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Article

Chemical Composition of the Dufour Gland Secretion in Queens of *Melipona bicolor* (Hymenoptera, Meliponini)

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Utilizando cromatografia gasosa acoplada à espectrometria de massa, descrevemos as diferenças na composição química da secreção da glândula de Dufour de rainhas virgens e fisogástricas de *Melipona bicolor*. A secreção da glândula de rainhas virgens consiste de hidrocarbonetos, enquanto a das rainhas fisogástricas contém, além de hidrocarbonetos, uma variedade de outros compostos, tais como ésteres isobutíricos e acéticos. Tais diferenças devem indicar o estado de fecundidade da rainha e os compostos oxigenados da secreção das fisogástricas podem ajudar aumentar sua atratividade.

We describe differences in the chemical composition of the Dufour gland secretion of virgin and physogastric queens of *Melipona bicolor* through gas chromatography and mass spectrometry. The Dufour gland secretion of virgin queens consists only of hydrocarbons, while that of physogastric queens contains, besides these, a variety of other compounds, such as isobutyrate and acetate esters. Such differences may indicate the queen fecundity condition and the oxygenated compounds of the physogastrics secretion may help to increase their attractiveness.

Keywords: Dufour gland, *Melipona bicolor*, physogastric queen, stingless bee, pheromone, ester, hydrocarbon

Introduction

The Dufour gland is found in all female hymenopterans. In bees, the gland is located at the base of the sting apparatus, ventrally to the poison gland, and opens into the dorsal vaginal wall.[1] A range of substances, from macrolactones and terpenoid esters to hydrocarbons and triglycerides, has been found in the Dufour gland secretion of bees.[2-13]

In solitary bees, which build underground nests, the Dufour gland produces hydrophobic substances used to line the nest[9] and to protect eggs against extreme fluctuations in humidity and against microorganisms.[14] This lining also maintains a stable environment during the several phases of development of the immature bee.[14]

In the eusocial Apidae, the function of this gland is unknown. In *Bombus*, the Dufour gland secretion consists of hydrocarbons and esters, and is colony- and species-specific.[15,16] In *Apis mellifera*, the secretion is composed of hydrocarbons in workers, and in egg-laying workers and queens, hydrocarbons and esters.[17] Bioassays done first by Abdalla and Cruz-Landim[18] demonstrated that the virgin queen secretion provokes worker attraction. On the contrary, the worker secretion repulses their nestmate workers. Secretion of non-nestmate bees has no effect on the worker behavior. Recently, Katzav-Gozansky *et al.*[19] noticed that the esters of the Dufour gland secretion is the active fraction, being the egg-laying worker and queen secretion greatly attractive to nestmate workers. The non egg-laying worker gland secretion has no effect on this aspect of worker behavior.

In stingless bees (Meliponini), in only one species, *Nanatrigona testaceicornis* workers, the chemical composition of the Dufour gland secretion has been studied. In this species 64% of the secretion consisted of the ester diterpene all-*trans*-geranylgeranyl acetate.[20]

The Dufour gland is absent in workers of *Melipona bicolor*, but occurs in queens, being more developed in physogastric queens. Behavioral observations suggest that a pheromone is released by physogastric queens

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synchronously with the egg laying, and this may attracts and stimulates the workers to help the queen in the provisioning and oviposition processes.[21] The most likely source of this pheromone seems to be the Dufour gland. To examine this possibility, we have first analyzed the Dufour gland secretion of virgin and physogastric queens of *M. bicolor*.

Material and Methods

The Dufour glands of 20 virgin and 3 physogastric queens of *Melipona bicolor* Lepeletier, 1836 (Hymenoptera, Meliponini) were used. The virgin queens (VQ) were collected from colonies maintained by the Department of Biology, Institute of Biosciences of Rio Claro, Unesp. Two of the physogastric queens (PHQV1 and PHQV2) were active layers and the third (PHQV3) had oviposition problems and was harassed by workers.

All of the queens were collected from polygynous and different colonies. The small number of physogastric queens analyzed reflected the fact that this species is uncommon in the State of São Paulo, and that the few colonies maintained in the apiaries at the Universidade Estadual de São Paulo (USP) and at Universidade Estadual Paulista (UNESP) - Rio Claro are very fragile, and are used in several other research projects.

Sample collection and work up

After collection, the bees were immediately transferred to an amber flask and placed in a refrigerator (4 °C) for 5 min to prevent them emptying their glands during dissection. The bees were dissected in distilled water and the glands were transferred to a soda glass capillary 1 mm in diameter and sealed in a flame for subsequent analysis by gas chromatography and mass spectrometry (GC/MS), as described by Morgan and Wadhams.[22]

Sample preparation

The Dufour glands conditioned in capillaries at room temperature were transported to the School of Chemistry and Physics, Keele University (U.K.), where the analyses were done. Extracts of the glands were prepared by crushing the sealed capillary in 30 μL of hexane.

Chemical analyses

The sample were analyzed directly in a Hewlett-Packard series 5890A gas chromatograph coupled to a selective mass detector HP series 5970B (quadrupole spectrometer) operated at 70 eV of electric impact of ionization. The system was controlled and the data were stored on a Hewlett-Packard series 300 microcomputer connected to a HP 5971/5972 MSD Chemstation (Chemical Ecology Group, Department of Chemistry, Keele University). The analyses were done using a 30 m fused silica column Rtx-5 (30 m x 0.37 m x 0.5 μm) covered with poly(5%-biphenyl-95% dimethyl)siloxane.

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

Interpretation of the data

The chemical compounds were identified by comparing their mass spectra with MS-databases, ion fragmentation pattern and searching the NBS Library of Mass Spectra and the Mass Spectral Register (Stenhagen, Abrahamsson and McLafferty; J. Wilely and Sounds).

An external standard solution was prepared by mixing 12 synthetic hydrocarbons (C15, C16, C17, C18, C20, C21, C22, C23, C24, C25, C28, C30 – 0.1 mg mL^{-1}). Immediately after the injection of an extract, 1 μL of the external standard solution was injected to compare the retention times of the hydrocarbons detected in the extract.

Results

Composition of the Dufour gland secretion of virgin queens

No compounds were detected in seven of the virgin queen glands analyzed, since all of the glands had been dissected carefully and placed intact in the capillary, these results indicate that the Dufour gland was empty in approximately 35% of the virgin queens analyzed. It is possible that the absence of secretion was related to the recent emergence of some queens since morphological analysis of the glands of newly-emerged queens showed that they did not contain secretion in the gland lumen.[23] The virgin queens were collected directly from the colony with no consideration for their age.

The Dufour gland secretion of the other 13 virgin queens contained 27 hydrocarbons (Figure 1A), most of which were long chain (C17-C32) and unsaturated, with

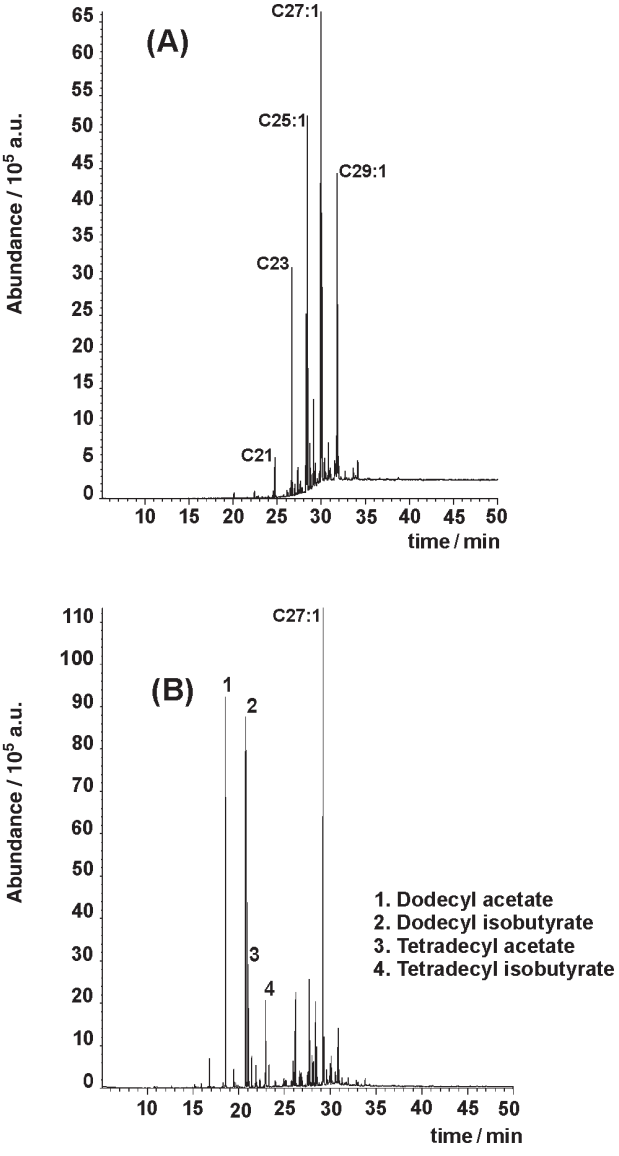

Figure 1. Chromatogram of the Dufour gland contents of a virgin (A) and physogastric queen (B) of *Melipona bicolor*.

an odd number of carbon atoms (Table 1). The most common and abundant hydrocarbons were: pentacosene-1, pentacosane, heptacosene-1, heptacosane and nonacosene-1. Of these, the most abundant was heptacosene-1 (Table 1).

Composition of the Dufour gland secretion of physogastric queens

The secretion of physogastric queens differed significantly from that of virgin queens (Figure 1B), containing esters, in addition to hydrocarbons (Table 1). In queens PHQV1 and PHQV2, acetate, isobutyrate and terpenoid esters were the major constituents; a significant quantity of linoleic acid was also detected. The hydrocarbons detected were very similar to those found in virgin queens (Table 1), the major component again being heptacosene-1.

No esters or acids were found in PHQV3 and, although all of the major hydrocarbons found in virgin and the other physogastric queens (PHQV1, PHQV2) were present, the most abundant compound was the pentacosene-1 (Table 1).

Discussion

The results of this study suggest that newly-emerged queens have empty glands, which then begin to synthesize hydrocarbon components. After mating, when the queen became physogastric, the compounds found in virgin queens continue to be synthesized in physogastric queens but significant quantities of esters are added to the secretion. Queens that lost their reproductive fitness (PHQV3) changed the composition of their gland secretion, losing the oxygenated compounds.

Many functions have been attributed to the Dufour gland secretion in bees, all of them directly or indirectly related to reproduction.[24] Our results showed that changes in the secretion composition were related to the queen stage or *status* in the colony. The hydrocarbons in the virgin queen may act as an attractant to males. However, it is possible that the Dufour gland secretion may only function after the queen reaches sexual maturity or after mating. Indirect support for this hypothesis is the observation that in *Apis mellifera* the Dufour gland is most developed when the queens are ready to mate and after mating, when the queen is in active egg-laying activity.[25]

In *M. bicolor* physogastric queens, as in *A. mellifera* queens, the active compounds would be the esters, which could function as a pheromone to attract workers for provisioning and oviposition processes (POP), as suggested by Velthuis *et al.*[21] In meliponines in general and specifically in *M. bicolor* the attractiveness of the physogastric queen is a high adaptative process since the queens depend on the workers to oviposite, controlling then her oviposition taxa and the population size, and in *M. bicolor* the colonies usually have more than one queen (polygyny). In a polygynic colony, the more attractive queen receives more trophic eggs by the workers, which increases the queen ovary development due the richer diet, being the queen able to oviposite more than other less attractive queens.[21]

In *M. bicolor*, virgin queens are tolerated in the colonies when newly emerged, but as they get age, they are eventually killed or must substitute the old physogastric queen.[26] In some meliponine species, the workers are not

Table 1. Relative abundance (%) of the main chemical compounds in the Dufour gland secretion of virgin and physogastric queens of *Melipona bicolor*

Retention time (min)	Compounds	PHQV1	PHQV2	PHQV3	VQ[a]
16.82	Pentadecene-1	1	1	———	———
17.09	Pentadecane	———	8	———	———
18.56	Dodecyl Acetate	13	9	———	———
20.10	Heptadecene-1	———	———	———	Traces
20.74	Dodecyl Isobutyrate	13	16	———	———
21.06-30.59	Terpenes**	5	6	———	———
20.97	Tetradecyl Acetate	6	3	———	———
21.44	Geranylgeranyl Acetate	1	1	———	———
22.94	Tetradecyl Isobutyrate	5	6	———	———
24.70	Henicosane	———	———	———	1
25.40	Docosane	———	———	———	2
25.70	11-Metildocosane	———	———	———	Traces
26.13	Farnesyl Acetate (2)	2	———	———	———
26.20	Linoleic Acid	5	6	———	———
26.60	Tricosene-1	1	———	———	Traces
26.70	Tricosane	———	———	———	5
27.38	Metiltricosane	———	———	———	Traces
27.40	Tetracosene-1	———	1	———	Traces
27.50	Tetracosane	1	———	———	1
27.65	11-Metiltetracosane	———	———	———	Traces
27.67	7-Metiltetracosane	Traces	———	———	———
28.28	Pentacosene-1	1	———	77	7
28.44	Pentacosane	5	5	2	11
28.72	9,11,15-triMetilpentacosane	———	———	———	Traces
28.75	11,13-diMetilpentacosane	———	1	———	1
28.86	5-Metilpentacosane	1	1	———	———
29.11	3-Metilpentacosane	5	———	———	Traces
29.12	Hexacosene-1	———	———	———	3
29.26	Hexacosane	2	———	———	1
29.30	15-Metilhexacosane	———	———	———	Traces
29.31	Heptacosene-1	20	27	15	41
29.32	Heptacosane	2	1	———	8
30.30	11,15-diMetilheptacosane	———	———	———	2
30.31	11-Metilheptacosane	1	———	———	———
30.71	5-Metilheptacosane	1	1	———	———
30.82	Octacosene-1	———	———	———	2
30.90	Octacosane	———	———	———	Traces
30.94	Nonacosene-1	3	3	5	10
33.99	Nonacosane	1	———	———	1
34.10	Hentriacontene-1	———	1	———	Traces
34.80	Dotriacontene-1	———	———	———	Traces

[a] Results of 13 virgin queen glands. Traces, < 0.4%; ———, chemical compound not found. PHQV1,2 and 3, see Material and Methods. Indeterminate substances were included in the total percentage.

interested in very young virgin queens and they are allowed to walk freely within the colony. Later, these queens must hide in storage pots to escape persecution by workers.[26] This behavior indicates a change in these individuals, which allows their identification by the workers. Since changes in the Dufour gland secretion composition were detected in the corresponding phases of the *M. bicolor* queens, and since the compounds produced may have pheromonal qualities, they may serve to communicate information about the queen reproductive stage or *status*. The absence of esters in virgin queen glands would make them imperceptible in the colony since the workers and virgin queens have similar cuticular hydrocarbon profiles.[27]

Small quantities of oxygenated compounds could be a signal of sexual maturation in virgin queens and could increase worker aggression towards such queens. This suggestion is supported by the fact that we observed that the gland of a virgin queen produced trace amounts of the esters found in physogastric queen.

Ratnieks[28,29] considered that the eggs laid by *A. mellifera* workers might be distinguished from queen eggs because the later could be marked with Dufour gland secretion. The chemical composition of the surface of eggs

laid by the *M. bicolor* queens was analyzed by Jungnickel *et al.*[31] Only hydrocarbons were found on the eggs. Although these hydrocarbons were the same as those found in the Dufour gland secretion, a function in egg-marking may be discounted, because these compounds are also found in the tegumentary cuticle of this species.[27] Katzav-Gozansky *et al.*[30] through bioassays, contested the conclusions of Ratnieks do not observing egg-marking action for the *A. mellifera* Dufour gland secretion, being it probably involved in queen *status* signalization for the workers.[19] Therefore, in *M. bicolor* we suggest that the secretion produced by the physogastric queen may also signalize her fecundity condition, as may serve to attract workers for POP, increasing her own fitness.

Based on this preliminary study of the composition of the Dufour gland secretion of *M. bicolor* queens, we have planned new GC/MS analyses in age-controlled virgin and physogastric queens and to set the double band position of the alkenes. Bioassays on the principal chemical compounds of the secretion and with gland extracts have being done in order to understand their biological function. Our preliminary results showed that physogastric queen gland is more attractive for workers than the virgin queen gland extract and that this later provokes more repulse behavior on the workers than the first.

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