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Cuticular hydrocarbons in the stingless bee *Schwarziana quadripunctata* (Hymenoptera, Apidae, Meliponini): differences between colonies, castes and age

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ABSTRACT. Chemical communication is of fundamental importance to maintain the integration of insect colonies. In honey bees, cuticular lipids differ in their composition between queens, workers and drones. Little is known, however, about cuticular hydrocarbons in stingless bees. We investigated chemical differences in cuticular hydrocarbons between different colonies, castes and individuals of different ages in *Schwarziana quadripunctata*. The epicuticle of the bees was extracted using the non-polar solvent hexane, and was analyzed by means of a gas chromatograph coupled with a mass spectrometer. The identified compounds were alkanes, branched-alkanes and alkenes with chains of 19 to 33 carbon atoms. Discriminant analyses showed clear differences between all the groups ana-

lyzed. There were significant differences between bees from different colonies, workers of different age and between workers and virgin queens.

Key words: Hydrocarbons; Chemical communication; Social insect; Stingless bees; *Schwarziana quadripunctata*

INTRODUCTION

Communication is the action of an organism that modifies the behavior of another organism (Wilson, 1971). In social insects, a great part of a colony's behavior is mediated through chemical signals and cues (Singer, 1998; Howard and Blomquist, 2005) such as hydrocarbons associated with the cuticle of colony members (Pfennig et al., 1983; Breed and Stiller, 1992; Singer and Espelie, 1992; Gamboa et al., 1996). The insect cuticle is covered by a thin waterproof layer of wax consisting of long chain lipids. According to Lockey (1988), these substances originally served as a protection against water loss. The pheromonal function of the hydrocarbons evolved later.

In social insects, the cuticle of each individual shows a unique chemical profile, carrying species-specific cues, as well as cues about its nest origin, age, genetic lineage, caste, sex, reproductive status, and its function in the colony (Arnold et al., 1996; Howard and Blomquist, 2005; Monnin, 2006).

There have been a steadily increasing number of studies on the chemical profiles of the cuticles of ants, social wasps, and honey bees (Vander Meer et al., 1998). So far, however, little is known about the composition and the function of cuticular hydrocarbons in stingless bees. This group of highly eusocial bees, which comprises several hundred species (Michener, 2000), displays a great diversity, not only concerning nest architecture, but also in the materials used for the nest construction (Wille and Michener, 1973). Among different species, we encounter a variety of different kinds of resins, mud and floral oils (Nogueira-Neto, 1997; Roubik, 2006). These materials might be the source of a richer chemical profile in comparison to other social bees.

Up to now, there have been few studies that have investigated the composition and function of cuticular hydrocarbons in stingless bees (Abdalla et al., 2003; Jungnickel et al., 2004). For *Melipona bicolor*, Abdalla et al. (2003) reported both qualitative and quantitative differences in the chemical profile of the cuticle between different castes and sexes. Moreover, they found no differences between newly emerged workers and virgin queens, though the chemical profile of the cuticle changed with the age of the individuals. Studies in *Scaptotrigona bipunctata* showed that each colony has its own chemical profile (Jungnickel et al., 2004). These results are concordant with the idea that recognition of nestmates is mediated by olfactory cues presented by cuticular lipids (Buchwals and Breed, 2005).

The main compounds of the epicuticular surface of stingless bees are linear alkanes, methyl-branched alkanes, and alkenes with chains from 23 to 35 carbon atoms (Abdalla et al., 2003; Jungnickel et al., 2004; Pianaro et al., 2007). Some of these compounds are common to other social insects and may have a structural function, while some, such as C25 and Z:C23, have been demonstrated to be used as chemical cues in recognition systems (Lockey, 1988; Buchwals and Breed, 2005).

We analyzed the composition of cuticular hydrocarbons in the stingless bee *Schwarziana quadripunctata.* We examined whether there are differences between colonies, and between individuals of different ages and castes.

MATERIAL AND METHODS

The study was performed in Ribeirão Preto, Brazil, from May to July 2006. For the chemical analyses, two colonies of *S. quadripunctata* (henceforth colonies A and B) were used. The colonies were housed in wooden boxes (20 x 32 x 13 cm) inside the laboratory. The boxes were connected to the external environment through plastic tubes, allowing the workers to forage. From colony A we collected four young workers, 11 old workers and 11 virgin queens. From colony B we collected five young workers, four old workers and three virgin queens. Young workers were individuals less than four days old, recognized by their bright coloration and by their activities on the brood cells. Old individuals, which were at least 10 days old, were darkly pigmented. Virgin queens were considered unmated queens with intermediary pigmentation and were less than 10 days old. For the chemical analysis, the collected bees were stored individually in small vials and killed by freezing.

The epicuticular compounds were extracted in hexane (1 mL per individual, for 1 min). After eliminating the solvent, the apolar extract was suspended in 50 µL hexane for the analysis with a gas chromatograph coupled with a mass spectrometer (GC-MS; Shimadzu, model QP2010). The GC-MS was equipped with a DB-5MS capillary column, using helium as the carrier gas. The temperature protocol was: 150° to 280°C at a rate of 3°C/min (held for 15 min). Analyses were performed in splitless mode. For the identification of the compounds, the mass spectra were compared to Wiley library data (software: GCMSsolutions for Windows, Shimadzu Corporation).

The areas under the peaks of the chromatograms were transformed according to Reyment's formula: $Z = \ln[A_p / g(A_p)]$, where A_p is the area of the peak, and $g(A_p)$ is the geometric mean peak area (Aitchison, 1986). The subsequent statistical analysis was applied only to those substances with three or more samples for each group. Principal components analysis was applied in order to reduce the number of variables. Only those peaks with the highest factorial weight on the first two roots were selected. Data were then analyzed by a stepwise discriminant analysis to look for segregations between the groups of the individuals analyzed. The statistical analyses were performed using the Statistica 6.0 for Windows software (StatSoft Inc.).

RESULTS

The chemical analysis of the cuticular hydrocarbons of *S. quadripunctata* identified 35 substances, divided into linear alkanes, methyl-branched alkanes and linear alkenes, with chains between 19 and 33 carbon atoms (Table 1). The most abundant component of the cuticle was linear alkanes, mainly tricosane (C23), pentacosane (C25) and heptacosane (C27). Young workers had a higher concentration of short chain hydrocarbons (e.g., tricosane) than did old workers and virgin queens (Table 1). The cuticle of young workers of colony A did not show several long chain hydrocarbons that were found in all other groups. The cuticle of old workers and virgin queens had a higher concentration of long chain hydrocarbons (such as heptacosane) than the cuticle of young workers.

Table 1. Mean area percentage of the peaks (± standard deviation) of the cuticular hydrocarbons of *Schwarziana quadripunctata.*

Retention time	Compound	Colony A			Colony B		
		Young workers	Old workers	Gynes	Young workers	Old workers	Gynes
13.12	Nonadecane (C19)	6.3 ± 2.1	0.2 ± 0.1	0.8 ± 0.5	3.1 ± 1.6	0.7 ± 0.8	0.4 ± 0.2
15.82	Eicosane (C20)	0.4 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.0 ± 0.0
18.64	Heneicosane (C21)	2.2 ± 0.6	0.2 ± 0.1	0.4 ± 0.2	1.9 ± 0.6	0.4 ± 0.3	0.2 ± 0.2
21.40	Docosane (C22)	0.3 ± 0.1	0.1 ± 0.0	0.5 ± 1.1	0.2 ± 0.1	0.1 ± 0.0	-
22.10	Octadecanal	0.2 ± 0.0	0.3 ± 0.2	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0
23.35	Tricosene	0.8 ± 0.2	-	0.2 ± 0.1	0.7 ± 0.2	0.1 ± 0.1	0.1 ± 0.1
23.55	Tricosene	0.2 ± 0.1	0.1 ± 0.0	0.2 ± 0.3	0.4 ± 0.1	0.2 ± 0.1	0.1 ± 0.0
24.15	Tricosane (C23)	13.5 ± 4.0	4.6 ± 2.2	6.7 ± 6.0	12.7 ± 1.9	6.8 ± 3.0	3.2 ± 1.4
25.05	5-Methyltricosane	1.3 ± 0.4	-	0.3 ± 0.1	1.3 ± 0.4	0.4 ± 0.0	0.2 ± 0.2
26.05	3-Methyltricosane	0.3 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.1 ± 0.1
26.70	Tetracosane (C24)	0.8 ± 0.2	0.2 ± 0.1	0.5 ± 0.2	0.8 ± 0.1	0.6 ± 0.1	0.4 ± 0.2
28.65	Pentacosene	1.1 ± 0.3	0.5 ± 0.4	0.3 ± 0.2	0.4 ± 0.2	1.3 ± 0.8	0.1 ± 0.1
28.86	Pentacosene	0.3 ± 0.1	0.3 ± 0.2	0.2 ± 0.3	0.4 ± 0.1	0.4 ± 0.4	0.1 ± 0.1
29.38	Pentacosane (C25)	17.9 ± 4.6	13.8 ± 9.1	23.2 ± 3.7	18.4 ± 0.0	21.5 ± 8.1	20.9 ± 3.5
30.20	11 and 13-methylpentacosene	3.3 ± 0.8	0.1 ± 0.0	1.2 ± 0.6	2.3 ± 0.6	0.3 ± 0.5	0.6 ± 0.5
31.95	Hexacosane (C26)	1.0 ± 0.6	1.5 ± 0.4	3.1 ± 0.6	2.1 ± 0.4	2.1 ± 0.6	3.3 ± 0.4
33.66	Heptacosene	0.4 ± 0.1	0.8 ± 0.9	0.3 ± 0.2	-	0.1 ± 0.1	-
33.75	Heptacosene	0.4 ± 0.1	1.6 ± 1.6	0.5 ± 0.4	0.2 ± 0.0	2.4 ± 2.4	0.1 ± 0.0
33.95	Heptacosene	0.4 ± 0.1	2.4 ± 2.6	0.6 ± 0.6	0.6 ± 0.1	2.8 ± 3.3	0.4 ± 0.1
34.40	Heptacosane (C27)	14.5 ± 2.0	38.4 ± 5.2	39.2 ± 9.2	25.7 ± 5.8	34.9 ± 1.8	45.2 ± 3.4
35.10	9 and 11 and 13 and 15-methylheptacosane	4.0 ± 0.7	0.4 ± 0.2	2.3 ± 0.8	3.0 ± 0.4	1.0 ± 1.1	1.6 ± 0.6
36.06	3-Methylheptacosane	3.3 ± 2.8	0.2 ± 0.1	0.3 ± 0.3	0.2 ± 0.0	0.2 ± 0.2	0.3 ± 0.3
36.75	Octacosane (C28)	0.5 ± 0.3	0.5 ± 0.2	0.8 ± 0.2	0.4 ± 0.1	0.6 ± 0.2	1.1 ± 0.2
38.20	Nonacosene	0.1 ± 0.0	1.5 ± 1.0	0.2 ± 0.1	0.1 ± 0.1	0.5 ± 0.5	0.3 ± 0.2
38.50	Nonacosene	0.4 ± 0.2	2.7 ± 3.5	0.2 ± 0.2	0.2 ± 0.1	3.9 ± 3.7	0.2 ± 0.2
39.05	Nonacosane (C29)	4.0 ± 2.5	7.3 ± 4.3	4.7 ± 1.0	2.7 ± 0.7	4.7 ± 1.8	5.8 ± 1.7
40.21	11,15 and 13,9 and 13,17-dimethylnonacosane	1.1 ± 0.2	0.1 ± 0.1	0.9 ± 1.3	0.6 ± 0.1	0.2 ± 0.2	0.4 ± 0.1
40.60	3-Methylnonacosane	3.4 ± 2.2	0.2 ± 0.1	1.7 ± 1.9	-	0.1 ± 0.0	0.4 ± 0.6
42.25	Hentriacontene	-	0.2 ± 0.1	-	-	-	-
42.86	Hentriacontene	-	3.6 ± 1.9	0.3 ± 0.2	0.2 ± 0.1	1.1 ± 1.3	0.6 ± 0.4
43.06	Hentriacontene	-	0.8 ± 1.0	0.1 ± 0.1	0.1 ± 0.0	0.4 ± 0.5	0.2 ± 0.2
43.38	Hentriacontane (C31)	0.7 ± 0.7	1.1 ± 1.1	0.4 ± 0.2	0.5 ± 0.4	0.4 ± 0.2	0.5 ± 0.3
44.00	11 and 13 and 15-methylhentriacontane	6.9 ± 3.6	0.7 ± 0.4	1.5 ± 0.6	1.6 ± 0.1	1.1 ± 1.2	2.0 ± 0.5
44.50	13,17-dimethylhentriacontane	0.5 ± 0.2	-	0.3 ± 0.2	0.3 ± 0.1	0.1 ± 0.1	0.3 ± 0.0
47.50	Tritriacontane (C33)	-	1.2 ± 1.0	0.2 ± 0.2	-	0.5 ± 0.8	0.3 ± 0.4

Data are reported as means ± SD.

Factor 1 of the principal component analysis using the 21 peaks analyzed described 30.3% of the observed variance. Factor 1 + factor 2 described 52.6%, and factor 1 + factor 2 + factor 3 described 68.8% of the total variance. Stepwise discriminant analysis of the main compounds significantly separated the individuals according to their group (Global model: Wilks's $\lambda = 0.008$, $F_{45,43} = 7.07$, $P < 0.0001$). The classification based on the discriminant analysis revealed 100% correct allocation of all individuals to their predicted group (Figure 1). The plot of the first versus the second roots of the discriminant analysis revealed that for both colonies all groups were completely segregated. The Mahalanobis distance between the groups, the respective F value and levels for each combination are listed in Table 2.

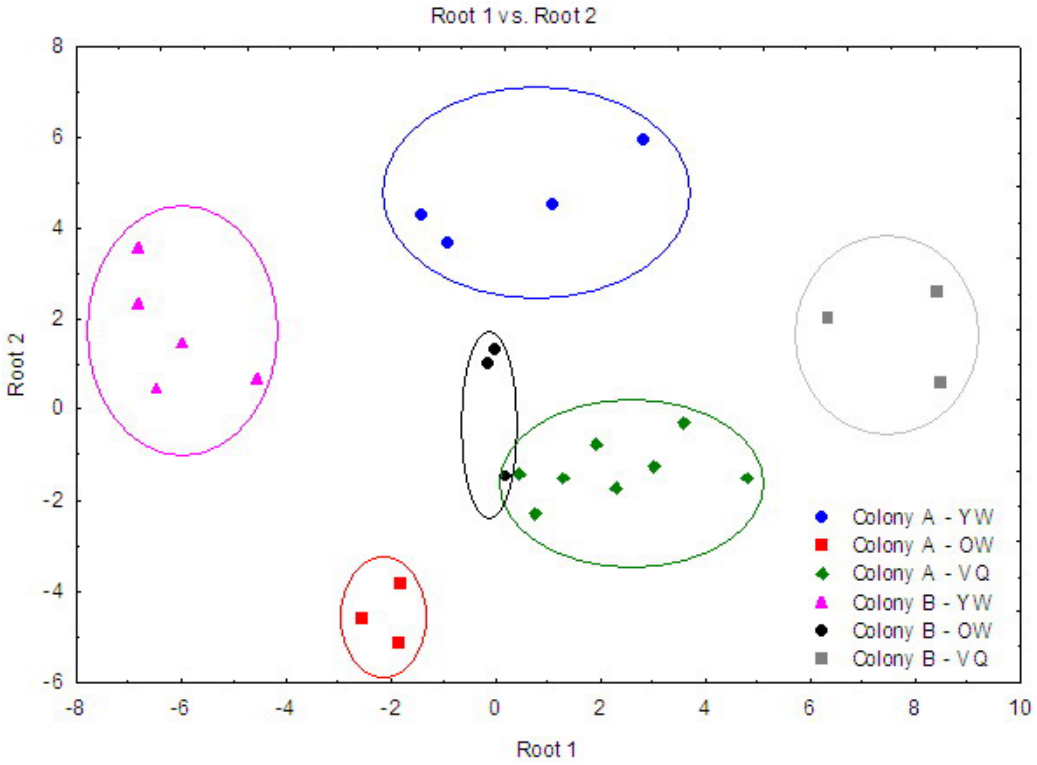

Figure 1. Plot of the first versus the second roots of the canonical analysis. YW, young workers; OW, old workers, and VQ, virgin queens.

Table 2. Result of discriminant analyses for two colonies of *Schwarziana quadripunctata*.

Colony			Mahalanobis distance	F values	P
A		B			
YW	x	YW	77.97	6.55	<0.01
YW	x	OW	56.69	4.00	0.02
YW	x	VQ	106.66	7.53	<0.01
OW	x	YW	61.90	6.83	<0.01
OW	x	OW	59.12	5.22	0.01
OW	x	VQ	142.92	12.61	<0.01
VQ	x	YW	79.71	13.68	<0.01
VQ	x	OW	27.49	3.40	0.04
VQ	x	VQ	59.21	7.31	<0.01
Colony B					
YW	x	OW	67.89	7.49	<0.01
YW	x	VQ	194.74	21.48	<0.01
OW	x	VQ	99.73	8.80	<0.01
Colony A					
YW	x	OW	94.84	6.69	<0.01
YW	x	VQ	50.92	4.66	0.02
OW	x	VQ	37.05	4.58	0.02

YW, young workers; OW, old workers, and VQ, virgin queens.

DISCUSSION

We found a great diversity in the chemical compounds of the epicuticle of *S. quadripunctata* (Table 1). Compounds found in the cuticle clearly separated individuals according to their colony, age and caste (Figure 1). The identified compounds as well as their relative concentrations, mainly the greater abundance of linear alkanes, such as tricosane, pentacosane and heptacosane, comply with the results for other stingless bees species (Abdalla et al., 2003; Jungnickel et al., 2004; Pianaro et al., 2007). Our study reveals hydrocarbons with less than 21 carbon atoms, such as nonadecane (C19) and eicosane (C20), for the first time in stingless bees. Similar short chain hydrocarbons have already been identified in other social insects, such as wasps and honey bees (Singer, 1998; Fröhlich et al., 2000). However, their function is still unknown.

The cuticle of old workers of both colonies presented a higher concentration of the alkenes heptacosene (eluted at 33.75 and 33.95), and hentriacontene (eluted at 42.86) than that of young workers and virgin queens. Such alkenes probably are important factors for nestmate recognition. This can be deduced from experiments with honey bees, where bees treated with alkenes were attacked more fiercely by nestmates than those treated with alkanes (Dani et al., 2005). Also, stingless bees of the species *Trigona fulviventris* treated with Z-Tricosene induced a high level of aggression by nestmates (Buchwald and Breed, 2005). Old workers usually leave the colony looking for food and other resources. Hence, a different composition of alkenes could be used as recognition cues at the nest entrance.

Studies on honey bees have shown that adults emerge with their cuticle in a "blank slate", lacking any recognition cue (Breed et al., 1995). The bees gain these cues (i.e., alkenes and fatty acids) after exposure to the comb wax of their colony (Breed et al., 2004a,b). Similarly, young workers of *S. quadripunctata* lacked some hydrocarbons that were identified in older bees. This difference is probably due to the fact that the young individuals were exposed to the wax of the nest for a shorter time than the old individuals, which resulted in a lower transfer of lipids between the nest material and the cuticle of young bees. A chemical analysis of the wax composition would give a deeper insight into this hypothesis; however, no overlap was found between wax and cuticle in *Melipona bicolor* (Koedam et al., 2002). Recognition experiments using younger workers would verify if these chemical cues are important for the recognition system.

We found a clear difference between the chemical profile of the two colonies. This distinct chemical profile of a colony can be a result of different factors. Arnold et al. (1996) studying honey bees showed that different genetic lineages present different hydrocarbon profiles. Furthermore, the chemical profiles of colonies may reflect differences in the collected material such as floral scents, and in secreted substances, like queen pheromones. The nest wax probably homogenizes the colony odor by absorbing the chemical information from different sources, and by subsequently redistributing the joint information to the colony members (Breed et al., 1992, 1995).

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