involved in several behavioral processes in social insects including recognition of nestmates, castes, and sex. The aim of the present study was to characterize and to verify differences in the chemical profiles of 1-day recently emerged workers, males, and queens in colonies of the stingless bee *Melipona marginata*. Gas chromatography–mass spectrometry and multivariate analyses of bee samples from three colonies clearly separated cuticular hydrocarbon profiles of males, queens, and workers. The results showed that cuticular hydrocarbon profiles were comprised of alkanes, alkenes, and methyl alkanes that varied quantitatively and qualitatively according to the castes and sex. Different from previous studies, our investigation also demonstrated that males and queens presented higher similarity in their epicuticular hydrocarbons than to workers.

stingless bees / *Melipona marginata* / castes and sex / cuticular hydrocarbons / gas chromatography–mass spectrometry

1. INTRODUCTION

The outer covering of terrestrial insects consists of a thin layer of lipids (Hadley 1985). The main compounds are hydrocarbons that prevent dehydration and serve as a protective barrier against microorganisms (Blomquist and Dillwith 1985; Lockey 1988; Nelson and Blomquist 1995). Some epicuticular hydrocarbons can play also a role as communication cues in social insects (Howard and Blomquist 1982; Breed and Bennett 1987; Blomquist et al. 1998; Singer et al. 1998; Howard

and Blomquist 2005; revised in Blomquist and Bagnères 2010). For instance, these variable compositions of these compounds allow for the conveyance of colony membership, age, caste, or sex of individuals (Blomquist et al. 1998; Monnin and Peters 1999; Sledge et al. 2001; Abdalla et al. 2003; Provost et al. 2008; Nunes et al. 2009; Tannure-Nascimento et al. 2009; Ferreira-Caliman et al. 2010).

Beyond the hydrocarbons, the epicuticle of insects is covered by other lipids as wax esters and fatty alcohols (Jackson and Baker 1970). In social hymenopterans, hydrocarbons comprise the main group of compounds present on the cuticle and they seem to be the essential compounds that serve as nestmate recognition cues (Howard and Blomquist 2005). In addition,

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fatty acids and esters found in honeybee's cuticle may also play an important role in this process (Fröhlich et al. 2001; Breed et al. 2004). Insect cuticular hydrocarbons have from 17 to 49 carbon atoms in the chain, frequently made up of alkanes, methyl-branched alkanes, and alkenes and they have been shown to be derived from fatty acids through chain lengthening and decarboxylation (Morgan 2004).

In stingless bees, hydrocarbons from 19 to 33 carbon atoms in the chain, comprising alkanes, methyl alkanes, dimethyl alkanes, and alkenes lengthening have been found (Nunes et al. 2008, 2009; Ferreira-Caliman et al. 2012). The hydrocarbons of the epicuticle are synthesized in the oenocyte cells associated to the epidermis (Piek 1964; Schal et al. 1998; Billeter et al. 2009), and they are transported through the hemolymph by a protein called lipophorin (Schal et al. 1998). This heritable source can be improved by environment (e.g., food, nest material) or by the cues from the queen into colonies of social insects (Zweden and d'Ettorre 2010) and temperature (Martin et al. 2012).

Experiments conducted in ants and in honeybees by Stuart (1988) and Breed (1983), respectively, showed that workers reared and kept under laboratory conditions use genetic cues to distinguish between nestmates and non-nestmates, suggesting that the heritable recognition cues produced by a younger worker are similar to those of nestmates. On the other hand, under natural conditions, the workers exposed to the comb wax replace these genetic cues, so that the bees come to rely on environmental recognition cues (Breed 1998). Indeed, in stingless bees, individuals can acquire chemical compounds from after emergence (environmental based acquisition) or these compounds can be incorporated during their development (endogenously based acquisition). Although recently emerged stingless bee workers had distinct chemical profiles, according to their original colony, they were not recognized when presented to non-nestmate guards (Nunes et al. 2011). Therefore, it would be interesting to understand the variation of the endogenously based cues in distinct individuals. Recent re-

ports on the cuticular chemical profiles in bees have revealed differences between castes and among workers performing different activities in both honey bees and stingless bees (Fröhlich et al. 2001; Nunes et al. 2009; Ferreira-Caliman et al. 2010; Kather et al. 2011).

The similarities and differences between castes and sex in stingless bees were previously studied through comparisons of morphology and behavior (Darchen 1969; Winston and Michener 1977). Some studies showed that workers resemble males more than queens in their external morphology (Kerr 1974, 1987; Kerr and Cunha 1990). But the chemical differences among the two female castes and the sex are inconclusive. Here, we tested which cuticular hydrocarbons were shared and which compounds provided information on castes and gender at the moment of emergence.

2. MATERIAL AND METHODS

2.1. Species

Melipona marginata Lepeletier, 1836 (Hymenoptera, Meliponini) is a Brazilian species of stingless bees and is distributed throughout the states of São Paulo, Paraná, Santa Catarina, and Bahia (Silveira et al. 2002). In order to characterize the cuticular profiles of newly emerged individuals, we collected 1-day-old males ($N=45$), gynes (virgin queens, $N=45$), and workers ($N=45$) from three *M. marginata* colonies. Hives were from the municipality of Cunha (São Paulo, Brazil) and kept in wooden boxes at the meliponary of Universidade de São Paulo, Ribeirão Preto. The females (workers and queens) and males were identified according to drawing boards published by Camargo et al. (1967), where the authors showed the morphological differences for this species. The individuals were collected in individual vials (2 mL), frozen, and the used to hydrocarbons extraction.

2.2. Chemical analyses

The epicuticular compounds for each bee were extracted in 1 mL hexane for 1 min. After evaporating the solvent under N_2 flow, the apolar extract was resuspended in 100 μ L of hexane, and 1 μ L was injected in a combined gas chromatography–mass spectrometry

(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 150°C and increased by 3°C min^{-1} until it reached 280°C . Analyses were performed in splitless mode. The mass spectra were obtained by 70 eV ionization.

For the analysis of data, we used the program GC-MS solutions for Windows version 2.6 (Shimadzu Corporation). The alkanes were identified based on their mass spectra by comparison with Nist Library data and with a standard solution of different synthetic hydrocarbons (Fluka). The branched alkanes were identified based on comparisons with mass spectral data from Monnin et al. (1998) and Carlson et al. (1998, 1999). The double bond

positions of alkenes were identified through derivatization of hexane extracts from 15 individuals of each group (queens, males and workers) according methodology proposed by Carlson et al. (1989). Extracts were dried with nitrogen and resuspended in $200 \mu\text{L}$ of hexane using magnetic stirring. Subsequently, $200 \mu\text{L}$ of dimethyl disulfide (DMDS) (Sigma-Aldrich) and $100 \mu\text{L}$ of iodine solution (dissolved in diethyl ether, $6\% \text{ m/v}$) were added. The vials then were purged with nitrogen, closed, and agitated at ambient temperature for 24 h. The mixtures were diluted in hexane and extracted with 5% sodium thiosulfate solution. The organic phase was subsequently dried with sodium sulfate and analyzed by GC-MS, following the method proposed

Table 1. Mean (%) and standard deviation of the relative concentrations of cuticular hydrocarbons in the males, workers and gynes of *Melipona marginata*. (N=45 for each group). RT=retention time. Diagnoses based on comparisons with mass spectral data from Monnin et al. (1998) and Carlson et al. (1998).

Hydrocarbon	Diagnostic mass spectral ion fragments	Workers	Gynes	Males
Heneicosane ($n\text{-C}_{21}$)	296	0.68 ± 0.55	0.48 ± 0.47	0.31 ± 0.19
Tricosane ($n\text{-C}_{23}$)	324	14.62 ± 3.90	10.49 ± 5.69	9.64 ± 2.66
Tetracosane	338	0.38 ± 0.11	0.32 ± 0.42	0.24 ± 0.19
Z-C25 (several)	DMDS ^a	1.88 ± 0.91	2.90 ± 3.60	0.87 ± 0.61
Pentacosane ($n\text{-C}_{25}$)	352	13.92 ± 1.86	13.32 ± 6.64	12.65 ± 5.63
5 Methyl C_{25}	85,281,351	—	0.09 ± 0.16	1.16 ± 3.95
Hexacosane ($n\text{-C}_{26}$)	366	0.42 ± 0.13	0.17 ± 0.25	0.45 ± 0.35
Not identified	—	—	0.20 ± 0.27	—
Z-C27 (several) ^b	DMDS ^b	31.02 ± 4.04	31.69 ± 10.16	26.20 ± 9.37
Heptacosane ($n\text{-C}_{27}$)	380	9.92 ± 3.12	9.94 ± 6.07	11.80 ± 7.16
Octacosane ($n\text{-C}_{28}$)	394	0.55 ± 0.28	6.48 ± 8.90	0.60 ± 1.11
Z-C29 (several) ^c	DMDS ^c	17.96 ± 2.20	13.28 ± 6.33	13.61 ± 9.27
Nonacosane ($n\text{-C}_{29}$)	408	1.44 ± 0.72	2.10 ± 3.29	2.76 ± 2.64
Z-C30 (several) ^d	DMDS ^d	5.99 ± 2.31	4.98 ± 3.16	2.13 ± 2.50
Hentriacontane ($n\text{-C}_{31}$)	436	0.41 ± 0.23	2.89 ± 3.15	1.01 ± 2.44
Dotriacontane ($n\text{-C}_{32}$)	450	—	—	0.70 ± 0.85
Tetratriacontane ($n\text{-C}_{34}$)	478	—	—	4.40 ± 4.51

DMDS diagnostic ions after derivatization with dimethyl disulfide

^a (Z)-7-C25 (145, 299, 444) (majority); (Z)-9-C25 (173, 271, 444) (majority); (Z)-11-C25 (201, 243, 444)

^b (Z)-5-C27 (117, 355, 472) (majority); (Z)-7-C27 (145, 327, 472); (Z)-9-C27 (173, 299, 472); (Z)-11-C27 (201, 271, 472); (Z)-13-C27 (229, 243, 472)

^c (Z)-5-C29 (117, 383, 500); (Z)-7-C29 (145, 355, 500) (majority); (Z)-9-C29 (173, 327, 500); (Z)-11-C29 (201, 299, 500) (majority); (Z)-12-C29 (215, 285, 500); (Z)-13-C29 (229, 271, 500); (Z)-14-C29 (243, 257, 500)

^d (Z)-7-C31 (145, 383, 528) (majority); (Z)-9-C31 (173, 355, 528); (Z)-11-C31 (201, 327, 528) (majority); (Z)-12-C31 (215, 313, 528); (Z)-13-C31 (229, 299, 528); (Z)-15-C31 (257, 271, 528)

by Carlson et al. (1989) in which the oven temperature was initially set to 80 °C (hold for 2 min), increased by 30 °C min⁻¹ until it reached 180 °C, and increased by 3 °C min⁻¹ until it reached 300 °C (hold for 80 min).

2.3. Statistical analyses

Principal components analysis (PCA) was used to recognize which compounds were common among predicted groups (“common cues”) and which compound were candidates to discriminate caste and gender. PCA therefore indicated the compounds that contributed less than 5 % to separate the predicted groups in the first two most important factors. Thus, these compounds were excluded from the canonical analysis and the relative concentrations of the remaining compounds were readjusted to 100 %. In canonical analysis, a forward stepwise discriminant function analysis was used to observe if combinations of variables could be useful in the predicting group. In this method, variables are successively added to the model based on the higher *F* to enter values, adding no more variables when the *F* ratio is no longer significant. Wilks’ λ values were used to verify the individual contribution of each variable to the model. The Wilks’ λ statistic for the overall discrimination is computed as the ratio of the determinant of the within-

groups variance/covariance matrix to the determinant of the total variance/covariance matrix. When this value is close to 1.0, then the residual is high and the variable is not a good discriminator, while a value closer to 0 means that the residual is low and the variable is a good discriminator (Rao 1973).

Squared Mahalanobis distance (D^2) was used as an indicator of chemical profile similarity. According to McLachlan (1992), the Mahalanobis squared distance is defined by $D^2 = (\mu_1 - \mu_2)^T \Sigma^{-1} (\mu_1 - \mu_2)$, (1) where μ_1 and μ_2 are the estimated means from random sample sizes. (2) The superfix *Z* denotes matrix transpose, Σ denotes the common (nonsingular) covariance matrix of *X* that represents a random vector that contains the measurements made on a given individual or entity under study. It can be seen that since Σ is a (nonsingular) covariance matrix, it is positive definite and hence *D* is a metric. In this sense, the squared Mahalanobis distance gives the distance from each of the group centroids. The *P* values are based on posterior probabilities indicating that the chance of each group is correctly classified. We also applied Kruskal–Wallis ANOVA test between castes and genders intending to analyze which hydrocarbons and group of hydrocarbons (alkanes and alkenes) are statistically changing their proportions ($P < 0.05$). The statistical analyses were performed

Figure 1. Chromatograms of cuticular hydrocarbons in *M. marginata* individuals. Main peaks: (1) heneicosane, (2) tricosane, (3) tetracosane, (4) Z-pentacosenes, (5) pentacosane, (6) Z-heptacosenes, (7) heptacosane, (8) octacosane, (9) Z-nonacosenes, (10) nonacosane, (11) Z-hentriacontenes, (12) hentriacontane, (13) dotriacontane, and (14) tetratriacontane.

Table II. Classification matrix of males, workers, and gynes of *M. marginata* ($N=45$ for each group).

	Percent correct	Workers	Gynes	Males
Workers	100	45	0	0
Gynes	95.56	0	43	2
Males	91.11	0	4	41
Total	95.55	45	47	43

using the software Statistic 7.0 for Windows (Statsoft, Inc).

3. RESULTS

Analyses of the cuticular hydrocarbons of 1-day recently emerged workers, gynes, and males showed a total of 17 hydrocarbons peaks, varying according to the female castes (gynes and workers) and males. These compounds were classified as linear alkanes and linear alkenes, varying between 21 and 34 atoms of carbon (Table I). In addition, we found a methyl alkane and one unidentified hydrocarbon. In general, the most abundant compounds in all groups were heptacosene, nonacosene, hentriacontene, tricosane, pentacosane, and heptacosane. Dotriacontane and tetratriacontane, compounds with higher molecular weight, were present only in males (Figure 1).

For the discriminant analyses, we used the compounds indicated by PCA as the variables that contributed more than 5 % to separate the predicted groups in the first two most important factors (tricosane, pentacosenes, pentacosane, heptacosenes, heptacosane, octacosane, nonacosenes, hentriacontenes, hentriacontane). Forward stepwise discriminant analyses of these compounds significantly separated the individuals according to their caste/sex (Global Model: Wilks' lambda, 0.04476; $F_{18, 248}=51.34$; $P<0.00001$). All compounds used in PCA analysis were important for this separation ($P<0.0005$).

In PCA, three eigenvalues of correlation matrix were higher than 1 (cumulative variation=66.16 %). The value number 1 (eigenvalues=2.77) described 30.82 % of the variation among groups, while the value number 2 (eigenvalues=1.87) and 3 (eigenvalues=1.31) described 20.74 and 14.60 % of the variation

Figure 2. Canonical analysis of pooled data of cuticular compounds from three *M. marginata* colonies. $N=45$ individuals per group.

among them, respectively. The classification matrix based on the discriminant analysis classified all workers correctly into their respective group. Two gynes and four males were not allocated to original group (Table II).

The log plot of discriminant analysis showed that the worker group was completely segregated from the other two groups (Figure 2). According to the Mahalanobis distances, the chemical profiles of workers were more similar to the males' chemical profiles than to the gynes' profiles (Table III). Moreover, the distance between gynes and males was lower than the distance between gynes and workers (Figure 3).

Tricosane, tetracosane, hexacosane, and Z-nonacosenes were significantly distinct when we compared workers to gynes. Workers showed greater differences to males concerning the hydrocarbons tricosane, tetracosane, Z-heptacosenes, Z-nonacosenes, dotriacontane, and tetratriacontane. Z-heptacosenes, octacosane, nonacosane, dotriacontane, and tetratriacontane presented distinct relative proportions when we compared gynes to males. When comparing classes of compounds, only alkenes presented significant difference between males and females (Figure 3).

Identification of alkenes by DMDS method showed that 1-day-old males and females (gynes and workers) had the same alkenes (Table I). The majority compounds for each alkenes were pentacosene = (Z)-7-C₂₅ and (Z)-9-C₂₅, heptacosene = (Z)-5-C₂₇, nonacosene = (Z)-7-C₂₉ and (Z)-11-C₂₉, and hentriacontene = (Z)-7-C₃₁ and (Z)-11-C₃₁.

4. DISCUSSION

Our study demonstrated for the first time that although individuals are only 1-day recently

Figure 3. Comparison of proportion of *n*-alkanes and alkenes between castes and genders. The bars are the average of the group of hydrocarbons and the error bars are the standard deviation. In accordance to the Kruskal–Wallis ANOVA test, same letters indicate no statistical significance between groups ($P<0.05$).

emerged, they presented clear-cut differences concerning their chemical cuticular profiles. This result is especially interesting because males and females, queens, and workers of *Melipona* bees are not raised in any special type of cells and, apparently, receive the same quantity and quality of food. Nevertheless, we can see a marked difference between the time of ontogenetic development in species of this genus, with queens having shorter development time, followed by workers and by males (Moo-Valle et al. 2004).

Our results are in agreement with other previous reports that showed that older males, queens, and workers have different patterns of cuticular hydrocarbon profiles (Abdalla et al. 2003; Kerr et al. 2004). In *Melipona bicolor*, Abdalla et al. (2003) presented that males had their chemical cuticular profiles more similar to workers than to the queens' profiles.

Table III. Results of discriminate analysis based on cuticular hydrocarbon profiles among workers, gynes, and males of *M. marginata* ($N=45$ for each group).

	Mahalanobis distance	$F_{9, 124}$	P value
Workers $\times$ gynes	92.24	216.63	<0.0001
Workers $\times$ males	49.21	115.57	<0.0001
Gynes $\times$ males	8.37	19.64	<0.0001

However, except for the fact that the bees were virgin queens and males, in Abdalla et al. (2003), the age of the bees was not precisely controlled and they just used the wings for the hydrocarbons analysis. Moreover, comparing the hydrocarbons profiles in cuticle of older individuals of *Melipona scutellaris*, Kerr et al. (2004) found that the pattern of workers is much closer to that of males than it is to queens.

In contrast to Abdalla et al. (2003) and Kerr et al. (2004), in this study, we found that gynes are closer to males than workers at the same age by cuticular hydrocarbon analysis of 1-day recently emerged individuals. Furthermore, the number of samples in both studies (Abdalla et al. 2003; Kerr et al. 2004) was small, not allowing a robust statistical approach to discriminate the groups according to the cuticular hydrocarbons profiles. In this sense, the origin and age variation could be the cause of differences between their chemical profiles. It is interesting to state that the within-nest environment would provide a more similar chemical profile after emergence, but previous studies showed that castes, age, and sex experience a temporal divergence in their respective chemical signatures (see Ferreira-Caliman et al. 2010).

We found both qualitative and quantitative differences between males and females related to hydrocarbons containing more than 30 carbons. Workers' cuticle showed a lower relative percentage of hentriacontenes when compared with males and gynes. The presence of hentriacontane and tetratriacontane only in males and gynes may mean potential cues for reproduction, which would also agree with our finding that males and gynes are more similar to each other than to workers in this species. Males are often expelled from colony early on or slaughtered (Sakagami 1982). This could explain the necessity of large linear alkanes for cuticular impermeabilization and protection (e.g., in insects: Ramsay 1935; Gibbs 2002) since they are earlier exposed to the external environment and these compounds are usually associated to the structure of surface as they are

not informative as alkenes to be used as a recognition cue (Martin et al. 2004; Châline et al. 2005).

In conclusion, we demonstrated in this study that 1-day emerged castes and genders of *M. marginata* share a great number of endogenous cuticular hydrocarbons, but that they already show a distinct variation in their relative proportions that may be perceived by older members of the colonies.

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Identité chimique, juste après l'émergence, des ouvrières, mâles et reines de l'abeille sans aiguillon, *Melipona marginata*

Meliponini / caste / sexe / hydrocarbure cuticulaire / chromatographie en phase gazeuse couplée à la spectrométrie de masse

Chemische Identität von frischgeschlüpften Arbeiterinnen, Männchen und Königinnen der Stachellosen Biene *Melipona marginata*

Stachellose Bienen / *Melipona marginata* / Kaste und Sex / kutikuläre Kohlenwasserstoffe / Gaschromatographie-Massenspektrometrie

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