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Comparative study of the cuticular hydrocarbon composition of *Melipona bicolor* Lepeletier, 1836 (Hymenoptera, Meliponini) workers and queens

Fábio C. Abdalla[1], Graeme R. Jones[2], Erik D. Morgan[2] and Carminda da Cruz-Landim[1]

[1]Departamento de Biologia, Instituto de Biociências, Universidade Estadual Paulista (UNESP), Rio Claro, SP, Brasil
[2]School of Chemistry and Physics, Keele University, Staffordshire, England
Corresponding author: F.C. Abdalla
E-mail: fabdalla@rc.unesp.br

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ABSTRACT. In social insects, cuticular hydrocarbons are involved in species, kin, caste and nestmate recognition. Gas chromatography and mass spectrometry were used to compare the cuticular hydrocarbon composition of workers, males and queens of *Melipona bicolor*. The cuticular hydrocarbon composition of this species was found to consist mainly of C23, C25:1, C25, C27:1, C27, C29:1 and C29, which are already present in imagoes that have not yet abandoned the brood cell. This composition varied quantitatively and qualitatively between and within the castes and sexes. The newly emerged workers and young queens (virgins) had similar cuticular hydrocarbon profiles, which were different from those of the males. When the females start executing their tasks in the colony, the cuticular hydrocarbon profile differences appear. The workers have less variety, while the queens conserve or increase the number of cuticular hydrocarbon compounds. The queens have more abdominal tegumentary glands than the workers, which apparently are the source of the new cuticular compounds.

Key words: Dufour's gland, *Melipona bicolor*, Kin recognition, Stingless bee, Pheromone, Cuticular hydrocarbons

INTRODUCTION

Semiochemicals are substances that convey information between organisms (Law and Regnier, 1971). This group of chemicals includes pheromones, which have an intraspecific action (Karlson and Butenandt, 1959; Karlson and Lüscher, 1959; Brown et al., 1970). In addition to the primary functions of preventing desiccation and regulating cuticular permeability, cuticular hydrocarbons also have semiochemical functions and may be the principal constituent of surface pheromones in insects (Howard, 1993; Smith and Breed, 1995; Dani et al., 1996; Arnold et al., 2000; Ayasse et al., 2001; Ruther et al., 1998, 2002).

Insect pheromones may be "primers" or "releasers" (Wilson, 1963). The releasers cause an immediate, reversible change in the behavior of the target organism and are often classified by the type of behavior they evoke (Shorey, 1973). Cuticular hydrocarbons are included in the class of releasers known as "surface pheromones". Such pheromones are important for social insects because they allow the recognition of conspecifics, nest mates, kin, or even members of different castes. Surface pheromones generally remain adsorbed to the body surface and are perceived by other insects through direct contact or may be detected over a short distance (Shorey, 1973).

Since nothing is known about the cuticular pheromone composition of stingless bees, we have investigated the presence of these compounds in *Melipona bicolor*, in order to determine whether there are differences in the composition among castes and among individuals of the same caste, but of different ages and developing different tasks.

MATERIAL AND METHODS

Material

The wings of workers (12 unemerged imagoes, 11 newly emerged, 14 nurse bees and 15 foragers), 11 males and 29 queens (26 virgin and 3 physogastric) were used to determinate the body surface composition by gas chromatography and mass spectrometry (GC/MS). All of the bees, except for the physogastric queens, were collected from polygynic colonies at the meliponary of the Department of Biology (UNESP, Rio Claro, SP, Brazil). The physogastric queens were collected from polygynic colonies at the meliponary of the Federal University of Viçosa (Viçosa, MG, Brazil).

Material collection

Foraging workers were collected as they returned to the colony with pollen in the corbicula, by placing an Ependorf tube in the nest entrance. One worker was collected per tube. Newly emerged and nurse workers were collected *in situ*, as they were emerging from the brood cell and when they were provisioning the brood cells, respectively. Unemerged workers were collected directly from brood cells containing workers ready to emerge. Males were collected in the colonies and were easily distinguished from the females by the yellow spot on the clypeus.

Sample preparation

The wings of each bee were removed and transferred to a 1-mm diameter soft soda glass capillary and sealed with a flame for subsequent analysis by GC/MS, according to

Morgan and Wadhams (1972) and Morgan (1990). The analyses were done at the School of Chemistry and Physics, Keele University (Staffordshire, England).

Sample analysis

The wings were analyzed by solid injection, by which the capillaries were injected through a solid injection adapter fitted to the injection port of the gas chromatograph. Analytic separation was done in a Hewlett-Packard series 5890A gas chromatograph coupled to a selective mass detector (series 5970B quadrupole spectrometer with an electric impact of ionization of 70eV). The system was controlled by and the data accumulated in a Hewlett-Packard series 300 microcomputer connected to an HP 5971/5972 MSD Chemstation (Chemical Ecology Group, Department of Chemistry, Keele University), which continuously recorded the data. The analyses were done using a 30 m Rtx-5 (30 m x 0.37 m x 0.5 μm) fused silica column covered with poly (5%-biphenyl-95% dimethyl) siloxane.

The oven was programmed to reach a final temperature of 325ºC, starting from an initial temperature of 60ºC and rising at a rate of 10ºC/min. Helium was used as the carrier gas at a flow rate of 1 ml/min. Injections were made in the splitless mode with a purge off time of 0.75 min and a solvent delay time of 5 min, before the mass spectrometer was switched on. The mass detector was programmed to detect a minimum mass of 35 kDa and a maximum mass of 550 kDa.

Data interpretation

The chemical compounds were identified based on their mass spectra, by comparison with MS-Databases and by searching the NBS Library of Mass Spectra and Mass Spectral Register.

The external standard solution was a mixture of 12 different synthetic hydrocarbons (C16, C17, C18, C19, C20, C21, C22, C23, C24, C25, C28, C30). Immediately after the injection of a sample, 1 μl of the external standard solution was also injected to compare the retention times of the hydrocarbons detected in the sample.

RESULTS AND DISCUSSION

Are workers born with a well-established cuticular hydrocarbon pattern?

Before emergence, workers (unemerged imagoes) already had a very well-defined cuticular hydrocarbon profile, containing the same principal hydrocarbons found on the body surface of emerged workers and queens (Table 1). However, these unemerged workers had a greater percentage of saturated hydrocarbons than did queens, newly emerged and foraging workers (Table 2).

The hydrocarbons, C23, C25:1, C25, C27:1, C27, C29:1 and C29, are probably structural hydrocarbons (Wigglesworth, 1970) that give the species its identity, since they were found in the cuticle of queens, workers and males, and in unemerged workers. In support of this interpretation, based on preliminary results, we found that the cuticular hydrocarbons in other species of meliponines (*M. marginata* and *M. quadrifasciata*) differ qualitatively and quantitatively from those of *M. bicolor* (data not shown).

Table 1. Cuticular composition of workers (unemerged - une, newly emerged - ne, nurse - nu and forager - fo), males and queens (virgin - vg and physogastric - pg) of *Melipona bicolor* (Hymenoptera, Meliponini).

Peaks	Compounds	une	ne	nu	fo	males	vg	pg
1	7-Dodecenol (C12ol)*	-	-	-	-	-	-	1
2	6-Dodecenol (C12ol)*	-	-	-	-	-	-	1
3	Uncosene (C21:1)	Traces	Traces	-	-	-	Traces	-
4	Uncosane (C21)	2	1	Traces	-	-	2	-
5	Docosene (C22:1)	-	Traces	-	Traces	-	-	-
6	Docosane (C22)	-	Traces	-	-	-	Traces	-
7	Tricosene (C23:1)	2	Traces	-	-	Traces	1	-
8	Tricosane (C23)	16	8	8	8	2	9	1
9	6,9,11-triMethyltricosane	-	Traces	-	-	-	Traces	-
10	5-Methyltricosane	Traces	2	-	Traces	-	-	-
11	3-Methyltricosane	Traces	Traces	-	-	-	Traces	-
12	Tetracosene (C24:1)	-	-	-	Traces	Traces	-	-
13	Tetracosane (C24)	1	Traces	-	-	Traces	Traces	-
14	Pentacosene (C25:1)	12	3	3	3	4	6	4
15	Pentacosane (C25)	14	10	20	11	12	10	5
16	5,11-diMethylpentacosane	-	-	-	-	-	-	2
17	5,19-diMethylpentacosane	-	-	-	-	-	-	2
18	9,15-diMethylpentacosane	1	Traces	-	-	-	Traces	-
19	11,13-diMethylpentacosane	-	Traces	-	-	-	Traces	1
20	11-Methylpentacosane	-	1	-	-	-	Traces	-
21	5-Methylpentacosane	1	1	-	-	-	Traces	-
22	3-Methylpentacosane	1	-	-	-	-	-	Traces
23	9-Methylpentacosane	-	Traces	-	-	-	-	Traces
24	Hexacosene (C26:1)	1	1	-	Traces	-	Traces	4
25	Hexacosane (C26)	-	2	1	-	1	Traces	2
26	Heptacosene (C27:1)	19	36	1	48	17	40	36
27	Heptacosane (C27)	11	9	37	8	21	13	4
28	11,13,15-triHeptacosane	-	2	-	-	-	Traces	3
29	14-Methylheptacosane	-	-	-	-	-	-	Traces
30	7-Methylheptacosane	1	-	16	-	-	-	-
31	5-Methylheptacosane	1	1	-	-	-	-	3
32	3-Methylheptacosane	1	-	-	-	-	-	-
33	Octacosene (C28:1)	-	1	Traces	Traces	-	Traces	-
34	Octacosane (C28)	-	2	-	-	-	Traces	6
35	15-Methyloctacosane	-	-	-	-	-	-	2
36	14-Methyloctacosane	-	Traces	-	-	-	-	1
37	10-Methyloctacosane	-	-	-	-	-	-	1
38	Nonacosene (C29:1)	2	9	6	10	15	8	17
39	Nonacosane (C29)	5	3	8	1	11	8	1
40	11,13,15-triMethylnonacosane	-	Traces	-	-	-	Traces	1
41	15-Methylnonacosane	-	-	-	-	-	-	2
42	11-Methylnonacosane	1	-	-	-	-	-	-
43	7-Methylnonacosane	-	-	-	-	-	-	Traces
44	5-Methylnonacosane	1	Traces	-	-	-	-	1
45	Triacontene (C30:1)	-	Traces	-	-	-	Traces	-
46	Triacontane (C30)	-	-	-	-	-	-	1
47	Untriacontene (C31:1)	-	-	-	Traces	6	Traces	1
48	Untriacontane (C31)	2	Traces	-	-	10	Traces	-
49	Dotriacontene (C32:1)	-	Traces	Traces	-	-	-	-
50	Dotriacontane (C32)	3	Traces	-	-	-	-	1

* Unconfirmed, traces <1%. - = Absent. Data are reported as percent of cuticular composition.

Table 2. Total percentage of saturated, unsaturated and branched hydrocarbons in workers, males and queens of *Melipona bicolor* (Hymenoptera, Meliponini).

Castes / Individuals	Hydrocarbons (%)*		
	Saturated	Unsaturated	Branched
Unemerged workers	54	37	9
Newly emerged workers	37	52	11
Nurse workers	74	10	16
Foraging workers	28	63	Traces
Males	56	43	Absent
Virgin queens	44	57	3
Physogastric queens	20	60	7

*The percentages were calculated from Table 1.

In honey bees, the cuticular hydrocarbon profiles are partly genetically determined and they differ among subfamilies, which suggests that they may be used by workers as "signatures" for subfamily recognition (Arnold et al., 2000).

Do young workers in the brood area (newly emerged and nurses) have a cuticular hydrocarbon pattern different from that of foragers?

Newly emerged workers had a great diversity of cuticular hydrocarbons, with a profile resembling that of queens (Table 1). The percentage of unsaturated hydrocarbons was higher than saturated ones, differing from the unemerged and nurse workers (Table 2). Newly emerged workers had a grater percentage of branched hydrocarbons than unemerged workers (Tables 2). The percentage of C27:1 was greater than in unemerged workers and resembled that of foraging workers and queens (Table 1).

Nurse workers contained a high percentage of branched hydrocarbons (mainly due to 7-MeC27, 16%) and had a predominance of saturated hydrocarbons (Table 2), but were poor in a variety of hydrocarbons, when compared to newly emerged workers and queens. In this regard, nurse workers resembled foraging workers and males (Table 1). However, the percentage of C27:1 was much lower in nurse workers than in other age workers and queens (Table 1). Similarly, the percentage of C27 was much higher in nurse workers than in the latter two groups. Since nurse workers have close interactions with physogastric queens (Bego, 1989), the inversion in the percentage of C27 and the high percentage of saturated hydrocarbons could allow the bees to distinguish physogastric queens from workers present in the brood area.

Some workers in the brood area will eat the queen's eggs and lay their own eggs in the brood cells, therefore competing with the queen for reproduction (Bego, 1989). If the lack of pheromonal activity of the alkanes in wasps (Dani et al., 1996) also applies to bees, then the absence or "suppression" of unsaturated hydrocarbons in the cuticle of nurse bees could be a "strategy" of such workers to obtain more opportunities for oviposition, since their "smell" would be much too weak for the queen to perceive it as coming from a competing nestmate.

Foraging workers had fewer branched and less diverse hydrocarbons than the other workers (Tables 1 and 2). Unemerged and newly emerged workers generally had a great diversity of saturated and unsaturated hydrocarbons, and a relatively large percentage of branched hydrocarbons. Nurse bees had a very different pattern from the other workers. In addition to

the high percentage of saturated hydrocarbons (also seen in unemerged workers), nurse bees had a high percentage of C27 and branched hydrocarbons, mainly because of the presence of 7-MeC27. The percentages of structural hydrocarbons in foraging workers were similar to those found in newly emerged workers, but the foragers had less diversity of other types of hydrocarbons.

Do workers, queens and males have different cuticular hydrocarbons in their cuticle?

Virgin queens were very similar to newly emerged workers in the percentage and types of cuticular hydrocarbons they contained (Tables 1 and 2). Physogastric queens differed from the workers and had a hydrocarbon composition that was the opposite of that of nurse bees (Tables 1 and 2). This difference may serve to distinguish these two groups in the brood area.

Unemerged workers had structural hydrocarbons characteristic of the species, although the higher percentage of unsaturated versus saturated hydrocarbons that characterized the other classes of workers was not observed (Tables 1 and 2). The proportion of these hydrocarbons begins to be defined after emergence, especially in newly emerged and foraging workers, as well as in queens (Table 2). Nurse bees and foragers were found to lack certain hydrocarbons that queens possess (Table 1). In physogastric queens the short chain-branched hydrocarbons of virgin queens were substituted by long chain-branched hydrocarbons (Table 1). In nurse bees, there was an inversion of the C27/C27:1 proportion and in physogastric queens this proportion favored C27:1 (Table 1). Unemerged workers, nurse bees and foragers, as well as males, were found to be very different from queens.

Males had the principal hydrocarbons found in the females (C23, C25:1, C25, C27:1, C27, C29:1 and C29), which are characteristic of the species (Table 1). They also had a high percentage of long chain (C31:1 and C31) and saturated hydrocarbons, but no branched hydrocarbons (Tables 1 and 2). This lack of diversity and the higher percentage of saturated hydrocarbons placed the males in an intermediate group with a hydrocarbon profile between those of foragers and unemerged workers. This hydrocarbon pattern may be due to the fact that since their social function in the colony is weak, males do not need a well-defined identity, except for sexual identification (long chain C31:1 and C31 and the absence of branched hydrocarbons).

Linear alkanes have no effect on the recognition response in wasps (Dani et al., 1996), whereas methyl-branched hydrocarbons or alkenes do. If, as in wasps, the alkanes in *M. bicolor* have no pheromonal activity, then males may have no "personal" identity, particularly since it would be preferable for the queens to mate with foreign males rather than with males of the same colony.

Do virgin queens have different cuticular hydrocarbon patterns from physogastrics?

Virgin and physogastric queens had very similar percentages of cuticular hydrocarbons, with a predominance of unsaturated hydrocarbons. However, physogastric queens had a greater diversity of hydrocarbons, especially of long chain-branched hydrocarbons (Tables 1 and 2). All saturated hydrocarbons in physogastric queens were found in lower percentages than in virgin queens. In addition, physogastric queens had alcohol as a cuticular compound (Table 1). Therefore, there is a blend difference between virgin and physogastric queens. The hydrocarbon composition indicates that the physogastric queens would be easily identified as such in the colony whereas virgin queens would be perceived as newly emerged workers. The relative

abundance of unsaturated hydrocarbons may indicate their importance in recognition, as observed by Dani et al. (1996) for wasps.

What is the origin of cuticular hydrocarbons?

Bee cuticle contains structural and facultative hydrocarbons. Since the cuticular hydrocarbons C23, C25:1, C25, C27:1, C27, C29:1 and C29 were present in all individuals analyzed and were the most abundant (Table 1), they probably represent structural hydrocarbons.

The origin of these compounds is unclear. Although workers have epidermal wax glands and hydrocarbons are part of the wax, there is evidence that normal epidermal cells synthesize these compounds and that oenocytes produce cuticular hydrocarbons (Kramer and Wigglesworth, 1950; Wigglesworth, 1965, 1970; Dielh, 1973, 1975; Cassier and Lensky, 1994), that are passed to the epidermal cells. Ruvolo and Cruz-Landim (1995a,b) found no correlation between oenocyte and wax gland development, contrary to the observations of Wigglesworth (1970). These compounds may also be produced by class III tegumentary glands, which have a well-developed smooth endoplasmic reticulum and lipid-like deposits in the cytoplasm (Guerino, 1999). It seems reasonable to suppose that structural hydrocarbons are produced in the oenocytes or tegumentary glands under a genetic program carried out in these ectodermal cells, whose expression could be controlled by environmental clues.

Similarities between the cuticular hydrocarbon composition and the hydrocarbons of the Dufour's gland (an accessory gland of the female reproductive tract) secretions have been observed in wasps and bees (Oldham et al., 1994; Dani et al., 2001). Therefore the hydrocarbons of the Dufour's gland secretions may be spread over the body by grooming behavior, thereby contributing to the cuticular identity and recognition of nestmates and castes (Ayasse et al., 2001). In *A. mellifera*, there is evidence that the wax cells and the Dufour's gland cells, both of which are modified epidermal cells, can take up hydrocarbons from the hemolymph (Cassier and Lensky, 1994; Katzav-Gozansky et al., 2000). The origin of these hydrocarbons is unknown, but they may come from the oenocytes (via hemolymph), or from ingested food (Hefetz et al., 1993).

Since the Dufour's gland is a fold or invagination of the epidermis, it has the same properties as epidermal cells, and is able to absorb the same substances from the hemolymph that epidermal cells absorb. If this is true, then there is no need for the Dufour's gland secretions to be spread over the body surface. Nevertheless, such grooming behaviour may provide additional information or reinforce the cuticular hydrocarbon information. The hydrocarbons found in the Dufour's gland may be present in the cuticle lining its lumen, which is continuous with the tegumentary cuticle.

The exact source of the cuticular hydrocarbons remains imprecise, but the most probable candidate for their production is the oenocytes and tegumentary glands. At least in *M. bicolor* workers, the cuticular hydrocarbons do not originate from the Dufour's gland, since workers do not have this gland. The Dufour's gland secretion of physogastric queens contains esters as the principal compounds (Abdalla, 2002), and these do not occur as cuticular components (Table 1).

CONCLUSIONS

The cuticle of *M. bicolor* has two types of hydrocarbons: structural and non-structural. The epithelial cells, following a specific genetic program, may produce the structural hydrocar-

bons, while the non-structural ones may be produced by these cells, and/or absorbed by them probably from the hemolymph.

The hydrocarbons C23, C25:1, C25, C27:1, C27, C29:1 and C29 probably are structural hydrocarbons of the cuticle, since they were present in all bees analyzed and also occurred in the Dufour's gland of *M. bicolor* (Abdalla, 2002) and may also be absorbed by the epithelial cells, since these vary quantitatively, maybe as a result of temporal or behavioral alterations in gene expression and/or as a result of differences in the capacity for structural and non-structural hydrocarbon uptake from hemolymph among the various classes of individuals, life stages, or depending on the types of tasks done in the colony. Environmental factor differences, the availability and type of food found in nature and stored in the colony, and the diet of individual bees may also affect the hydrocarbon composition of the secretion, as observed in *Bombus* by Hefetz et al. (1993).

The variability of the composition of cuticular hydrocarbons in the bees that we analyzed, producing different patterns according to the castes and sexes, as well as within castes, reinforces the role of these compounds in recognition in social bees.

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