Composition of the Labial Gland Secretion of the Bumblebee Males *Bombus pomorum*

Irena Valterová*, Klára Urbanová, Oldřich Hovorka and Jiří Kindl

Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo náměstí 2, 166 10 Praha 6, The Czech Republic.
Fax: 420 2 24310177. E-mail: irena@uochb.cas.cz

* Author for correspondence and reprint requests

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Labial gland secretions of 22 males of the bumblebee *Bombus pomorum,* collected in the Czech Republic, were analysed separately for each individual. The secretions contained 70 compounds among which saturated and unsaturated hydrocarbons strongly dominated. The proportion of hydrocarbons in the secretion was unusually high (85–100%) compared to other bumblebee species studied so far (3–15%). Methyl and ethyl esters of fatty acids, known from many other bumblebee species, formed only minor components (less than 1% in sum) of the secretions of several *B. pomorum* individuals. No terpenic compounds, typical for males' marking secretion of many bumblebee species, were detected in *B. pomorum*. The absolute quantities of hydrocarbons present in the labial gland extracts were comparable with those usually present in other species. The composition of hydrocarbons found in the labial glands was different from the profile of the cuticular hydrocarbons. Despite our expectations in species exhibiting a regular patrolling and scent-marking behaviour, the labial gland extracts obtained from *B. pomorum* males were unusually low concentrated and their chemical composition was atypical with respect of the proportions of hydrocarbons when compared with other patrolling species. This is the first report on the analysis of the labial gland secretion of the *B. pomorum* males.

Introduction

Premating behaviour of bumblebee males has been studied for a long time by different authors. The species can be classified into three main groups according to this behaviour, as species exhibiting 1) patrolling behaviour (males establish flight paths with scent marks on prominent objects and fly along these routes awaiting the appearance of females, attracted by a pheromone), 2) perching behaviour (males wait at prominent objects – perches – and dart at passing virgin queens – gynes – or other objects that resemble them), and 3) nest-waiting behaviour (males wait for emerging gynes at a nest entrance) (Schremmer, 1972; Lloyd, 1981; Bergman, 1997; Hovorka *et al.*, 1998). The patrolling behaviour is the far most common type of premating behaviour among the bumblebee and cuckoo-bumblebee species. The types of behaviour ad 1) and 2) are accompanied by scent marking either of several spots on a flight path (patrolling type, Bergström *et al.*, 1981) or of a perch (perching type, Hovorka *et al.*, 1998). This pheromone is produced by a pair gland in the head, the cephalic part of the labial gland (Kullenberg *et al.*, 1973; Bergman and Bergström, 1997). In the species exhibiting the nest-waiting behaviour, males' labial gland contains typical compounds known for male-marking pheromone, too (Bergström *et al.*, 1996). However, it is uncertain whether these compounds are used by the nest-waiting males for scent marking (Bergman, 1997; Lloyd, 1981).

The composition of the males' marking signal is species-specific (Bergström *et al.*, 1981). The compounds used by the bumblebee and cuckoo-bumblebee males for scent marking are of two types. First type represent long-chain (C_{12}-C_{20}) aliphatic alcohols, aldehydes, and methyl or ethyl esters of fatty acids. Second type of compounds form acyclic mono-, sesqui- and diterpenic alcohols or aldehydes. The labial gland secretion is always a mixture of compounds, usually a very rich mixture of components. Chemodiversity in different bumblebee species is accomplished by different chain length, different degree of unsaturation (one or more double bonds) or by different double bond positions in unsaturated aliphatic pheromone

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components. Quantitative differences in composition also contribute to distinguishing among the species. Typical compounds used by the bumblebee and cuckoo-bumblebee males for scent marking are summarised in the recent review (Valterová and Urbanová, 1997).

Bombus pomorum Panzer, 1805 is distributed in Europe from Belgium on west and Denmark on north through the whole Europe to the Southern Ural. There is one uncertain record from Sweden (Løken, 1973). Males of the species *B. pomorum* exhibit patrolling behaviour. This species prefers steppe habitats without trees and bushes, where males patrol at the height of herbs – approximately 30 –80 cm above the ground (Krüger, 1951; Haas, 1949; our own observation).

Patrolling behaviour of *B. pomorum* is similar to other patrolling bumblebees. Their patrolling routes usually measure from several tens to several hundreds meters. Males were also observed to mark grass stems on their route during first morning flight. No characteristic scent could be recognised by human observer (Haas, 1949; our own observation). The chemical composition of the labial gland secretion of the species *B. pomorum* has not been studied before. The purpose of our study was to analyse these secretions obtained from a series of males collected in the Czech territory and to describe its composition in detail.

Material and Methods

Insects

Males (22 individuals) of the bumblebee species *Bombus pomorum* were collected in summer seasons 1994–1999 in the Czech Central Mountains (elevation 510 m, map field code 5548). The insect material is deposited in the collection of one of the authors (O. H.). For the chemical analysis, the collected living insects were transported to the laboratory and then kept in the freezer until the labial glands were dissected. The dissected glands were extracted with hexane (50 µl per gland). After 15 minutes of shaking and 2 h standing in the refrigerator, the hexane extract was filtered off and stored in a freezer before analysis. Each sample was analysed separately without further concentration step.

For the analysis of cuticular hydrocarbons, male's abdomen was washed with hexane (3 × 200 µl). The solution was concentrated carefully at room temperature to the volume of 20 µl prior to the GC-MS analysis.

Chemical analyses

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 × 0.25 mm, film thickness 0.25 µm, J & W Scientific) and helium gas (flow 0.55 ml/min at 50 °C) were used for the separations. The temperature program started at 60 °C (1 minute delay) after which the temperature of the oven was increased to 280 °C at the rate of 10 °C/min. The identification of compounds was based mostly on their mass spectra compared to those in 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. The DMDS adducts were prepared using a modified published procedure (Attygalle *et al.*, 1993). The reaction time was shortened to 4 h which enabled obtaining monoadducts of the dienes present in the extracts. The monoadducts formed were easier to detect in the mixture. The products were analysed by GC-MS using the same temperature program as for original extracts. The double bond configurations in unsaturated hydrocarbons were determined from infrared spectra measured on Bruker IFS-88 instrument in a KBr micropellet (1.5 mm diameter). The elution order of the isomers of unsaturated fatty acids methyl esters was determined from the analogy with the literature data (Christie, 1988; Stránský *et al.*, 1997).

Results

Extracts of the labial glands of *B. pomorum* males consisted of 70 components (Table I). The main part of the extracts of all males formed unbranched aliphatic hydrocarbons. The sum of hydrocarbons in different specimens varied from 85% to 100%. Hydrocarbons with odd number of carbon atoms predominated over those with even number of carbons. No terpenic compounds were

Table I. Composition of the labial gland secretion of 22 *B. pomorum* males; relative proportions of components and structural evidence.

Compound	Mean (%)	Standard deviation	Median (%)	Double bond position	MS fragments in DMDS adducts (*m/z*)
Acids					
Octanoic acid	1.956	2.755	0		
Decanoic acid	0.146	0.336	0		
Dodecanoic acid	0.393	0.790	0.778		
Tetradecanoic acid	0.116	0.267	0		
Ethyl esters					
Ethyl hexanoate	0.018	0.042	0		
Ethyl octanoate	0.039	0.089	0		
Ethyl dodecanoate	0.038	0.094	0		
Ethyl tetradecanoate	0.097	0.428	0		
Ethyl hexadecenoate	0.022	0.050	0	11	117, 259, $M^{+\cdot}$ 376
Ethyl hexadecanoate	0.003	0.009	0		
Ethyl octadecenoate	0.029	0.067	0	?	adduct not found
Ethyl octadecenoate	0.040	0.080	0	?	adduct not found
Methyl esters					
Methyl hexanoate	0.054	0.112	0		
Methyl octenoate	0.060	0.158	0	5	89, 161, $M^{+\cdot}$ 250
Methyl octanoate	0.188	0.570	0		
Methyl decenoate	0.027	0.113	0	7	89, 189, $M^{+\cdot}$ 278
Methyl decanoate	0.012	0.049	0		
Methyl dodecenoate	0.013	0.039	0	9	89, 217, $M^{+\cdot}$ 306
Methyl dodecanoate	0.037	0.125	0		
Methyl tetradecenoate	Trace			11	89, 245, $M^{+\cdot}$ 334
Methyl tetradecanoate	0.029	0.076	0		
Methyl hexadecenoate	0.062	0.139	0	11	117, 245, $M^{+\cdot}$ 362
Methyl hexadecanoate	0.019	0.048	0		
Methyl octadecenoate	0.086	0.196	0	11	145, 245, $M^{+\cdot}$ 390
Methyl octadecenoate	0.074	0.148	0	13	117, 273, $M^{+\cdot}$ 390
Methyl octadecanoate	0.010	0.023	0		
Alcohols					
1-Hexadecanol	0.140	0.430	0		
Hydrocarbons					
Dodecane	0.056	0.177	0		
Henicosene	0.052	0.088	0	?	adduct not found
Henicosane	0.039	0.076	0		
Tricosadiene	0.059	0.140	0	?	adduct not found
Tricosadiene	0.024	0.069	0	?	adduct not found
Tricosene	0.584	0.672	0.373	?	adduct not found
Tricosene	0.943	0.759	0.698	7	145, 271, $M^{+\cdot}$ 416
Tricosane	2.057	1.562	1.744		
Tetracosene	0.253	0.234	0.205	?	adduct not found
Tetracosene	0.040	0.077	0	?	adduct not found
Pentacosadiene	0.590	0.557	0.431	?	adduct not found
Pentacosadiene	0.580	0.525	0.427	?	adduct not found
Pentacosene	2.784	1.781	2.890	9	173, 271 (a)
Pentacosene	11.515	7.750	8.416	7	145, 299, $M^{+\cdot}$ 444
Pentacosane	2.135	1.225	1.893		
Hexacosene	0.338	0.225	0.344	?	adduct not found
Heptacosadiene	1.243	0.760	1.272	?	adduct not found
Heptacosadiene	1.452	0.844	1.420	7,19	145, 325 (a); adduct A
Heptacosene	5.340	2.024	5.726	mixture 8–13	159, 311 (a); adduct B (b)
Heptacosene	11.805	2.929	12.316	7	145, 327, $M^{+\cdot}$ 472
Heptacosane	1.319	0.493	1.226		
Octacosadiene	0.099	0.192	0	?	adduct not found

Table I. (contiued).

Compound	Mean (%)	Standard deviation	Median (%)	Double bond position	MS fragments in DMDS adducts (*m/z*)
Octacosadiene	0.145	0.188	0.090	?	adduct not found
Octacosene	0.170	0.214	0.167	?	adduct not found
Octacosene	0.219	0.317	0.121	?	adduct not found
Nonacosadiene	3.059	1.263	2.687	?	adduct not found
Nonacosadiene	7.224	1.902	7.347	7,21	145, 353 (a); adduct A 159, 323 (a); adduct B
Nonacosene	7.692	2.556	6.957	mixture 8–14	(c)
Nonacosene	6.682	2.493	6.276	7	145, 355, $M^{+\cdot}$ 500
Nonacosane	0.668	0.451	0.598		
Triacontadiene	0.045	0.208	0	?	adduct not found
Triacontadiene	0.459	0.464	0.544	?	adduct not found
Triacontene	0.463	1.107	0.194	?	adduct not found
Triacontene	0.034	0.073	0	?	adduct not found
Hentriacontadiene	0.226	0.594	0	?	adduct not found
Hentriacontadiene	3.619	5.457	2.233	8,22	159, 367, 526; adduct A 173, 353, 526; adduct B
Hentriacontadiene	12.420	6.133	11.572	7,21	145, 381, 526; adduct A 187, 339, 526; adduct B
Hentriacontene	7.357	3.599	6.776	mixture 8–15	(d)
Hentriacontene	1.957	1.406	1.457	7	145, 383 (a)
Hentriacontane	0.051	0.153	0		
Tritriacontadiene	0.302	0.796	0	?	adduct not found
Tritriacontadiene	0.144	0.396	0	?	adduct not found
Tritriacontene	0.050	0.127	0	?	adduct not found

(a) Molecular ion $M^{+\cdot}$ is not visible in the mass spectrum of the dimethyl disulphide adduct.
(b) *m/z* (relative % in the peak of heptacosene, the mixture of Δ^8-Δ^{13} being 100%): Δ^8: 159, 313 (1.6%); Δ^9: 173, 299 (80%); Δ^{10}: 187, 285 (9.6%); Δ^{11}: 201, 271 (4.8%); Δ^{12}: 215, 257 (2.4%); Δ^{13}: 229, 243 (1.6%); $M^{+\cdot}$ missing.
(c) *m/z* (relative % in the peak of nonacosene, the mixture of Δ^8-Δ^{14} being 100%): Δ^8: 159, 341 (7.7%); Δ^9: 173, 327 (32.2%); Δ^{10}: 187, 313 (25.4%); Δ^{11}: 201, 299 (7.4%); Δ^{12}: 215, 285 (7.7%); Δ^{13}: 229, 271 (12.9%); Δ^{14}: 243, 257 (6.8%); $M^{+\cdot}$ 500.
(d) *m/z* (relative % in the peak of hentriacontene, the mixture of Δ^8-Δ^{15} being 100%): Δ^8: 159, 369 (2.4%); Δ^9: 173, 355 (47.8%); Δ^{10}: 187, 341 (33.0%); Δ^{11}: 201, 327 (2.9%); Δ^{12}: 215, 313 (5.7%); Δ^{13}: 229, 299 (2.9%); Δ^{14}: 243, 285 (1.9%); Δ^{15}: 257, 271 (3.3%); $M^{+\cdot}$ 528.

detected in the samples of any male studied. Some individuals produced very small amounts (< 0.1%) of methyl and ethyl esters of fatty acids (C_6-C_{18}) and hexadecanol. Two samples contained traces of nonanal and decanal.

In Table I, mean values for the secretion components are given together with standard deviations. It can be seen from these values that the variations between individuals are rather large (standard deviation value often exceeds the mean value). This is true especially for the esters of fatty acids that were present in few samples only. Therefore, the median value is given in the Table I, too. The comparison of the mean and median values gives a better picture of the characteristic peaks present in the secretion and of the variations in its composition among the individuals.

The double bond positions in unsaturated secretion components were determined wherever it was possible. The very low proportions of some components did not allow the determination of the double bond position in these compounds. Most of the compounds had double bonds in positions 7 or 9 which is also the most frequent case in the bumblebee secretions of other species examined so far. In alkadienes that were more abundant, we were able to find DMDS adducts and to determine the double bond positions. In the very long-chain alkenes (C_{27}-C_{31}), a mixture of isomers was found and their proportions were determined from characteristic fragments in mass spectra of the DMDS adducts. The configurations of the double bonds, as determined from infrared spectra (presence of stretching =C-H at 3005 cm^{-1} and absence of the

band 965 cm^{-1}, which is intensive and typical for *E*-isomers), were *Z* in all cases.

For comparison, cuticular hydrocarbons from a male's body were analysed (Fig. 1). As can be seen from Fig. 1, the profile was different from that in the labial gland extract. Saturated hydrocarbons strongly dominated in the mixture of cuticular hydrocarbons (13% tricosane, 39% pentacosane, 16% heptacosane, 6% nonacosane, 2% hentriacontane, 0.5% tetracosane and hexacosane, *i.e.* 77% in sum), while monoenes and dienes formed only its smaller part (20% alkenes and 3% alkadienes).

Discussion

In spite of the fact that *B. pomorum* belongs to the patrolling species, their labial gland secretion did not contain compounds that are normally found in other patrolling bumblebee species (terpenes or larger amounts of aliphatic esters, alcohols, or aldehydes; Valterová and Urbanová, 1997). Hydrocarbons are always present in the secretion of bumblebee males, however, they normally form a smaller part of the secretion (3–15%; Valterová, unpublished results). Thus, the composition of the secretion of *B. pomorum* males is exceptional among bumblebee species studied so far. External calibration indicates that absolute amounts of hydrocarbons in the glands of *B. pomorum* are of the same range as in other bumblebee species. It means that hydrocarbons in *B. pomorum* most probably do not replace those compounds that are in other species considered to be the active components of the secretion.

Compared to so far studied bumblebee species, unusually large relative amounts of alkadienes of the chain length C_{27}-C_{31} were found in the labial gland of *B. pomorum* (up to 12.4%) compared to 0.2% in *B. confusus* (Hovorka *et al.*, 1998). 7,17-Pentacosadiene was described as medium-abundant component (5%) in another paper on *B. confusus* (Kindl *et al.*, 1999). There are only few literature reports on the presence of alkadienes in the males' labial gland secretions. In *Psithyrus vestalis*, one of the main components was icosadienal (Bergman *et al.*, 1996), incorrectly interpreted in one paper as henicosadiene (Valterová *et al.*, 1996). However, the secretion of *B. pratorum* males' labial gland contains a substantial amount (up to 21%) of 7,17-pentacosadiene (Valterová *et al.*, unpublished results).

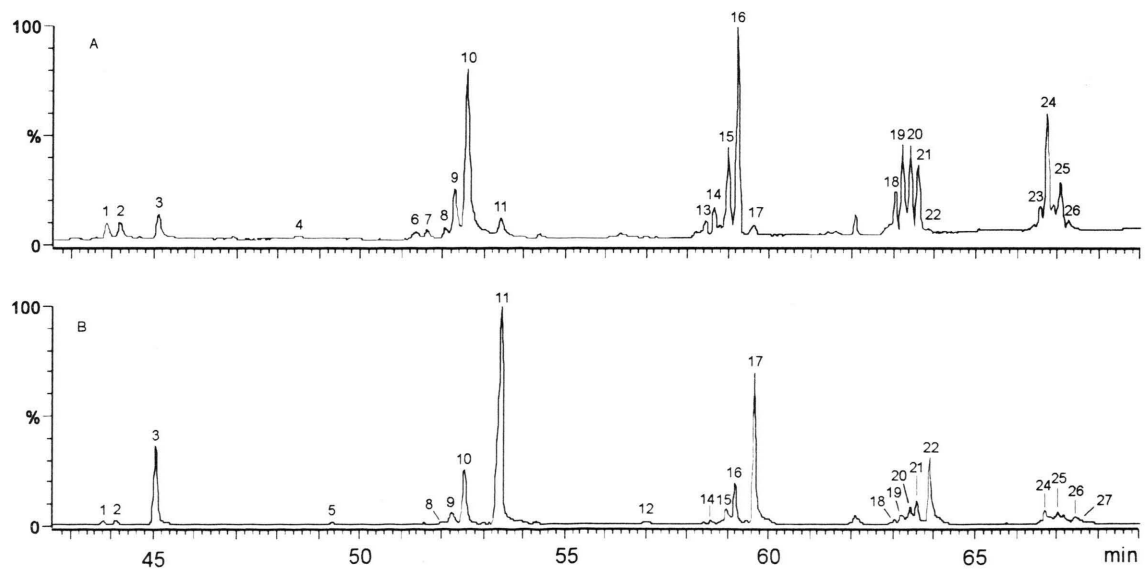

Fig. 1. Gas chromatogram of the labial gland extract (**A**) and cuticular hydrocarbons (**B**) of *B. pomorum* males. Components: 1, 2 – tricosenes; 3 – tricosane; 4- tetracosene; 5 – tetracosane; 6, 7, 8 – pentacosadienes; 9, 10 – pentacosenes; 11 – pentacosane; 12 – hexacosane; 13, 14 – heptacosadienes; 15, 16 – heptacosenes; 17 – heptacosane; 18, 19 – nonacosadienes; 20, 21 – nonacosenes; 22 – nonacosane; 23, 24 – hentriacontadienes; 25, 26 – hentriacontenes; 27 – hentriacontane.

The hydrocarbons present in the labial gland extracts are generally not considered to belong to the active components of the secretion. Bergman (1997) tested electrophysiological responses of queens *B. terrestris* to different components of the males' labial gland secretion. He did not find any responses to hydrocarbons. In workers, Oldham *et al.* (1994) showed on several species (*B. terrestris*, *B. pratorum*, *B. pascuorum*, and *B. lapidarius*) that the same hydrocarbons as found in the Dufour's gland dominated in the epidermal cuticle of bumblebees. This is not the case with the labial gland of *B. pomorum* (Fig. 1) where different proportions of saturated and unsaturated hydrocarbons have been found. Oldham *et al.* (1994) claims that the composition of cuticular hydrocarbons is species-specific. It is in agreement with a generally accepted role of cuticular hydrocarbons in recognition.

The atypical pattern of the labial gland secretion of *B. pomorum* may be connected with the unusually late occurrence of reproductive individuals of this species in the season (late August/September), time when most other bumblebee species have already finished their mating activities. Although labial glands of males of *B. pomorum* contained predominantly/only hydrocarbons, further research would be needed for understanding whether some of the hydrocarbons play a role in chemical communication of this species.

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