Pergamon

Journal of Insect Physiology 48 (2002) 249–254

Journal of Insect Physiology

www.elsevier.com/locate/jinsphys

The propolis of stingless bees: terpenes from the tibia of three *Frieseomelitta* species

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Received 26 June 2001; received in revised form 7 November 2001; accepted 8 November 2001

Abstract

The posterior tibia of foraging workers of three species of *Frieseomelitta* (Hymenoptera: Meliponinae) stingless bees have been shown to carry complex mixtures of plant-derived mono-, sesqui-, di- and tri-terpenes. These subtances were not found on the fore- or mid-legs, nor on other parts of the hind legs. *F. silvestrii* and *F. silvestrii languida,* when collecting, appear to exploit different plants for their resin even when housed in the same area. *F. varia* were found to be not collecting resin at the time of the initial sampling and were therefore sampled later. Mature foragers carry the resin. In the samples studied here, particularly prominent were the monoterpene α-pinene, the sesquiterpenes β-caryophyllene, α-cubebene, α- and γ-muurolene, γ-cadinene, germacrene-D, and elemol and the diterpenes manool and totarol The collected material is used for the resin placed around the entrance to their nests and is also mixed with wax, to produce the cerum used for the structures in the nest.

Keywords: Stingless bees; Propolis; Plant resin; Monoterpenes; Sesquiterpenes; Diterpenes; Triterpenes; β-caryophyllene; α-cubebene; δ-cadinene; Manool; Totarol

1. Introduction

Frieseomelita species of stingless bees from Brazil build their nests in hollows in rotten logs or any small and protected space. The entrance hole to the nest is no larger than 0.3 to 0.5 cm diameter, which permits only one bee at a time to enter or leave. They do not have guard bees to protect the nest entrance, but spread a sticky resin around the opening, thus preventing any insects from settling near the entrance. Even the cleptobiotic *Lestrimelitta* species do not attack the *Frieseomelitta* nests (Bego et al., 1991). The sticky material is a resin known as propolis, or bee-glue, collected from plants by the foraging workers (Bankova et al., 2000). The *Frieseomelitta* have large, flattened tibia on their third pair of legs, with poorly developed rastellum and corbicula (Figure 1), understood here as a space in the hind legs, surrounded by a fringe of hairs, used mostly to carry moistened pollen (Thorpe, 1979). The rastellum and corbiculae are found on the tibia of the hindlegs only in corbiculate Apidae (Michener, 1999). Worker bees also carry plant resin in these basket-like structures and in the case of *Frieseomelitta* species, the resin helps to hold the pollen grains together in the corbicula, which have only a few hairs to attach and bind the pollen. They possibly mix the resin with products from glands found on all segments of their legs (Patricio and Silva de Morais, 1995). They spread the resin around the entrance to their hive and mix it with wax to form cerum, to make the structures inside the nest.

In some species the hind tibia have other functions. For example, in the euglossine bees (Apidae: Euglossini), the hind tibia bear an elongated hole filled with long hairs which is used to store fragrances collected from flowers (Calaway et al., 1969). Some solitary

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PII: S0022-1910(01)00170-6

Fig. 1. Photograph of the left posterior leg of a foraging worker of *Frieseomelitta varia.* T, tibia with concave surface, the arrow indicates resin on the surface; A, abdomen; W, wing.

bees of the *Centris* genus have a tibial gland that produces chemicals apparently used in marking territory (Williams et al., 1984). We have examined the legs of three *Frieseomelitta* species to show that they carry resin only on their hind tibia, to analyse its composition, to discover whether different species had the same resin composition, and to see to what extent the resin resembles the cerum of the nest. Names here can be confusing. What is plant resin when collected by the bees becomes propolis inside the nest, and when mixed with wax becomes cerum or cerumen as parts of the nest structure.

2. Methods and materials

2.1. Insects

Samples of *F. varia* Lepeletier 1836 and *F. silvestrii* Ihering 1912 were provided from the collection of Professor Paulo Nogueira Neto and from the Laboratório de Abelhas, Instituto de Biociências, Universidade de Saõ Paulo, Saõ Paulo, Brasil. *F. silvestrii languida,* Moure 1989 were provided by Professor Marina Staurengo da Cunha from Instituto de Biociências, UNESP, Rio Claro SP Brasil. *F. varia* were collected at Riberão Preto in northern São Paulo State in October 1992 and in Rio Grande do Norte in February 1997. *F. silvestrii* came originally from Luiziania in Goias State, near Brazilia and *F. silvestrii languida* came from the Cerrado near Belo Horizonte in Minas Gerais State. Both these species were kept in artificial nests at São Paulo, where samples were collected in October 1992. Unless otherwise stated, mature foraging workers were taken for analysis

2.2. Preparation of sample for analysis

Individual workers of *F. silvestrii* were cooled in a refrigerator. Dissection was then carried out under a Vicker MG3 Zoomax binocular microscope (magnification range 5x to 45x) using two pairs of fine forceps (Inox No. 5 Pontax Junker S. A., Switzerland). Individual legs and segments of hind legs were dropped into thin-walled soft glass tubes (1.8 mm x 20 mm) previously sealed at one end and the open end was then sealed in a micro-flame (Morgan, 1990). For all species, tibia from the hind legs were sealed in capillaries at Rio Claro for analysis at Keele. Samples of fresh cerum were taken from inside the nests of the same colonies, and similarly sealed in glass capillaries and transported to Keele.

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 HP59970C Chemstation software. The chromatographic column for the analysis was a fused silica capillary column (12 m x 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). All samples were analysed with the column held initially at 30°C for 2 min and then heated to 250°C at a rate of 8°min^{-1}, and maintained at the upper temperature for up to 30 min.

Analysis of cuticular hydrocarbons was carried out as described by Bagnères and Morgan (1990).

The identification of the substances was confirmed by comparison of the mass spectra and retention time with those of authentic samples. Juniper berry oil of known composition, provided by C. Mansfield, International Flavours and Fragrances Ltd, was used as a reference to identify compounds in the hind legs of *F. silvestrii.* Totarol was the gift of Professor I. Kubo; manool was obtained from Sigma (Gillingham, Dorset, UK). Good mass spectral matches for some compounds (including sugiol) could be found in the Wiley-NBS computer library, version 1994.

3. Results

Solid-sampling chromatography of the whole forelegs and midlegs of *F. silvestrii* showed only hydrocarbons dominated by pentacosane and heptacosane. Similarly, the forelegs and midlegs of *F. varia* workers from São Paulo and Rio Grande do Norte showed a simple pattern of cuticular hydrocarbons dominated by heptacosane, and nonacosane. Chromatograms of the coxa, tarsal, basitarsa, arolium and femur from the hind legs were also obtained. These also showed only traces of compounds other than the cuticular hydrocarbons, but the tibia of all three species gave complex chromatograms with a series of peaks identified as mono-, sesqui-, di- and tri-terpene compounds. Some of the more familiar compounds could be identified with the help of a computer library.

The tibia of foragers of *F. silvestrii* showed a mixture of mono-, sesqui- and di-terpenes (Figure 2, Table 1). The most abundant monoterpene was α-phellandrene with a small amount of borneol and rather more bornyl acetate. Among the sesquiterpenes β-caryophyllene, γ-muurolene, γ-cadinene and δ-cadinene were the major compounds. Retention times and mass spectra were compared with authentic compounds in juniper berry oil. Diterpenes were evident from the retention times, molecular ions and general appearance of the mass spectra, but none could be identified with certainty. Triterpenes were not identified.

The most abundant tibial substances of *F. silvestrii languida* that were identified were again terpenes, but also 2-undecanol and 2-tridecanone, myristic acid and palmitic acid were found (Figure 3, Table 1). The main compound was the tricyclic diterpene totarol, with smaller amounts of sugiol. An authentic specimen of totarol was obtained for confirmation. Its mass spectrum has been analysed in detail (Enzell, 1966). Another constituent had a mass spectrum close to that of totarol, but with higher intensities of the ions at m/z 286 and 189. The spectrum of this unidentified compound was also close to that of ferruginol, according to published values (Scheffrahn et al., 1988). Many other diterpenes in the mixture could not be identified. In this case there was some contamination of the first and second pairs of legs with a little of the same mixture.

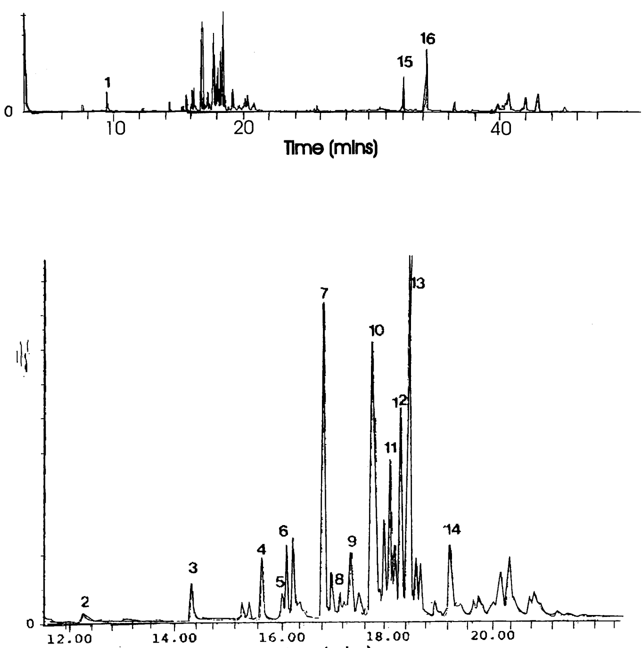

Fig. 2. Gas chromatogram of the volatile material on the hind tibia of workers of *F. silvestrii*. Above is the total ion chromatogram and below an expansion of the region from 12 to 24 minutes, containing sesquiterpenes. Numbers on peaks correspond to the numbers in Table 1: note that 15, pentacosane and 16, heptacosane are cuticular hydrocarbons found on all legs.

A list of the identified compounds from *F. varia* collected at Rio Grande do Norte in February 1997 is given in Table 1. Six out of ten foragers analysed contained terpenes on their legs, the other four were empty. There were large amounts of α-pinene; the most abundant sesquiterpenes were α-cubebene and δ-cadinene. An ion chromatogram search failed to disclose any totarol or ferruginol, found on *F. s. languida*, or sugiol found on *F. silvestri*, and only small amounts of manool were seen. The peaks due to triterpenes were broad and poorly resolved, but α-amyrin, the most abundant of the group, and β-amyrin could be identified from their mass spectra. It was noticeable that the *pattern* of peaks for the triterpenes in the gas chromatogram was the same for each of the samples of tibia collected and for the cerum. Only mature foragers contained the terpenes; workers of intermediate age had very little on their legs, consistent with what might adhere as they moved about the nest, and callow workers inside the nest contained little or none. The sample of *F. varia* workers collected at São Paulo in October 1992 showed no terpenes on the tibia and only small amounts of alkylpyrazines.

The cerum of *F. silvestrii* contained chiefly sesquiterpenes, diterpenes and hydrocarbons, of which δ-cadinene, isomers of manool and pentacosane, heptacosane, nonacosane and hentricontane were the most important, the last three in almost equal amounts (Figure 4). The hydrocarbons found on the cuticle were almost exclusively pentacosane and heptacosane. The cerum of *F. varia* consisted of some sesquiterpenes, particularly cadinene, and more diterpenes. Heptacosane was found in larger quantity, but the chromatography was not extended enough to identify further compounds.

4. Discussion

The propolis of stingless bees has received considerable attention, with several reports of its analysis (Velikova et al., 2000) but how it is carried by workers, what is the fresh composition of the resin, how this compares with the cerum of the nest, and other aspects have not been studied. We found that only mature foragers carried resin on their legs and only on the third pair of tibia in each of the three species. Analysis showed it to be a complex mixture of terpenes typical of plant resins. Each species had different groups of terpenes. Although

Table 1
Substances identified on the third tibia of three species of *Frieseomelitta* bees. The numbers refer to the numbered peaks in Figures 2 and 3. The symbol ∗ indicates where comparison was made with authentic material.

Number in Figure 2	*F. silvestrii*(*n*=4)	Number in Figure 3	*F. s. languida*(*n*=4)	*F. varia*(*n*=6)
1	α-Phellandrene∗	1	Acetic acid∗	α-Pinene∗
2	Borneol∗	2	Unknown	Camphene∗
3	Bornyl acetate∗	3	α-Phellandrene∗	β-Pinene∗
4	α-Cubebene∗	4	Sabinene	3-Carene∗
5	Ylangene∗	5	α-Terpinene∗	Terpinen-4-ol
6	α-Copaene∗	6	γ-Terpinene∗	α-Ylangene
7	β-Caryophyllene∗	7	2-Undecanol∗	α-Cubebene∗
8	α-Bergamotene∗	8	α-Terpineol∗	α-Copaene∗
9	α-Humulene∗	9	2-Tridecanone∗	β-Cubebene
10	γ-Muurolene∗	10	2-Pentadecanone∗	α-Gurjunene
11	α-Muurolene	11	Unknown sesquiterpene	γ-Elemene
12	γ-Cadinene∗	12	Myristic acid∗	Simularene
13	δ-Cadinene	13	Palmitic acid∗	*trans*-β-Farnesene∗
14	Spathulenol	14	Manool∗	α-Amorphene
15	Pentacosane∗	15	Unknown diterpene	Germacrene-D
16	Heptacosane∗	16	Totarol∗	δ-Cadinene
		17	Ferruginol	Cadina-1,4-diene
		18–20	Unknown diterpenes	Elemol
		21	Sugiol	Spathulenol
		22	Heptacosane∗	β-Amyrin
		23	Nonacosane∗	α-Amyrin
		24	Hentriacontane	
		25	Branched hydrocarbon	
		26	Dotriacontane	
		27,28	Branched hydrocarbons	
		29–31	Unidentified triterpenes	
		32–34	Branched hydrocarbons	

each species has a different home range, at the time of collection they were housed at the same place. It is interesting therefore that they were foraging on different plants (unpublished observations) and two (*F. silvestrii* and *F. s. languida*) were collecting different resins. The *F. varia* collected at the same time had no resin, and were presumably collecting nectar. The sample of *F. varia* for which details are given were collected at Rio Grande do Norte at a later time. That four of the ten bees in that sample did not contain resin is consistent with the report of Biesmeijer and Toth (1998). They found that workers of *Melipona beecheii* specialized in one commodity, either pollen, nectar or resin, in a single day. It has been noted that the propolis of meliponine bees is very variable in composition (Velikova et al., 2000), as we might expect if the bees are exploiting different plants as the season changes.

The list of compounds given in Table 1 does not permit any guess at the plant source of the propolis. Manool, which was confirmed as a constituent carried by *F. silvestrii*, was first isolated from the wood of the yellow pine *Dacrydium biforme*. The presence of 2-nonanol, 2-undecanone, myristic and palmitic acids on the legs of *F. s. languida* is noteworthy. Francke et al. (2000) found 2-nonanol and the acids as major compounds in the heads of this species, but did not find 2-undecanone present. Ghisalberti (1979) has reported that the main components of the propolis of the nest include mono-, sesqui- and di-terpenes, but triterpenes and other compounds beyond the range of our chromatographic conditions are also known (Velikova et al., 2000).

Most of these volatile compounds are present in plants and have been identified as insect kairomones (Metcalf, 1987). For example, α-copaene and α-ylangene are widely distributed in plants, chiefly as ingredients of essential oils (Teranishi et al., 1987). They are highly attractive to fruit flies of the family Tephritidae (Metcalf, 1987). Many diterpenes display a wide range of biological properties, including antibacterial action. Manool and ferruginol in particular have shown insect feeding-deterrent properties (Scheffrahn et al., 1988; Bobzin and Faulkner, 1992). Propolis is generally regarded as having antibacterial properties, valuable inside the nest (Velikova et al., 2000; Drago et al., 2000). *Plebia* species make large deposits of resin inside their nests. When a colony is attacked by the robber *Lestrimelitta* bees, the workers use the resin to trap and glue their enemies (Perico, personal communication). Other possible functions may be for protection; it is known that some monoterpenes act as repellents to ants (Eisner et al., 1986).

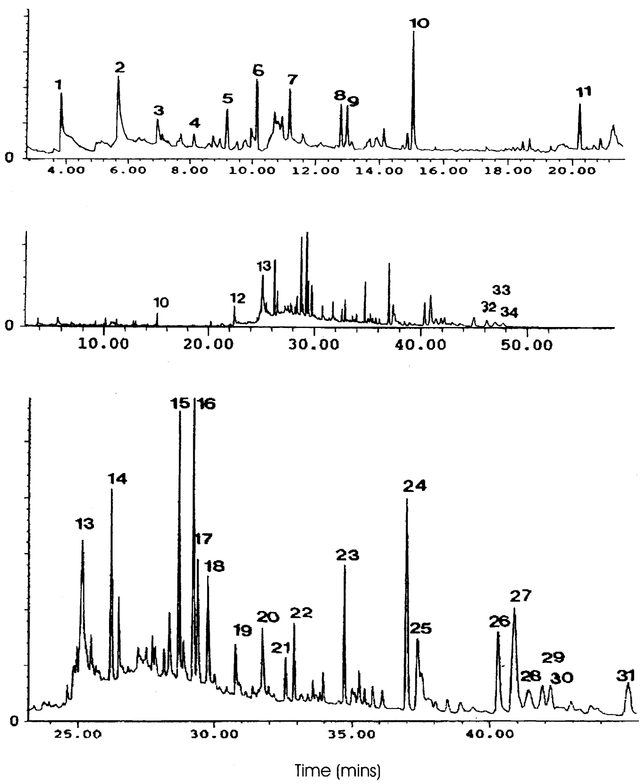

Fig. 3. Gas chromatogram of the volatile material on the hind tibia of workers of *F. silvestrii languida*. In the middle is the total ion chromatograph, at the top an expansion of the monoterpene region from 4 to 20 minutes, and below an expansion of the sesquiterpene and diterpene region. The numbers on the peaks correspond to the compound numbers in Table 1.

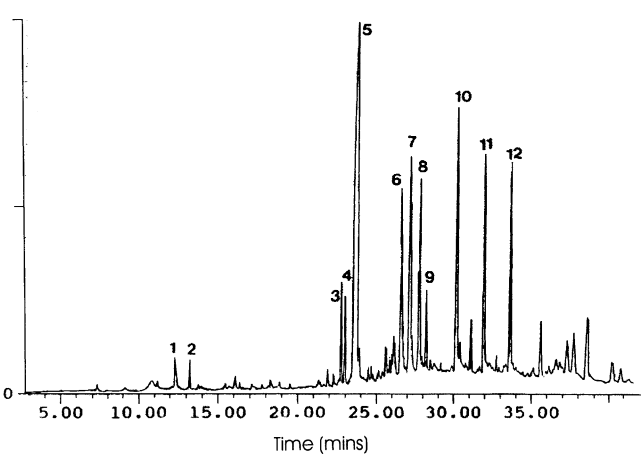

Fig. 4. Total ion chromatogram of the cerum from the nest of *F. silvestrii*, consisting of terpenes from the propolis and hydrocarbons from the wax glands. Numbered peaks are 1, unidentified sesquiterpene; 2, γ-cadinene; 3,4, isomers of manool; 5, manool; 6,7,8, unidentified diterpenes; 9, pentacosane; 10, heptacosane; 11, nonacosane; 12, hentriacontane.

The antenna of *F. silvestrii* and *F. varia* showed a significant EAG response to the hind legs extracts and to some pure compounds. *F. silvestrii* responded strongly to α-cubebene and less strongly to humulene and (-)-β-caryophyllene, pure compounds found among the terpenes on the hind legs (Cruz López et al., 2002). Similarly, the antennae of *F. varia* workers in a less complete study also gave a strong response to the cerum extract (Cruz López et al., 2002). This result may suggest that the secretion is involved in communication among workers inside the nest, in which the members of the colony use the odour of the returned foragers to discover the source of forage as suggested by Roubik (1989). Engels et al. (1987) observed that cerum is attractive to workers of *Scaptotrigona postica.*

The cerum for each species consisted of similar terpenes and wax hydrocarbons. There was less of monoterpenes in the cerum, due to their volatility and loss by evaporation. All of the terpenes are unsaturated and slowly oxidize and polymerize through the action of oxygen. The initially colourless-to-yellow propolis slowly becomes darker, viscous and tacky. Much of the material in the cerum will not have submitted to gas chromatography. The more unsaturated compounds will already have polymerized. The cerum composition also represents material collected over time from different sources, therefore one does not expect to find just the same mixture as in the newly collected resin (compare Figs. 2 and 4).

The wax of stingless bees is very different from that of honeybees (Blomquist et al., 1985; Milborrow et al., 1987). It is softer, has a lower melting point and contains a high percentage of hydrocarbons and less esters (Koedam et al., 2002). Therefore, stingless bees mix their wax with propolis to strengthen it for building cells and other structures in the nest. There are no reported analyses of *Frieseomelitta* wax, but it is evident from the analysis of the cerum of *F. silvestrii* that it is different from the cuticular hydrocarbons of that species. Analysis of other leg segments showed that the cuticular hydrocarbons were almost entirely pentacosane and heptacosane, while in the cerum heptacosane, nonacosane and hentriacontane were in approximately equal proportions with much less pentacosane. This presumably refects the composition of the wax from their wax glands. There was no evidence of any material from the Dufour gland in the cerum (Cruz López et al., 2002).

Acknowledgements

We thank C. Mansfield, International Flavours and Fragrances Ltd, Suffolk, UK, for Juniper Berry oil samples, Dr. I Kubo of the University of California for the sample of totarol. This work was partly supported by CONACYT, México through a studentship to L.C.L., partly by CAPES, Brasil through a research studentship to E. F. L. R. A. P. and partly by the European Commission through the Training and Mobility of Researchers (TMR) programme (Contract ERBFMRXCT 960072) “Social Evolution”, a co-operative programme of seven European universities. We thank Pe. J. S. Moure for the identification of the specimens.

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