Male Cephalic Labial Gland Secretions of Two Bumblebee Species of the Subgenus *Cullumanobombus* (Hymenoptera: Apidae: *Bombus* Latreille) and Their Distribution in Central Europe

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The cephalic labial gland secretion of *Bombus semenoviellus* males was analyzed, and its chemical composition is reported for the first time. The secretion functions as sex or marking pheromone. Eighty compounds were identified in the secretion, the main one being all-*trans*-geranylgeranyl acetate (48%). The same compound was shown to form 87% of the labial gland secretion of *B. cullumanus* males. Both species are closely related and belong to the subgenus *Cullumanobombus*.

1. Introduction. – Bumblebees have been studied intensively for a long time, both as important pollinators and as objects attractive for collectors. They are common objects of entomological studies in classical disciplines such as insect morphology, taxonomy, faunistics, as well as objects of physiological, ecological, ethological, and other kinds of research including disciplines from applied entomology and of modern interdisciplinary areas such as chemical ecology.

The studies of chemical communication of bumblebees led to many interesting results in last decades; however, even the basic descriptive research of chemical composition of secretions of different bumblebee pheromonal glands is still in its beginning. Male marking (sexual) pheromones have been described for *ca.* 40 bumblebee species, which is *ca.* 13% of the total number of bumblebee species known [1]. The situation is even worse in case of other glands producing chemical signals in other bumblebee casts. The study of function of individual pheromonal components is nowadays a highly interesting field. As *Terzo* and co-workers [2] showed recently, chemical composition of the male marking pheromone is often a more reliable trait than some morphological differences (*e.g.*, color) for determination of a bumblebee species. While variability in color is sometimes great [3], the pheromonal composition is more or less stable within a species regardless of the locality of occurrence. Thus, a thorough redescription of the compositions of male sexual pheromones for their use in taxonomical and phylogenetical studies is needed. This necessity became greater with the development of sensitive analytical tools that allow identification of minor and trace components of complex mixtures, the gland extracts. The older literature often reported only one or a few major pheromonal components, and these data are not satisfactory for chemotaxonomical purposes [4].

The subgenus *Cullumanobombus* was established by *Vogt* [5] for a group of palaearctic species. On the basis mainly of color coat characters, four taxa are to be considered in the West-Palaearctic region: *cullumanus* (KIRBY 1802), *serrisquama* MORAWITZ 1888, *apollineus* SKORIKOV 1910, and *semenoviellus* SKORIKOV 1910. *Bombus serrisquama* MORAWITZ and *B. apollineus* SKORIKOV are considered by some authors as *cullumanus* subspecies [6] and as good species by others [7]. Out of the West-Palaearctic region, Williams [6] takes into account *Bombus unicus* MORAWITZ 1883 (East-Palaearctic) and *B. rufocinctus* CRESSON 1863 (Nearctic).

The marking pheromone is produced by the cephalic part of the male labial gland [8]. It is used by males for marking their territories while searching for females (patrolling behavior) and to attract females for mating. The aims of this paper were to describe chemical composition of male marking pheromone of *Bombus* (*Cullumanobombus*) *semenoviellus* for the first time, and to complete the data for *Bombus* (*Cullumanobombus*) *cullumanus* reported earlier by *Kullenberg et al.* [9]. Furthermore, we give recent information about distribution of the former bumblebee species in the Czech Republic.

2. Results and Discussion. – 2.1. *Composition of the Labial Gland Secretions.* Both species studied produce the same compound, all-*trans*-geranylgeranyl acetate, as the main component of the labial gland secretion (*Fig.*). The identification was confirmed by comparison of the retention time and mass spectrum with a synthetically prepared standard. The secretion of *B. cullumanus* is much simpler in composition (*Table*) than that of *B. semenoviellus*. Beside the main component (87%), mainly hydrocarbons were present in the gland extract of *B. cullumanus* males. These hydrocarbons are always present in labial gland extracts of all species, and, most probably, they do not belong to their active components [10]. Four other diterpenic compounds were detected in the *B. cullumanus* gland extract, all of them in minor or trace proportions (0.1% or less). For comparison, the qualitative data on *B. cullumanus* from the literature [9] are given in the *Table*. Our results agree with the literature data in the description of the main component, geranylgeranyl acetate. However, in our *cullumanus* sample, we have not detected mono- and sesquiterpenes reported by *Kullenberg et al.* [9]. The absence of minor components may be due to the natural intraspecific variability within the species as it has been observed, *e.g.*, for *Bombus* (*Thoracobombus*) *ruderarius* (MÜLLER) [11]. On the other hand, hydrocarbons that make almost 12% of the whole gland *cullumanus* extract are not mentioned by the Swedish authors [9]. The difference might be due to the focus of Swedish authors on the main terpenic compounds only.

The sample of *B. cullumanus* reported by *Kullenberg et al.* [9] originated from Öland, Sweden and it was reconfirmed as *B. cullumanus* s.s. [12]. Mistaking for closely related taxa *B. serrisquama* or *B apollineus*, absent in Northern Europe, can be excluded. Thus, the presently detected differences in the labial gland secretion are not to be interpreted as interspecific. *Bombus cullumanus* s.s. has disappeared from Scandinavia as well as from other countries since then, except for very few localities in Southwest France and North Spain [13].

No literature data exist so far on the chemistry of the labial gland of *B. semenoviellus* males. Geranylgeranyl acetate, the same isomer as in *B. cullumanus*,

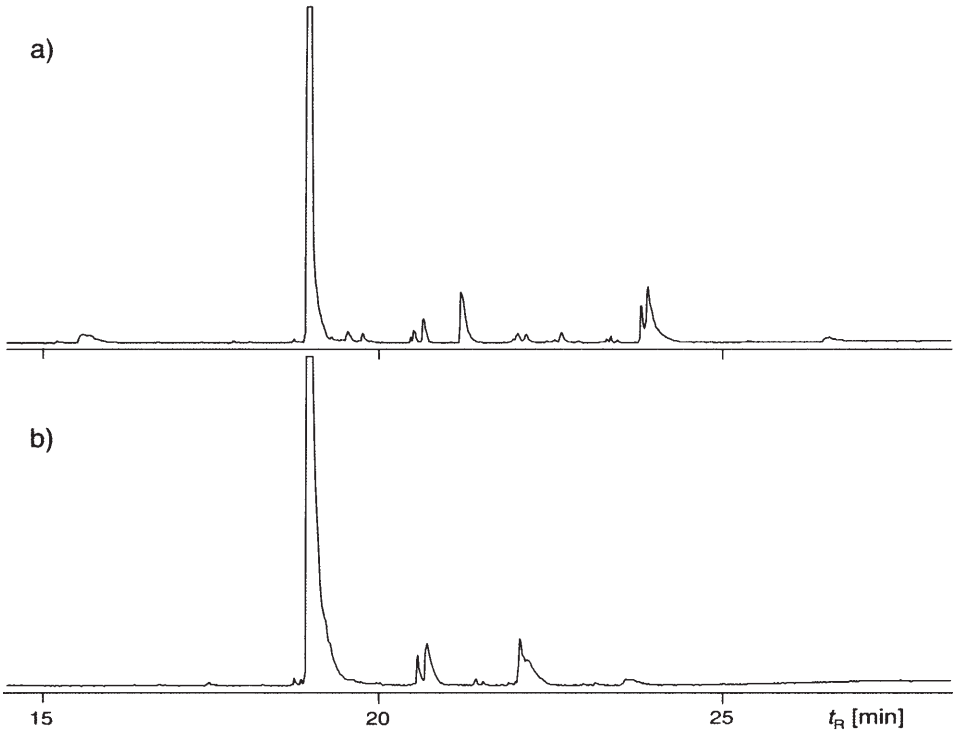

Figure. *Chromatograms of the labial gland extracts of* a) Bombus semenoviellus *and* b) Bombus cullumanus *males*

formed 37–59% of the gland extract. Beside this compound, hexadecanol, fatty acids, and higher esters were identified in substantial proportions (2–8%). While monoterpenes were completely absent in our *cullumanus* sample, they were, on the other hand, identified in our samples of *B. semenoviellus*. Thus, the two species studied, although closely related, differ in the presence of medium and minor components (*Table*). These components probably play a role in the species recognition by conspecific bumblebee specimens. A similar case occurs in *Bombus* (*s. str.*) *cryptarum* (FABRICIUS) and *B.* (*s. str.*) *magnus* VOGT with ethyl dodecanoate as the main component [14][15]. While *B. cryptarum* produces only hydrocarbons beside the main component, *B. magnus* contains several other minor components in the labial gland secretion. These components distinguish males of both species clearly from one another.

2.2. *Occurrence of the Main Component in Other Sources.* Geranylgeranyl acetate has been observed in the labial gland secretion of males of several bumblebee and cuckoo bumblebee species. It dominates in *Bombus* (*Kallobombus*) *soroensis* (FABRICIUS) [9], *B.* (*Alpigenobombus*) *wurflenii* (RADOSZKOWSKI) [9], and a North American species *B.* (*Separatobombus*) *griseocollis* DE GEER [16]. As a medium-abundant component, it is produced by *B.* (*Psithyrus*) *rupestris* (FABRICIUS) [17][18]

Table. *Compounds Identified in the Labial Gland Extracts of Males of Two* Cullumanobombus *Species*

Compound	*B. semenoviellus*[a])	*B. cullumanus*[b])	*B. cullumanus*, literature data[c]) [9]
Isoprenoids:			
Geraniol	< 0.01	–	x
Geranyl acetate	0.02	–	x
Geranyl decanoate	0.31	–	
Geranyl dodecanoate	0.98	–	
Geranyl tetradecanoate	0.01	–	
Geranyl hexadec-7-enoate	0.24	–	
Geranyl octadecenoate[d])	< 0.01	–	
Farnesol	< 0.01	–	
Farnesal	< 0.01	–	
Farnesyl acetate	0.31	–	x
7,11,15-Trimethyl-3-methylidenhexadeca-1,6,10,14-tetraene	0.47	–	
(*E*,*E*,*E*)-3,7,11,15-Tetramethylhexadeca-1,3,6,10,14-pentaene	0.18	–	
Geranylgeraniol	–	–	x
Geranylcitronellal	–	0.04	
Geranylcitronellol, isomer I	–	0.11	
Geranylcitronellol, isomer II	–	0.05	
Geranylgeranyl acetate, isomer I	0.11	0.10	
Geranylgeranyl acetate, isomer II (all-*trans*)	47.79	87.06	xxx
Geranylgeranyl butyrate	0.08	–	
Geranylfarnesyl acetate	0.34	–	
Squalene	0.02	–	
β-Sitosterol	0.02	–	
Aliphatic alcohols:			
Dodecan-1-ol	0.67	–	
Hexadecen-1-ol[d])	< 0.01	–	
Hexadecan-1-ol	3.30	–	
Aliphatic aldehydes and ketones:			
Dodecanal	< 0.01	–	
Tridecan-2-one	< 0.01	–	
Pentadecan-2-one	0.01	–	
Fatty acids:			
Decanoic acid	< 0.01	–	
Dodecanoic acid	0.07	–	
Tetradecanoic acid	0.01	–	
Hexadec-9-enoic acid	6.45	–	
Hexadecanoic acid	2.39	–	
Octadec-11-enoic acid	< 0.01	–	
Octadec-9-enoic acid	1.72	–	
Esters:			
Pent-4-en-1-yl decanoate	< 0.01	–	
Pent-4-en-1-yl dodecanoate	< 0.01	–	
Decyl hexadec-7-enoate	0.05	–	
Dodecyl acetate	0.03	–	
Dodecyl butyrate	< 0.01	–	
Dodecyl octanoate	0.14	–	
Dodecyl decanoate	1.21	–	

Table (cont.)

Compound	*B. semenoviellus*[a])	*B. cullumanus*[b])	*B. cullumanus*, literature data[c]) [9]
Dodecyl dodecanoate	4.82	–	
Dodecyl tetradecanoate	1.18	–	
Dodecyl hexadec-9-enoate	7.98	–	
Dodecyl hexadecanoate	7.59	–	
Dodecyl octadecenoate[d])	0.31	–	
Dodecyl octadecenoate[d])	0.52	–	
Hexadecyl acetate	0.12	–	
Hexadecyl tetradecanoate	0.21	–	
Hexadec-9-enyl undecanoate	0.09	–	
Hexadecyl hexadec-9-enoate	2.88	–	
Hexadecyl hexadecanoate	0.50	–	
Hexadecyl octadecenoate[d])	0.09	–	
Hexadecyl octadecenoate[d])	0.02	–	
Hexadecyl octadecanoate	0.01	–	
Hydrocarbons:			
Tridecane	<0.01	–	
Pentadecane	<0.01	–	
Henicosane	0.06	0.04	
Docosane	0.03	0.03	
Tricos-9-ene	–	0.01	
Tricos-7-ene	–	0.08	
Tricosane	–	0.90	
Tetracos-7-ene	–	<0.01	
Tetracosane	0.11	0.05	
Pentacosadiene[d])	–	<0.01	
Pentacos-9-ene	0.17	0.03	
Pentacos-7-ene	0.50	0.88	
Pentacos-5-ene	0.02	–	
Pentacosane	2.08	2.28	
Hexacos-7-ene	0.02	0.12	
Hexacosane	0.07	0.06	
Heptacosadiene[d])	0.04	0.06	
Heptacosadiene[d])	–	0.06	
Heptacos-11-ene	0.01	–	
Heptacos-9-ene	0.19	0.13	
Heptacos-7-ene	0.66	5.19	
Heptacosane	1.02	1.17	
Octacos-7-ene	0.02	0.04	
Octacosane	–	0.02	
Nonacosadiene[d])	<0.01	0.03	
Nonacos-11-ene	0.02	0.02	
Nonacos-9-ene	0.21	0.12	
Nonacos-7-ene	0.43	0.87	
Nonacosane	0.21	0.25	
Hentriacont-9-ene	0.12	0.05	
Hentriacont-7-ene	–	0.04	
Hentriacontane	–	<0.01	

[a]) Median value ($N=3$); five minor components remain unidentified (0.22% of total extract). [b]) One sample only; four minor components remain unidentified (0.08% of total extract). [c]) Literature does not give exact figures; xxx: main component, x: minor component. [d]) The C=C bond position not determined (neither CI fragments nor DMDS adducts found).

and *B.* (*Pyrobombus*) *pratorum* (L.) [15][19][20], while three other species, *B.* (*Pyrobombus*) *hypnorum* (L.) [15][19], *B.* (*Confusibombus*) *confusus* SCHENCK [21], and *B.* (*s. str.*) *lucorum* (L.) [22], contained this compound in minor or trace quantities. The older literature does not report on the specific isomer present in the above mentioned species. In the samples we had in hand (*B. soroensis*, *B. wurflenii*, *B. pratorum*, *B. rupestris*, and *B. confusus*), the same isomer of geranylgeranyl acetate (all-*trans*) was present. In the samples of labial gland extracts of *B. lucorum* and *B. hypnorum* males, collected in the Czech territory, geranylgeranyl acetate has not been detected. Thus, the presence of all-*trans*- or another geranygeranyl acetate isomer could not be verified in these two species.

Geranylgeranyl acetate is a compound that often plays a role in chemical communication in the order Hymenoptera. Beside bumblebees, it is used for territorial marking by the male bee-wolf *Philanthus pulcher* (Hymenoptera, Sphecidae) [23]. The *Dufour* glands of stingless bees *Nannotrigona testaceicornis* or of some ant species (*Formica*) also contain geranylgeranyl acetate [24–26]. The compound has been found as a component of egg-marking mixture in honeybee [27]. all-trans-Geranylgeranyl acetate and geranylgeraniol are components of the recruitment pheromone of the ponerine ant *Ectatomma ruidum* [28]. Beside Hymenopteran family, the compound has been found in the female sex pheromone of click beetles (Coleoptera, Elateridae) [29] and even in a mammalian secretion, dorsal glands of the collared peccary (Mammalia, Tayassuidae) [30]. Thus, a large number of even unrelated insect species is able to synthesize geranylgeranyl acetate and, in specific combinations with other components, use it for chemical communication.

2.3. *Distribution of* Bombus semenoviellus. The occurrence of *Bombus* (*Cullumanobombus*) *semenoviellus* SKORIKOV, 1910, in the Czech Republic was published just recently [31]. This species has been described from central and eastern parts of European Russia [32]; its redescription and distribution has been reported by *Panfilov* [33]. Easternmost (and so far also northernmost) known locality given by *Panfilov* is Zhigansk (west bank of Lena river, 66° 48′ N, 123° 30′ E), rest of localities lies between 52°–58° N from Baical lake to Byelorussia. Later, *Van der Smissen* and *Rasmont* [34] summarized the data on the species occurrence from Finland, Lithuania, and Poland, and reported its occurrence in three localities in Northern Germany. Additional records from Germany and two records from the Czech Republic were published by *Přidal* and *Tkalců* [31].

B. semenoviellus occurs in a relatively low altitude in the Czech Republic [31] (240 m and 465 m a.s.l., resp.), one locality has even a xerotherm character. This is not very typical for a species mainly known from Asiatic and East (+North) European taiga. However, a rapid westward spreading of this species indicates a possible shift in its ecological valence. All specimens of *B. semenoviellus* used for this study were collected on flowers along forest path in spruce forest in eastern part of Giant Mountains. The elevation of the locality is *ca.* 850 m a.s.l., the sum of yearlong precipitation is 868 mm, and the average yearlong temperature is 5.2°. Different biotopes surround the spot of collection, *i.e.*, clearings, pastures, wet mountain meadows, wet grounds, *etc*. The species diversity is responding to this wide variability of biotopes and of plants, and it is a dynamic process: some species are missing in comparison with literature data [35], *e.g.*, *Bombus* (*Rhodobombus*) *mesomelas*

GERSTAECKER 1869, or *Bombus* (*Allopsithyrus*) *barbutellus* (KIRBY 1802), but some others, including *B. semenoviellus* as a recent immigrant, are new for this part of Giant Mountains. Generally, there is a distinct shift of vertical distributional limits of some species that were known formerly from altitudes below 800 m a.s.l. only, and during last decade they were found repeatedly in altitudes 900–1000 m a.s.l.

Experimental Part

Bombus (*Cullumanobombus*) *semenoviellus* (three specimens) were collected during the first decades of August 2003 and 2004 in eastern part of Giant Mountains (Krkonoše), near Horní Albeřice village (50°41′ N, 15°51′E), on west slopes of Rýchory Mt. Ridge (altitude *ca.* 800–850 m a.s.l.). *Bombus* (*Cullumanobombus*) *cullumanus* (one specimen) was collected in France, Pyrénées-Orientales, Eyne, Serra del Bosc (42°28′ N, 02°05′ E, 1700 m) in July 2001. The insect material is deposited in the collections of the authors (*O. H.* and *M. T.*).

For the chemical analysis, living insects were transported to the laboratory, and their labial glands were dissected. The glands were extracted with hexane (*UniSolv* hexane for org. trace analysis, *Merck*; 50 µl per gland). After 15 min. of shaking and 2 h standing in the refrigerator, the hexane extract was filtered and stored in a freezer before analysis. Each sample was analyzed separately.

The extracts were analyzed with a *Finnigan Focus* GC (*Thermo Finnigan*) coupled to *Fisons MD 800* detector (*Fisons*). The apparatus was equipped with a *DB-5ms* fused-silica column (30 m × 0.25 mm, film thickness 0.25 µm). He Carrier gas (constant flow 1 ml/min) was used for the separations. The temp. program started at 70° (2-min delay) after which the temp. of the oven was increased to 320° at the rate of 10°/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 standard of all-*trans*-geranylgeranyl acetate was prepared from all-*trans*-geranylgeraniol (*ICN Pharmaceuticals*) *via* a standard acetylation method.

The C=C bond positions in unsaturated components were determined either from chemical-ionization mass spectra on *Varian Saturn 2000* (ion trap) using MeCN as collision gas [36] or from mass spectra of dimethyl disulfide (DMDS) adducts [37]. The DMDS adducts were analyzed by GC/MS using the same temp. program as for original extracts.

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