Exocrine Gland Secretions of Virgin Queens of Five Bumblebee Species (Hymenoptera: Apidae, Bombini)[§]

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Secretions of three different glands (mandibular gland, labial gland, and Dufour's gland) of virgin queens of five bumblebee species (*Bombus lucorum*, *B. lapidarius*, *B. hypnorum*, *B. pascuorum*, and *B. terrestris*) were analysed. Around 200 compounds were identified in the secretions. The compositions of the secretions of labial and mandibular glands were species-specific. Dufour's gland of all species produced mainly hydrocarbons, both saturated and unsaturated, the proportions of which differed quantitatively between the species studied.

Key words: Bombus, Virgin Queens, Sex Pheromone

Introduction

The life in a bee society is directed by chemical signals produced by individuals of each cast. All life-important activities such as foraging, taking care of the brood as well as mating depend on pheromones to a large extent. Thus, the exchange of information between individuals is chemically mediated.

Males' marking pheromones have been studied extensively. During the summer period, the bumblebee males of the majority of species fly on long paths and mark spots along their flight routes with a pheromone secreted from their labial gland (patrolling behaviour; Kullenberg *et al.*, 1970; Morse, 1982). The pheromone-marked places attract females of the same species for mating. Marking pheromones function primarily as an attractant and arrestant for females and, moreover, as a short-term aphrodisiac for males themselves (Kullenberg *et al.*, 1973; Bergman, 1997).

The chemical composition of the Dufour's gland secretion and/or its function has been studied *e.g.* by Foster and Gamboa (1989), Tengö *et al.* (1990, 1991), Hefetz *et al.* (1993), Oldham *et al.* (1994), Pouvreau (1996) and Ayasse *et al.* (1999). The main functions reported are marking of the nest entrance or construction of an odour trail from nest entrance into the core of the nest in underground nesting species, nest mate (kin) recognition and egg-marking. Social nest parasites (*Psithyrus*) are known to recognize and prefer the nest odour of their host bumblebee species (Cederberg, 1979; Fisher, 1984; Fisher and Greenwood, 1990).

For better understanding the mechanisms of communication in a bumblebee colony, we need to know which chemicals are produced and shared by the colony members. Similarly to a colony of honeybees, the bumblebee queen was postulated to produce a pheromone the main function of which is to inhibit the ovarian development in workers (Röseler *et al.*, 1981). Van Honk *et al.* (1980) suggested that the queen's pheromone is a product of the mandibular gland. Later Hefetz *et al.* (1996) analysed 5 glands of the body of mated and egg-laying *Bombus terrestris* queens and identified over 500 compounds, mostly of the aliphatic type. Many of the secretion components of different glands were identical. Thus, in contrast to the honeybee, the key compounds important for bumblebee queen dominance remain unknown.

Very little is known about the chemical signals of young females showing their receptivity for mating. Van Honk *et al.* (1978) reported that the mandibular secretion of young virgin queens con-

[§] This paper is dedicated to the memory of Dr. Jan Vrkoč and his significant contribution to chemical ecology.

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tains a sex pheromone that releases mating behaviour of conspecific males. So far, there are no literature data on the composition of the mandibular gland secretion of virgin queens. The first analysis of *B. terrestris* virgin queens' extracts was presented only recently (Krieger *et al.*, 2003). We are giving here detailed description of the composition of secretions of three different glands of virgin females of five bumblebee species.

Material and Methods

Bumblebees

Colonies of five bumblebee species (*Bombus lucorum*, *B. lapidarius*, *B. hypnorum*, *B. pascuorum*, and *B. terrestris*) were the source of the experimental material. Except for *B. hypnorum*, the nests were established in the laboratory. All mother queens were taken from nature during the nest searching period in order to minimise a possible negative influence of artificial conditions on their progeny production. To stimulate egg-laying, the two-queen cascade method (Ptáček *et al.*, 2000) or combined methods (Ptáček, 2001) were used. The nests of *B. lapidarius*, *B. lucorum* and *B. terrestris* remained in the laboratory till the young queen production. The colonies of *B. pascuorum*, started in the laboratory, were placed outside for the developmental period after they had first worker progeny. They were given supplemental food *ad libitum* to enhance the queen production. Colonies of *B. hypnorum* were initiated deliberately in hives placed outside and later on, after they had several worker generations, they were transferred into laboratory and managed under the laboratory conditions as described above.

When colonies turned to queen production, the queen cocoons were removed from hives and left to ripen out of the parental colonies, supplied with the care of several workers and quality food (Ptáček, 1999). Freshly emerged queens were removed and kept assorted according to their age. The rearing conditions were darkness, a temperature of 28 °C, quality food. The test queens originated from one colony of each respective species. Virgin queens, 5–6 d old, were killed by freezing and kept deep frozen prior to dissection. Mandibular, labial, and Dufour's glands were dissected and extracted with hexane (30 μl per gland) by shaking (15 min) and standing at room temperature (2 h). After that, the extract was removed and kept at – 18 °C prior analysis.

Chromatography

The extracts were analysed using a gas chromatograph with a splitless injector (200 °C) and a mass detector (Fisons MD 800), working in electron impact ionisation mode. A DB-5 ms column (30 m x 0.25 mm, film thickness 0.25 μm; Agilent Technologies) and helium gas (starting flow 0.94 ml/min at 70 °C) were used for the separations. The temperature program started at 70 °C (2 min delay) after which the temperature of the oven was increased to 320 °C at the rate of 10 °C/min. Enantioselective separations were done on a permethylated β-cyclodextrin column (HP Chiral, 30 m x 0.25 mm, film thickness 0.25 μm) using a HP6850 instrument (Agilent Technologies). The temperature program was as follows: 40 °C (1 min), 40 °C/min to 95 °C (20 min), 2 °C/min to 105 °C (15 min), 20 °C/min to 180 °C (10 min); carrier gas hydrogen (constant flow 2.3 ml/min).

Identification of compounds

The identification of compounds was based mostly on their mass spectra compared to those of the National Institute of Standards and Technology Library (NIST, U.S.A.) and on the co-chromatography with synthetic or commercially available standards.

The double bond positions were determined from mass spectra of dimethyl disulphide (DMDS) adducts of unsaturated components (Francis, 1981; Vincenti *et al.*, 1987) or from chemical ionisation (CI) with acetonitrile as a reaction gas (Oldham and Svatoš, 1999). Acids and hydroxy acids in the mandibular gland extracts were methylated with diazomethane (10 μl per gland extract; Klimetzek *et al.*, 1989). The elution order of the isomers of unsaturated ethyl and methyl esters was determined from equivalent chain length values according to literature data (Christie, 1988; Stránský *et al.*, 1997).

Standards

Standards used for identifications were either commercially available or prepared in our laboratory. Geranylgeraniol, a series of aliphatic alcohols and esters, 3-hydroxyoctanoic acid, and 3-hydroxydecanoic acid were bought from Sigma. Among earlier synthesized standards were geranylcitronellol (Valterová *et al.*, 1996), geranylcitronellal, and geranylcitronellyl acetate. Propyl and isopropyl esters of tetradecanoic, hexadecanoic, and oc-

tadecanoic acid were prepared in the laboratory using a standard acid-catalysed esterification.

Results and Discussion

Over 200 compounds were identified in the extracts of three different glands of virgin queens of five *Bombus* species. These compounds cover the whole spectrum of chemical types from hydrocarbons (both saturated and unsaturated), aliphatic alcohols, aldehydes, fatty acids and their esters to isoprenoids, mostly diterpenic alcohols and their esters (Table I and supporting information, which can be obtained from the authors).

Mandibular glands

Mandibular glands extracted with hexane gave very low concentrated samples compared to labial or Dufour's glands. However, we detected groups of alkanes, alkenes, alkadienes, aliphatic alcohols, fatty acids, their ethyl esters, hydroxy acids, and higher esters (Table I). Since the mandibular gland extracts contained high amounts of acids, it made it difficult to get a good chromatographic separation and a reliable integration. Therefore, the samples were esterified with diazomethane and the quantification was done with the methylated samples.

Table I. Main components (> 2%) identified in glands of virgin queens of five *Bombus* species.

Compound/species	Relative proportions (%, median)				
	B. hypnorum	*B. lapidarius*	*B. lucorum*	*B. pascuorum*	*B. terrestris*
Mandibular gland	N = 4	N = 3	N = 3	N = 4	N = 3
Dodecane	0.34	2.08	–	6.38	1.93
Tridecane	0.08	0.60	–	2.22	0.61
Nonacos-9-ene	0.55	0.34	4.87	–	–
Tetradecanoic acid	2.80	0.66	0.38	0.54	2.95
Hexadec-7-enoic acid	4.63	1.32	–	2.48	2.90
Hexadec-9-enoic acid	5.66	1.00	1.58	1.12	0.24
Hexadecanoic acid	7.56	3.63	1.55	3.88	8.48
Octadecatrienoic acid[a]	–	7.04	4.52	8.41	8.89
Octadecadienoic acid[a]	12.04	2.16	7.00	5.75	5.09
Octadec-9-enoic acid	43.70	28.61	10.29	55.24	26.20
Octadec-11-enoic acid	2.30	1.08	–	1.71	2.70
Octadecanoic acid	6.67	15.12	5.52	8.19	11.65
(*S*)-3-Hydroxyoctanoic acid	–	8.65	–	–	4.92
(*S*)-3-Hydroxydecanoic acid	1.44[g]	14.31	–	–	12.85
Dodecyl dodecanoate	–	–	6.20	–	–
Dodecyl tetradecanoate	–	–	3.99	–	–
Dodecenyl hexadecenoate[a]	–	–	2.99	–	–
Dodecyl hexadecenoate[a]	–	–	7.62	–	–
Dodecenyl octadecenoate[a]	–	–	10.42	–	–
Dodecyl octadecenoate[a]	–	–	13.10	–	–
Squalene	3.12	1.56	0.60	–	0.46
Labial gland	N = 3	N = 3	N = 3	N = 4	N = 3
Tricosane	1.50	5.39	0.80	3.58	4.39
Tricos-7-ene	–	14.86	–	–	–
Tricos-9-ene	0.13	6.67	0.08	0.79	0.14
Pentacos-7-ene	0.14	18.12	0.21	1.74	–
Pentacos-9-ene	1.02	12.60	0.71	12.74	0.37
Heptacos-7-ene	1.45	5.05	1.24	0.89	0.64
Heptacos-9-ene	6.63	1.97	2.13	6.33	0.92
Nonacos-9-ene	5.28	0.39	5.18	1.60	2.17
Nonacosadiene[a]	6.39	–	2.16	0.32	1.24
Hexadec-7-enal	–	–	–	2.50	–
Hexadec-7-enoic acid	2.22	–	–	–	–
Hexadec-9-enoic acid	4.11	1.19	0.62	–	–
Hexadecanoic acid	8.10	0.26	0.08	–	2.03
Octadec-9-enoic acid	2.76	3.81	1.39	–	3.04
Methyl hexadec-7-enoate	–	0.41	–	–	2.06
Methyl octadecatrienoate[a]	–	–	–	–	3.70

Table I (cont.).

Compound/species	Relative proportions (%, median)				
	B. hypnorum	*B. lapidarius*	*B. lucorum*	*B. pascuorum*	*B. terrestris*
Labial gland	N = 4	N = 3	N = 3	N = 4	N = 3
Methyl octadecadienoate[a]	–	–	–	–	3.08
Methyl octadec-9-enoate	–	5.46	0.10	–	6.96
Methyl octadec-11-enoate	–	0.29	0.08	–	2.31
Dodecyl dodecanoate	–	–	7.21	–	6.88
Dodecyl tetradecanoate	–	–	3.97	–	3.67
Dodecenyl hexadecenoate[a]	–	–	5.01	–	–
Dodecyl hexadec-11-enoate	–	–	9.36	–	2.09
Dodecenyl octadecenoate[a]	–	–	12.41	–	–
Dodecyl octadecatrienoate[a]	–	–	–	–	3.75
Dodecyl octadec-9-enoate	–	–	9.01	–	1.80
Dodecyl octadec-11-enoate	–	–	5.53	–	4.86
Hexadecenyl tetradecenoate[a]	–	–	–	2.98	–
Hexadecenyl hexadecenoate[a]	3.11	–	–	39.30	–
Hexadecyl octadecenoate[a]	–	0.19	0.24	2.26	–
Octadecenyl hexadecenoate[a]	1.71	–	–	9.68	–
Octadecenyl octadecenoate[a]	–	0.76	–	3.59	–
Octadecyl octadecanoate	–	2.00	0.35	–	–
7,11,15-Trimethyl-3-methylen-hexadeca-1,6,10,14-tetraene	5.49	–	–	–	–
Squalene	11.99	0.07	–	–	0.25
Geranylcitronellol	–	–	–	–	8.45
Geranylcitronellyl hexadecenoate[a]	4.45	–	–	–	0.12
Geranylcitronellyl hexadecanoate	5.51	–	–	–	0.12
Geranylcitronellyl octadecenoate[a]	2.53	–	–	–	2.09
Dufour's gland	N = 6	N = 6	N = 5	N = 4	N = 3
Henicosane	0.40	0.90	–	0.32	4.86
Tricosane	6.04	8.10	5.51	7.03	17.07
Pentacosane	5.05	3.33	3.30	5.01	5.68
Heptacosane	5.78	0.92	2.06	2.11	3.66
Tricos-7-ene	0.03	21.89	0.12	0.74	–
Tricos-8-ene	–	–	–	2.12	0.19
Tricos-9-ene	0.24	7.91	1.39	–	2.10
Pentacos-7-ene	0.34	23.39	0.87	4.10	0.22
Pentacos-9-ene	2.76	16.43	6.48	13.89	0.90
Heptacos-7-ene	3.88	3.36	3.26	4.12	2.21
Heptacos-8-ene	0.97	–	1.62	0.98	3.09
Heptacos-9-ene	11.87	2.03	6.72	10.25	4.19
Heptacos-10-ene	0.77	–	2.71	0.15	4.19
Heptacos-12-ene	0.58	–	1.99	0.15	3.30
Heptacos-13-ene	0.19	–	0.54	–	3.50
Nonacos-7-ene	2.28	0.47	0.55	0.99	–
Nonacos-9-ene	16.53	0.39	6.60	5.06	7.11
Nonacos-10-ene	3.05	–	1.86	1.60	4.49
Nonacos-13-ene	0.51	–	0.21	0.19	2.06
Hetriacont-9-ene	3.95	0.15	1.22	2.27	0.45
Heptacosadiene	2.91	–	3.90[b]	3.33[d]	1.46
Nonacosadiene	10.28	–	4.92[c]	3.54[e]	4.33
Hentriacontadiene	2.60	–	0.99	3.97[f]	0.57
Methyl octadec-9-enoate	–	0.09	6.24	–	0.25
Ethyl octadec-9-enoate	0.26	0.33	2.53	0.01[a]	1.50
Hexadecenyl hexadecenoate[a]	–	–	0.01	5.88	–
Octadecyl octadec-9-enoate	–	0.01[a]	5.64	0.73[a]	0.57[a]
Geranylcitronellyl octadecenoate[a]	0.16	–	3.14	–	0.57

[a] Double bond position not determined (DMDS nor acetonitrile adducts not found) if not otherwise stated. [b] Position of double bonds 9,17 and 8,18. [c] Position of double bonds 7,19. [d] Position of double bonds 8,18. [e] Position of double bonds 9,19. [f] Position of double bonds 8,22. [g] Absolute configuration not determined.

The proportion of the sum of fatty acids varied from 31% in *B. lucorum* to 87% in both *B. hypnorum* and *B. pascuorum.* Acids of the chain length C-18, both saturated and unsaturated, were most abundant. Octadec-9-enoic acid dominated in all species, the other C-18 acids varied in different species (Table I). *B. hypnorum* and *B. terrestris* contained higher amounts of C-16 acids than the other species (8% of hexadecanoic acid compared to 1.5–4% in other species). Traces of odd-numbered fatty acids were detected in some species.

Main species-specific patterns were found among other types of compounds. *B. lapidarius* and *B. terrestris* contained substantial amounts (19–24%) of oxygenated fatty acids such as 3-hydroxy- and 3-methoxy acids. The positions of hydroxy or methoxy groups were determined from characteristic fragments in mass spectra (Hefetz *et al.*, 1996). Even-numbered acids from C-6 to C-14 were found in both species, with 3-hydroxydecanoic acid dominating (13–14%). The absolute configuration of 3-hydroxyoctanoic acid and 3-hydroxydecanoic acid in *B. terrestris* and *B. lapidarius* was found to be (*S*) in both cases as determined from the enantioselective gas chromatography according to the literature (de Roo *et al.*, 2002). *B. hypnorum* produced small amounts of 3-hydroxydecanoid acid, too. The other species contained no traces of hydroxy or methoxy acids.

Specific compounds for *B. lucorum* were higher esters (wax-type esters), all derived from C-12 alcohol (dodecyl or dodecenyl esters) and acids of the chain length C-6 to C-18. These compounds were not detected in other species. Although they were present in substantial amounts (up to 13%), we did not succeed in finding their DMDS adducts to be able to determine positions of the double bonds.

Bombus pascuorum is characterised by a very simple composition of the mandibular gland secretion. Beside fatty acids (sum 87%), only three alkanes were present such as undecane (2%), dodecane (6%), and tridecane (2%). No other types of compounds were detected in the gland extract.

Van Honk *et al.* (1978) reported on the function of the mandibular gland in virgin queens. Mandibular gland extract released mating behaviour in conspecific males. Recently, Krieger *et al.* (2003) found 21 EAG-active components in the head extracts of *B. terrestris.* Some of them were identical with our identified compounds.

Van Honk *et al.* (1980) and Röseler *et al.* (1981) published the evidence for a production of queen's pheromone in mandibular glands of egg-laying queens. Later Bloch and Hefetz (1999) published their reevaluation of the role of the mandibular gland in the regulation of the development of a colony by a bumblebee queen. The authors have not confirmed the inhibitory effect of the mandibular gland secretion on the juvenile hormone biosynthesis by *corpora allata* in workers. Thus, the source of the queen's pheromone in bumblebees remains unknown.

Labial glands

The compositions of the labial gland secretions were different in different species, both from qualitative and quantitative point of view (Fig. 1, Table I). The most obvious difference was in the presence of isoprenoids. Thus, *B. lucorum*, *B. pascuorum*, and *B. lapidarius* contained no isoprenoid

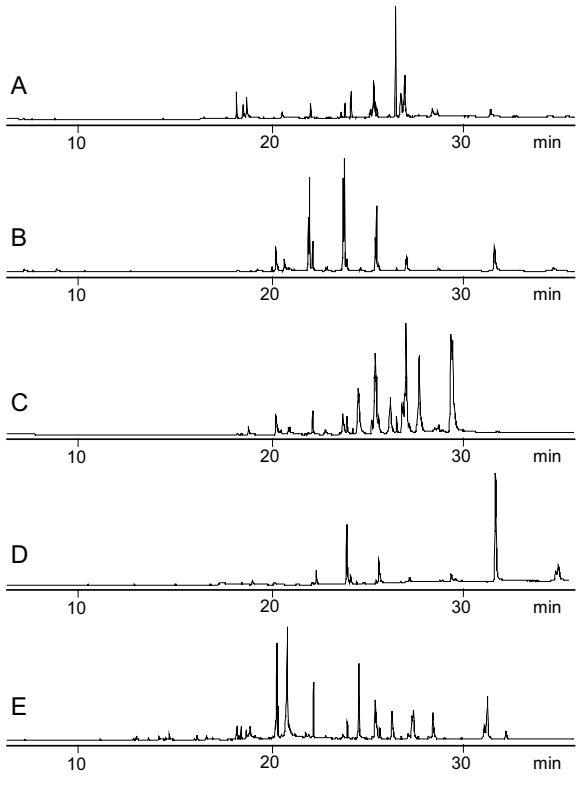

Fig. 1. Chromatograms of the queens' labial gland extracts of *B. hypnorum* (A), *B. lapidarius* (B), *B. lucorum* (C), *B. pascuorum* (D), and *B. terrestris* (E).

compounds, while *B. hypnorum* and *B. terrestris* produced substantial amounts (31% and 13%, respectively). Furthermore, the main components of the isoprenoid fraction were different. While *B. hypnorum* produced higher amounts of squalene (12%) and higher geranylcitronellol esters (14%), *B. terrestris* was characterized by a high amount of geranylcitronellol (8%). Egg-laying queens studied by Hefetz *et al.* (1996) produced no geranylcitronellol in their labial glands. *B. hypnorum* produced a diterpenic hydrocarbon, 7,11,15-trimethyl-3-methylenhexadeca-1,6,10,14-tetraene, that was not present in any other species studied.

B. hypnorum was characterized by the presence of highest amounts of free fatty acids (20%) and, on the other hand, by a lack of lower esters and only a small proportion of higher esters (6%). The opposite was observed in *B. pascuorum.* No free fatty acids were present in the secretion and no lower esters, but the proportion of higher esters was substantial (59%). Similarly as in case of the mandibular gland, the labial gland secretion of *B. pascuorum* females was less complex than those of other species studied. *B. lucorum* was similar to *B. pascuorum* considering the production of fatty acids and their esters. Thus, the proportion of free fatty acids in *B. lucorum* was around 5%, lower esters were present in traces only, while the amounts of higher esters reached 58%. However, the fraction of higher esters differed qualitatively in *B. pascuorum* and *B. lucorum.* Dodecyl and dodecenyl esters dominated in *B. lucorum*, while hexadecenyl and octadecenyl esters were prevailing in *B. pascuorum.* Thus, the species-specificity is reached by both quantitative and qualitative means.

The differences in the content of hydrocarbons are also worth mentioning, especially when we look at the unsaturated ones. While the amounts of alkanes varied from 3% to 10% between the species, the amounts of alkenes and alkadienes varied between 18% in *B. lucorum* or *B. terrestris* to 64% in *B. lapidarius.* Again, the qualitative composition of hydrocarbons differed substantially between the species (Table I).

Dufour's gland

The majority of compounds present in the Dufour's glands of all species were hydrocarbons (up to 97%). More unsaturated hydrocarbons were present, both alkenes and alkadienes (up to 83%). Alkanes represented proportions between 12% and 34%. The pattern of alkanes was similar in all species, with tricosane and other odd-numbered alkanes predominating. Odd-numbered chains were prevailing also in alkenes. The double bonds were located mostly in positions 7 and 9. There were quantitative differences between the species studied. Thus, *B. lapidarius* produced 83% of alkenes (tricosenes and pentacosenes forming the majority) and only traces of alkadienes. *B. terrestris* produced higher proportions of alkanes (sum 34%, tricosane predominating) and 51% of alkenes (nonacos-9-ene predominating). Pentacos-9-ene and heptacos-9-ene were found as main components in *B. pascuorum* (14% and 10%, respectively), while heptacos-9-ene and nonacos-9-ene were highly abundant in *B. hypnorum* (12% and 17%, respectively) together with nonacosadiene (10%). The Dufour's gland extract of egg-laying queens was studied earlier by Ayasse *et al.* (1995). The composition was comparable with our results on virgin *B. hypnorum* queens. *B. lucorum* produced similar main hydrocarbons, however, the total proportion of hydrocarbons was lower (59%) and there were other types of compounds present in substantial amounts in this species.

The proportions of esters varied from 1% in *B. hypnorum* up to 20% in *B. lucorum.* Methyl octadec-9-enoate dominated among methyl esters in *B. lucorum* (6%), octadecyl octadecenoate was highest abundant among higher esters in this species (6%). Hexadecenyl hexadecenoate was the most abundant among esters in *B. pascuorum* (6%). *B. lapidarius* and *B. terrestris* produced small amounts of esters only.

The amount of isoprenoids in *B. lucorum* was the highest of all species studied (5%). The main component in the isoprenoid fraction was geranylcitronellyl octadecenoate (3%). Other species produced only minor amounts of geranylcitronellyl esters.

The literature reports on chemical analyses of Dufour's glands of workers in *B. lapidarius*, *B. lucorum* (Tengö *et al.*, 1991), and *B. pascuorum* (Oldham *et al.*, 1994). The identified compounds in workers were significantly different from those in virgin queens.

The role of the Dufour's gland was described in workers as a source of nest-marking scent (Hefetz, 1987). However, the function of the Dufour's gland in virgin queens is unknown. The biological

function of labial glands in queens has not been reported. A thorough biological investigation needs to follow to answer questions on the role of different queens' glands and of individual compounds that were identified in glands of virgin queens.

Supporting Information available

Data on minor components can be obtained from the authors.

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