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Original article

Secretions of stingless bees: the Dufour glands of some *Frieseomelitta* species (Apidae, Meliponinae)

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Abstract – The first recorded analyses of meliponine bee Dufour gland secretions by gas chromatographic-mass spectrometry on three species of *Frieseomelitta* showed these glands contain a mixture of oxygenated compounds and terpenoids with some hydrocarbons. In *F. varia* the major substances are eicosenal, 1-eicosenol and 2-pentadecanone. In *F. sylvestrii* they are pentacosene, nonadecanal and heptacosene. *F. silvestrii languida,* with the largest glands and the most complex mixture, has geranylfarnesol, followed by 1-tetradecanol and tetradecanal, making it quite different from *F. silvestrii*.

***Frieseomelitta sylvestrii / Frieseomelitta varia / Frieseomelitta sylvestrii languida* / geranylfarnesol**

1. INTRODUCTION

The subfamily Meliponinae Ashmead 1899, or stingless bees, is divided into two tribes, the neotropical Meliponini Handlirsh 1924, and the pantropical Trigonini Moure 1946. The Dufour gland, an accessory gland of the venom apparatus, is generally accepted as present in the queens and workers of all aculeate Hymenoptera. However, Kerr and de Lello (1962) and de Lello (1976) examined 26 species of Trigonini and about 15 species of Meliponini. They found that the Dufour gland is normally present in workers of all Apoidea, but while the gland was present in all the queens they were able to examine, it was absent or vestigial in workers of all *Melipona* species. Likewise, it was absent from workers of *Trigona, Oxytrigona, Cephalotrigona, Scaptotrigona, Partamona* and *Lestrimelitta.*

The secretion of this gland has been found to modulate the behavior of some bee species, particularly solitary bees (Hefetz, 1987). Isoprenoid esters found in the Dufour gland of *Colletes* and *Andrena* might be employed to line the cells and if mixed with other odours, to mark the nest (Bergström and Tengö, 1974; Tengö and Bergström, 1975). The most important components of the Dufour gland of many Colletidae and Halictidae bees are macrocyclic lactones used to line the nest in soil (Hefetz et al., 1978, 1979). Terpenoid esters were found in the Dufour gland secretion of *Andrena haemorrhoa* and other bees of this group and in *Calliopus andreniformis* (Andrenidae), long chain alkanes and alkenes were used to line the cells without further

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modification (Hefetz, 1987). Butyrates, acetates and monounsaturated alcohols found in the Dufour gland secretion of *Melitta* (Melittidae) might be used in nest-building and interestingly, were also found in the heads of their male parasite *Nomada flavopicta* (Tengö and Bergström, 1976). Acetates have also been found in *Melissodes desponsa* (Anthophoridae), employed for the same purpose (Batra and Hefetz, 1979). For the Megachilidae the secretion seems not to be so important for the lining of the cells, since they use plant material as petals, leaves and resin besides mud and sand to build their nests (de Lello, 1971). Indeed in *Trachusa byssina* a derived component of *Pinus* was found in the lining of the nest (Cane, 1981).

The function of the gland in stingless bees is unknown. Only one report of the chemical analysis of this gland from a trigonine bee has been published (Cruz Lopez et al., 2001). In this we showed that the species *Nannotrigona testaceicornis* from as far apart as São Paulo in Brasil and Chiapas in México were the same chemically with respect to the contents of this gland. We report here an examination of the glands of workers from three species of *Frieseomelitta,* meliponine bees, from Brazil with unusually large glands. An interesting aspect of the *Frieseomelitta* group is the fact that they do not build cells in flat combs as most Meliponinae do, but group them in clusters. For this reason and because they possess large Dufour gland, they have been considered "primitive" stingless bees (Kerr, 1969). While workers of many Meliponini and Trigonini regularly produce trophic and reproductive eggs (trophic eggs act as a complementary item in the diet of the queen or the workers; and reproductive eggs give rise to males), the workers of *Frieseomelitta* species never lay eggs (Cunha and Campos, 1993).

The *Frieseomelitta* group is widely distributed from the Central and Southeastern areas of Brazil to southern Mexico. It belongs to the *Tetragona-Tetragonisca* complex, centered in the shield of Guianas-North of Brazil (Camargo and Pedro, 1992). *Frieseomelitta silvestrii* collected in Goiás near Brasilia can thrive reasonably well in wooden man-made boxes in the northern area of the State of São Paulo (Nogueira Neto, 1997). *F. silvestrii languida* has been described as a subspecies, for want of more proof that it is a separate species (Moure, personal communication). It is found only in the central Cerrado area of the State of Minas Gerais (Moure, 1989). This subspecies is characteristically larger, has relatively short hairs and fewer yellow markings than *F. silvestrii*. By appearance it may be mistaken for *F. silvestrii* but its geographical distribution is different. *F. varia* is very different from *F. silvestrii*; it is yellowish and widely distributed in Brazil, occurring from the State of Rio Grande do Norte to the northern region of the State of São Paulo.

The Dufour glands of *Frieseomelitta varia, F. silvestrii* and *F. s. languida* foraging workers are long ribbon-like structure, taking the shape of the letter U (Patricio, 1995). The secretion is stored in a gland reservoir lined with a columnar epithelium of type I cells (Noirot and Quennedey, 1974) and is discharged to the exterior through a glandular duct (Patricio, 1995). For the honeybee, it has been suggested that the hydrocarbons of the secretion are not actually produced in the gland but in the fat body (Katzav-Gozansky et al., 2000).

A compilation of the volatile compounds from the heads of a number of stingless bees, containing three species of *Frieseomelitta,* among them *F. s. languida,* has been published (Francke et al., 2000) but there are no reports of analyses of secretion of Dufour glands. Descriptions of the cephalic secretions of *F. silvestrii* and *F. varia* (Cruz López et al., 2002) and *F. silvestrii languida* (Francke et al., 2000) have been published, and preliminary studies by electroantennography have been made with the head extracts of *F. silvestrii* and *F. varia* and some substances found in those extracts (Cruz López et al., 2002). The plant resins (cerumen) carried on the corbicula of the hind tibia of these three species have also been analysed (Patricio et al., 2002). Simple electroantennographic studies have also been performed upon abdominal, leg and cerumen extracts, geranyl farnesol, and some of the pure substances found in the cerumen (unpublished). We also provide further chemical evidence for the differences between *F. silvestrii* and *F. silvestrii languida.*

2. MATERIALS AND METHODS

2.1. Insects

Samples of *F. varia* Lepeletier 1836 and *F. silvestrii* Friese 1902 were provided from the Laboratório de Abelhas, Instituto de Biociências, Universidade de São Paulo, Brasil and from Prof. Paulo Nogueira Neto's collections; *F. s. languida* Moure 1989 was provided by Prof. Marina Staurengo da Cunha (Instituto de Biociências, Rio Claro SP, Brasil). *F. varia* Lepeletier were originally collected at Riberão Preto in northern São Paulo State, *F. silvestrii* came originally from Luiziania in Goiás State, near Brasilia. *F. silvestrii languida* came from the central Cerrado area in Minas Gerais State. Unless otherwise stated, mature foraging workers, collected as they arrived at the hive entrance, were taken for analysis.

2.2. Preparation of samples for analysis

Individual workers of each species were cooled in a refrigerator. Dissections were then carried out under a Vicker MG3 Zoomax binocular microscope with two pairs of fine forceps. Individual Dufour glands were dissected under distilled water and placed in thin-walled soft glass tubes (1.8 mm × 20 mm) previously sealed at one end and the open end was then sealed in a micro-flame (Morgan, 1990).

2.3. Chemical analysis

Gas chromatography-mass spectrometry was carried out on a Hewlett Packard 5890 gas chromatograph coupled to a 5070B Mass Selective Detector. The operation parameters were controlled by a HP series 200 computer with HP5970C chemstation software. The chromatographic column for the analysis was a fused silica capillary column (12 m × 0.22 mm i.d.) coated with immobilized polydimethylsiloxane (SGE, Milton Keynes, UK). The carrier gas used was helium at a flow rate of 1 ml·min^{-1}. The glass capillaries were directly inserted into the injection area, heated and crushed as described by Morgan (1990). The column was maintained at 30 ºC for 2 min after injection of the sample and then heated at 8 ºC min^{-1} to 250 ºC. The injector port was kept at 200 ºC.

Identification of the substances was confirmed where possible by comparison of the mass spectra and retention time with those of synthetic samples. Geranylfarnesol was kindly provided by C. Mansfield, International Flavours and Fragrances Ltd. Geranyl palmitoleate and geranyl oleate were prepared in the laboratory from palmitoleyl chloride and oleyl chloride with geraniol as described by Attygalle and Morgan (1986). Octadecyl acetate was identified by comparison of the mass spectrum with that of a sample prepared in the laboratory, eicosen-1-ol was compared with samples of both (*E*)- and (*Z*)-11-eicosen-1-ol, provided by J.O. Schmidt, (*Z*)-11-octadecenal was prepared by pyridinium chlorochromate oxidation of commercial (*Z*)-11-octadecen-1-ol; linear alkanes, 1-alkanols and 2-alkanones were available commercially. Quantification was by comparison with an injection of a known amount of the same or a similar compound.

3. RESULTS

3.1. Contents of glands

The Dufour glands of *F. varia* foraging workers contained a mixture of oxygenated compounds, alcohols, aldehydes, ketones and acetates, of relatively constant composition, and one hydrocarbon (Tab. I). Compounds that were identified from their mass spectra and confirmed by comparison with retention times and mass spectra of authentic samples

Table I. The contents of the Dufour glands of workers of *Frieseomelitta varia* (N = 5). * Indicates compounds where identification has been confirmed by comparison with synthetic compounds.

	Compound	Mean amount		
		ng/bee	± SD	%
1	2-Tridecanone*	4.6	0.29	1.02
2	2-Pentadecanone*	87.4	8.95	19.3
3	2-Heptadecanone*	16.6	1.59	3.66
4	Oleyl aldehyde*	88.8	4.02	19.6
5	Unknown	2.2	0.38	0.48
6	Oleyl alcohol*	22.6	2.67	4.98
7	Nonadecenal (?)	5.3	0.51	1.16
8	Oleyl acetate*	3.9	1.4	0.85
9	Eicosenal*	98.8	2.2	21.8
10	Eicosen-1-ol*	96.5	9.59	21.3
11	Eicosen-1-yl acetate	2.5	0.78	0.55
12	Docosane*	11.3	2.48	2.50
	Mean amount (ng)	453	212	†

† Minor components account for 2.86%.

Table II. Compounds identified in the Dufour gland secretion of *Frieseomelitta silvestrii* ($N = 5$). * Indicates compounds where identification has been confirmed by comparison with synthetic compounds, t indicates trace component, less than 1% of total.

	Compound	Mean amount		
		ng/bee	± SD	%
1	Tetradecanal*	t	-	
2	Heptadecanal*	t	-	
3	Nonadecanal	24	2.4	9.6
4	Heneicosane*	14	1.8	5.6
5	Tricosene*	14	1.2	5.6
6	Tricosane*	7	1.0	2.8
7	Pentacosene	159	6.4	63.6
8	Pentacosane*	t	-	
9	Geranylfarnesol*	t	-	
10	Heptacosene	23	1.1	9.2
11	Geranyl palmitoleate*	t	-	
12	An isomer of $C_{30}H_{48}$	t	-	
13	An isomer of $C_{30}H_{48}$	t	-	
14	Geranyl oleate*	t	-	
	Total amount (ng)	230	12.1	†

† Minor components together equal 3.6%.

are so marked in the table. Insufficient material was available to determine double-bond positions by the dimethyl disulphide method. The major components were eicosenal and eicosen-1-ol, confirmed with authentic samples of (*Z*)-11-eicosenal and (*Z*)-11-eicosen-1-ol. In decreasing order of quantity were 2-pentadecanone and 9-octadecenal. In the first sample of *F. varia*, from another nest, not fully quantified because the bees were of mixed ages, 2-hexadecanone was the major component in some individuals, in others it was octadecenal (oleyl aldehyde) and still others octadecenyl acetate (oleyl acetate). These latter results have not been tabulated. While the mass spectra and retention times matched, total proof of the double bond position in the eicosenyl and octadecyl compounds has not been obtained.

The average contents of individually analysed Dufour glands of *F. silvestrii* workers are listed in Table II. The gland secretion of this species is composed mainly of a mixture of unsaturated and saturated hydrocarbons, from heneicosane to nonacosane. The alkenes were identified by their mass spectra and relative retention times, since they elute just before the corresponding alkanes. Pentacosene is the major component. In some samples a small amount of tetradecanal, geranyl palmitoleate and geranyl oleate were found and in one sample a large amount of geranylfarnesol was detected. Compounds 12 and 13 in Table II have the mass spectra of linear terpenes (like the lower homologues farnesene and springene), and appear to be two isomers of the unknown compound 3,7,11,15,19,23-hexamethyltetracosaheptaene ($C_{30}H_{48}$) but not squalene, which appears at slightly longer retention. The Dufour glands of young workers of *F. silvestrii* contained the same hydrocarbons and some geranylfarnesol, but in much smaller quantity and different proportions compared with those of foraging workers.

F. s. languida Dufour glands contained a more complex mixture of hydrocarbons and oxygenated compounds than either of the other two species. Although the proportions varied widely, as shown by the standard deviations (Tab. III), the qualitative composition did not vary much. Geranylfarnesol and 1-tetradecanol were the major substances. The identification of compounds was by comparison with a mass spectral library (NIST, 2001) and approximate knowledge of the appropriate retention times, confirmed for some substances by comparison with authentic specimens.

4. DISCUSSION

The only previously published analysis of the Dufour gland secretion from stingless bees is our analysis of the trigonine bee *Nannotrigona testaceicornis* (Cruz López et al., 2001), which showed a much more complex mixture of oxygenated substances and terpenoids, but with geranylgeranyl acetate as by far the major substance. In *Frieseomelitta* species the mixtures are much simpler, but with the addition

Table III. Compounds identified in the Dufour gland secretion of *Frieseomelitta silvestrii languida* (N = 5). * Indicates compounds where identification has been confirmed by comparison with synthetic compounds, t indicates trace component, less than 0.5% of total.

	Compound	Mean amount		
		ng/bee	± SD	%
1	Pentadecane*	27	8.2	1.4
2	Tetradecanal*	272	6.0	14.3
3	1-Tetradecanol*	307	182	16.2
4	Heptadecene	t	-	
5	Heptadecane*	18	5.6	0.95
6	Hexadecanal	203	101	10.7
7	1-Hexadecanol*	t	-	
8	2-Heptadecenone	t	-	
9	2-Heptadecanone	t	-	
10	Nonadecene	109	62.4	5.8
11	Nonadecane*	181	76.7	9.6
12	Unidentified	t	-	
13	Nonadecanal	t	-	
14	Eicosane*	t	-	
15	Heneicosane	165	147	8.7
16	Tricosene*	70	78	3.7
17	Tricosane*	t	-	
8	Pentacosene	117	130	6.2
19	Pentacosane*	t	-	
20	Geranylfarnesol*	358	192	18.9
21	Heptacosene	t	-	
22	Geranyl palmitoleate*	37	33	2.0
23	Geranyl oleate*	21	11	1.1
24	Unidentified aldehyde	t	-	
25	Branched hydrocarbon	t	-	
	Mean total amount (ng)	1875	1034	

of hydrocarbons. The compounds are of similar type, that is, oxygenated compounds in all three species and terpenes in *F. sylvestrii* and *F. s. languida*. There was some similarity between the secretions of *F. silvestrii* and *F. s. languida* in that both contained a number of hydrocarbons, aldehydes and geranyl esters of oleic and palmitoleic acids but, while geranylfarnesol is the major substance in *F. s. languida*, it was only a trace compound in *F. silvestrii*. These differences suggest a real species difference between *F. silvestrii* and *F. s. languida*. The glands of *Nannotrigona testaceicornis* were much larger, with about 5 µg of secretion (Cruz López et al., 2001), compared with 1.8 µg in *F. s. languida*, 230 ng in *F. sylvestrii*, and slightly more in *F. varia*. Nevertheless, the glands of *F. s. languida*, with nearly 2 µg of liquid in them, are large compared to those of many hymenopteran workers.

The saturated and unsaturated hydrocarbons are frequently found in the Dufour glands of bees and ants (Blum, 1981; Hefetz et al., 1986; Tengö et al., 1991; Oldham et al., 1994). Some of the oxygenated compounds too are found in other Hymenoptera (Blum, 1981; Wheeler and Duffield, 1985). It is noteworthy that 11-eicosen-1-ol is also found in the venom apparatus of *Apis* species (Schmidt et al., 1997), and 9-octadecen-1-ol and 9-octadecen-1-yl acetate have been reported in the labial glands of males of *Bombus muscorum* (Kullenberg et al., 1970). This is the first time that geranylfarnesol, geranyl oleate and geranyl palmitoleate have been found in the glandular secretions of bees, but geranylfarnesol has been earlier discovered in the wax of the insect *Ceroplastes albolineatus* (Rios and Perez, 1969).

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Résumé – Sécrétions des abeilles sans aiguillon : les glandes de Dufour de certaines espèces de *Frieseomelitta* (Apidae, Meliponinae). On considère que la glande de Dufour, glande exocrine de l'appareil venimeux, est présente chez toutes les femelles et les ouvrières des Hyménoptères Aculéates, mais elle a été peu étudiée chez les abeilles sans aiguillon (Hymenoptera, Apidae, Meliponinae) et la nature de la sécrétion n'a jamais été examinée. Les abeilles sans aiguillon se répartissent en deux groupes : les Trigonini et les Meliponini. Nous avons récemment publié la première analyse de la sécrétion de la glande de Dufour d'une espèce de trigone et nous poursuivons cette étude sur trois espèces de mélipones. Bien que les trois espèces appartiennent au même genre, *Frieseomelitta*, leurs sécrétions sont bien différentes (Tab. I à III). *F. varia* renferme du 2-pentadecanone et ses homologues en C_{13} et C_{17}, l'aldéhyde, l'alcool et l'acétate d'oleyle et les trois composés correspondants en C_{20} : l'éicosénal, l'éicosénol et l'acétate d'éicosényle. On trouve aussi un peu de docosane et d'autres composés mineurs. Chez *F. silvestrii* la sécrétion comporte principalement des hydrocarbures en C_{21} à C_{27}, avec des traces d'aldéhydes, de géranylfarnésol, d'esters de géranyle d'acides gras et un composé inconnu $C_{30}H_{48}$. *F. silvestrii languida* possède les plus grosses glandes et le mélange le plus riche avec des hydrocarbures en C_{21} à $C_{27,}$ du 1-tetradécanole, du géranylfarnésol et les mêmes esters de géranyle. La composition des sécrétions de la glande de Dufour spécifique de chacune de ces trois espèces de *Frieseomelitta* suggère qu'elles peuvent avoir une fonction de phéromone pour la colonie et rend nécessaires des études comportementales.

***Frieseomelitta sylvestrii / Frieseomelitta sylvestrii languida / Frieseomelitta varia* / geranylfarnesol / glande de Dufour**

Zusammenfassung – Sekrete von Stachellosen Bienen: Dufour Drüsen bei einige Arten von *Frieseomelitta* (Apidae, Meliponinae). Die Dufour Drüse ist eine exokrine Drüse am Stachelapparat, von der angenommen wird dass sie bei allen Weibchen und Arbeiterinnen der aculeaten Hymenoptera vorhanden ist. Diese wurde bei Stachellosen Bienen (Hymenoptera: Apidae: Meliponinae) bisher wenig untersucht. Bisher ist über die Zusammensetzung des Sekretes noch nichts bekannt. Die Stachellosen Bienen sind in 2 Stämme unterteilt, in Trigonini und Meliponini. Wir haben kürzlich über die ersten Analysen von Dufour Drüsensekreten von einigen Arten der Trigonen berichtet, hier beschreiben wir diese für drei Arten der Meliponinen. Obwohl sie zurselben Gattung gehören, waren die Sekrete sehr unterschiedlich (Tab. I bis III). *Frieseomelitta varia* enthält 2-Pentadecanon und seine C_{13} und C_{17} Homologe, Oleyl Aldehyd, Alkohol und Acetat und 3 sich entsprechenden C_{20}Verbindungen: Eicosenal, Eicosenol und Eicosenyl Acetat. Es gab geringen Mengen von Docosan und anderen untergeordneten Komponenten. In *F. silvestrii* bestand das Sekret hauptsächlich aus den Kohlenwasserstoffen C_{21} bis C_{27}, mit Spuren von Aldehyden, Geranylfarnesol, Geranyl Estern mit Fettsäuren und einer unbekannten Verbindung $C_{30}H_{48}$. *F. silvestrii languida* hatte die größten Drüsen und die reichhaltigste Mischung aus Kohlenwasserstoffen von C_{15} bis C_{27}, 1-Tetradecanol, Geranylfarnesol und den gleichen Geranyl Ester. Der artspezifische Gehalt der Dufour Drüsen dieser 3 Arten lässt vermuten, dass sie auch eine Pheromonfunktion im Volk haben könnten und macht Verhaltensuntersuchungen notwendig.

***Frieseomelitta sylvestrii / Frieseomelitta varia / Frieseomelitta sylvestrii languida* / Geranylfarnesol / Dufour Drüse**

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