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Chemotaxonomical characterisation of males of *Bombus lucorum* (Hymenoptera: Apidae) collected in the Czech Republic

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Abstract. Labial gland secretions of 26 males of the bumblebee *Bombus lucorum* (L.), collected in the Czech Republic, were analysed. The secretions consisted of 60 compounds; ethyl (*Z*)-9-tetradecenoate was the main component (average 53%). Although the males varied in colour, their labial gland secretions were similar in composition, which indicated they belonged to one species. Chemically the *B. lucorum* occurring in the Czech Republic correspond to the earlier described "blonde form" of this species.

INTRODUCTION

The species of bumblebees (*Bombus* Latreille, 1802) belonging to the subgenus *Bombus* s.str. are often difficult to determine. Colouration is very variable and other morphological differences are indistinct. Individuals of the "*lucorum* species group" are especially difficult to distinguish morphologically (Krüger, 1954). In West and Central Europe this group consists of *Bombus* (s.str.) *lucorum* (Linnaeus, 1761) and two other closely related species - *B.* (s.str.) *magnus* Vogt, 1911 and *B.* (s.str.) *cryptarum* (Fabricius, 1775).

Bombus lucorum in the broadest sense (the taxonomy and nomenclature is very confused - see Williams, 1991, p. 81–85) is a widely distributed bumblebee species, which occurs throughout the Palaearctic. It is one of the commonest bumblebee species in Central and Northern Europe (Rasmont, 1984). The males are very variable in colour throughout their distribution. Due to their morphological similarity *B. cryptarum* (Rasmont, 1984) and *B. magnus* (Løken, 1973) have either been regarded as distinct species or have been synonymised with *B. lucorum,* sometimes even with *B. terrestris* (Warncke, 1981, 1986). This has led several authors to use characteristics other than morphological and morphometrical ones to separate these taxa. Species can be distinguished using enzyme electrophoresis (Scholl et al., 1983, 1992) or the chemical composition of the marking pheromone of the males (Pamilo et al., 1997; Bertsch, 1997a, b). The second characteristic is known to be species-specific. In Scandinavia, the labial gland secretions of males were used to identify two forms of *Bombus lapponicus* (Bergström & Svensson, 1973), which were later classified as distinct species, *Bombus lapponicus* (blonde form) and *Bombus scandinavicus* (dark form) (Svensson & Bergström, 1977). Svensson (1979, 1980) later synonymised *Bombus scandinavicus* with *Bombus monticola.*

The first analysis of the chemical composition of the labial gland secretion of *B. lucorum* males was reported by Calam (1969). He identified the main component (ethyl 9-tetradecenoate) and several of the minor components. *B. lucorum* occurring in Sweden was studied extensively by Kullenberg et al. (1970) and Bergström et al. (1973). The labial gland secretions of individuals of a wide variety of colour, ranging from light yellow to dark, from the island Öland were analysed. While that of the blonde form contained mainly ethyl (*Z*)-9-tetradecenoate, the dark form produced mainly ethyl dodecanoate (Kullenberg et al., 1970). The "dark" form was later transferred to *B. cryptarum* (Rasmont et al., 1986; Bertsch, 1997a, b).

Bumblebees belonging to the "*B. lucorum* species group" occur in Czech Republic (Tkalců, 1974, 1999), but the chemical composition of the males' labial gland secretion is unknown. The purpose of this study was to make a detailed analysis of the labial secretions of specimens collected in different localities in the Czech Republic, to determine the species on the basis of both morphological and chemical traits and to compare our results with the data in the literature.

MATERIAL AND METHODS

Insects. Males (26 individuals) of the bumblebee species *Bombus lucorum* were collected in the summers of 1994–1999 at seven different localities in the Czech Republic (both in Bohemia and Moravia, Table 1). Some of the males came from colonies that were established artificially using mated females from the previousyear. The insect material is deposited in the collection of one of the authors (O.H.).

For the chemical analyses, living insects were transported to the laboratory and then kept in a freezer until the labial glands were dissected. The 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 and stored in a freezer before analysis. Each sample was analysed separately.

Gas chromatography. The extracts were analysed using a gas chromatograph with a splitless injector (200°C) and a mass detector (Fisons MD 800). A BPX5 column (5% phenyl methyl silicone; 30 m × 0.22 mm, film thickness 0.25 µm) and helium (flow 0.55 ml/min at 50°C) were used for the separations. The temperature program started at 70°C (2 minutes delay) after which the temperature was increased to 140°C at a rate of 40°C/min, then to 240°C at a rate of 2°C/min, and finally to 300°C at a rate of 5°C/min. Compounds were identified by comparing their mass spectra with those in the National Institute of Standards and Technology Library (NIST, U.S.A.) and by 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 configurations of the double bonds in the following compounds were determined (after their chroma-

tographic separation) from the retention times and co-chromatography with the corresponding *E*- and *Z*-standards (isothermally 160°C for ethyl 9-hexadecenoate, 200°C for ethyl 9-octadecenoate, 170°C for 9-hexadecenol and 11-octadecenol on a DB-1 column (methyl silicone - 30 m × 0.25 mm, film thickness 0.25 μm); hydrogen as carrier gas, flow 1.2 ml/min; Hewlett-Packard 5890A). The double bond configurations in unsaturated hydrocarbons were determined from infrared spectra measured on a Bruker IFS-88 instrument in a KBr micropellet (1.5 mm diameter). The elution order of the isomers of unsaturated ethyl esters was determined from the analogy with chromatographic properties (equivalent chain length values) of fatty acids methyl esters according to the data in the literature (Christie, 1988; Stránský et al., 1997). The identity of (*Z,Z*)-9,12-octadecadienol and (*Z,Z,Z*)-9,12,15-octadecatrienol was verified by co-chromatography with commercial standards, on two different columns, and by their mass spectra.

Preparative column chromatography and derivatisation. A hexane extract of one selected gland (50 μl) was chromatographed on silica gel Merck 60 (0.040–0.063 mm; 290 mg) in a Pasteur pipette. The elution of the sample started with pentane, followed by hexane/ether mixtures (1%–40% of ether). Fractions were checked by GC-MS and those containing unsaturated compounds were derivatised with DMDS according to the procedure published by Attygalle et al. (1993). The products were analysed by GC-MS using the same temperature program as for the original extracts.

Evaluation of GC data. The analytical results were evaluated by multivariate data analyses PCA (principal components analysis) and PLS-DA (projections to latent structures-discriminant analysis) (Wold et al., 1989), which are included in CODEX® (SumIT System AB, Sweden). The GC-data were pretreated as in earlier investigations (Sjödin et al., 1989; Valterová et al., 1995). The normalised data were logarithmically transformed and represented as a matrix X. Each variable was scaled to unit variance (autoscaling).

In PLS-DA, the Y matrix was made up as a single dummy variable, each male being given a value -1 or 1 depending on the group of males to which it belonged. The significance, i.e. the number of significant components, was determined by cross-validation. The importance of the constituents in differentiating between the two groups of bumblebees was determined from the loading plots (Wold et al., 1989).

RESULTS

All the males belonged to the "blonde" form, in particular the medium blonde form as figured in Bergström et al. (1973, Plate I, Figs 2–4). The material was slightly variable. The variability in the colouration of the Czech males corresponds to that cited for *B. lucorum* males by Rasmont et al. (1986).

The composition of the labial gland secretion of *B. lucorum* males is summarised in Table 2. The positions of the double bonds are specified in Table 2 except for minor components (content < 0.1%) and polyunsaturated compounds where the DMDS adducts were not found. The configurations of the double bonds, as determined either from the co-chromatography with standards or from infrared spectra (absence of the band 965 cm^{-1}, which is intensive and typical for *E*-isomers), were *Z* in all cases.

In all the Czech samples, ethyl (*Z*)-9-tetradecenoate dominated (mean value 52.9%) but was present in variable quantities. Present in the medium-abundant category (3–10%) were ethyl dodecanoate (5.9%), hexadecanol (3.6%), ethyl 9-hexadecenoate (3.6%), and tricosane (5.4%). In some samples, relatively high proportions of tetradecenoic acid (2.5%) and hexadecyl tetradecenoate (5.3%) were identified. In the minor category (1–2%) were ethyl tetradecanoate, 7-hexadecenal, 11-octadecenol, ethyl 9-octadecenoate, 9-pentacosene, pentacosane, and tetradecenyl octadecatrienoate. With the exception of 7-hexadecenal, 11-octadecenol and the wax-type esters, all of the above mentioned compounds were identified in the labial gland secretion of the Scandinavian "blonde form" (Bergström et al., 1973). Many trace components (less than 1%) not reported in the literature for *B. lucorum*, were detected in the Czech samples.

Traces of the sesquiterpenic alcohols farnesol and 2,3-dihydrofarnesol (below 0.1%) were detected in Czech bumblebees. The Scandinavian blonde form did not contain any isoprenoids (Bergström et al., 1973). We also detected several compounds with larger molecules than pentacosane. The majority were present in trace amounts, but substantial quantities of 9-heptacosene and wax-type esters were present, especially in some of the samples. It is possible that these compounds were not analysable by the older techniques, which may account for why they were not recorded previously.

The principal components analysis (PCA) showed a slight tendency for the data to separate into two groups, one containing the males collected in 1997 and one containing all the other males. However, cross-validation revealed no significant components (CSV/SD(1) = 0.97). A PCA, in which hydrocar-

Table 1. Data on where and when the *Bombus lucorum* males were collected.

Number of samples	Year of collection	Region in the Czech Republic	Locality	Type	Elevation (m)	Map field code
3	1994[a]	Central Moravia	Prostějov	edge of flooded forest	220	6568
1	1995	Central Bohemia	Žebrák	field margin	340	6149
1	1995	České středohoří Mountains	Oblík	steppe	510	5548
1	1995	Central Bohemia	Praha-Dejvice	garden	220	5852
1	1995	Krkonoše Mountains	Horní Albeřice	wet mountain meadow	750	5361
6	1997	Central Bohemia	Praha-Dejvice	garden	220	5852
3	1998	South Moravia	Pálava	steppe	420	7165
4	1998	Krkonoše Mountains	Horní Albeřice	wet mountain meadow	750	5361
2	1998	České středohoří Mountains	Oblík	steppe	510	5548
4	1999[b]	South Moravia	Brno-Královo Pole	garden	235	6765

[a]Males from a colony artificially established using a mated queen from the previous year
[b]Males from one nest

bons and fatty acids were excluded, resulted in a model with one component on the limit of significance (CSV/SD = 0.94, variance explained (1) = 22%) that showed the same pattern of groups. Hydrocarbons present in the gland extracts are not considered to be the active components (Bergman, 1997). Free fatty acids are most probably precursors of the components in the secretion (Bergman, personal communication). Therefore, hydrocarbons and free fatty acids were excluded from the following analysis.

A PLS-DA (for males 1997 Y = -1 and for the other males Y = 1) resulted in one significant component (CSV/SD = 0.69, variance explained in X = 21%, variance explained in Y = 58%). The loading plot showed that the most important constituents separating the males collected in 1997 from the rest were the wax-type esters. They produced higher quantities of hexadecyl dodecanoate (0.4–1.2%), octadecadienyl dodecanoate (0.2–0.7%), hexadecyl tetradecenoate (4.5–22.5%), and tetradecenyl octadecatrienoate (2.4–7.7%). The function of these "heavy" molecules in the secretion of some males remains unknown. No grouping based on origin was observed.

DISCUSSION

Although the males collected in 1997 differed from the rest of the males, the order of difference is very small compared to that between the Scandinavian dark and blonde forms described by Bergström et al. (1973). Therefore, *B. lucorum* in the Czech Republic are all chemically similar and more similar to the Scandinavian blonde form than to the dark form (Bergström et al., 1973). All the major components we found were also found by Bergström in the blonde form. Compounds reported only from the dark form (ethyl decanoate, ethyl octadecadienoate, geranylgeraniol, and geranylgeranyl acetate) were not found in the Czech males. The literature on "*lucorum* species group" and our results point to the conclusion that the Czech specimens belonged to *B.* (s.str.) *lucorum* and not to *B. cryptarum* or *B. magnus* (Bertsch, 1997a). Differences between individuals were most probably due to differences in age or physiological conditions rather than their origin.

The composition of the labial gland secretion of *B. cryptarum* (Bertsch, 1997a) corresponds to that of the "dark form" of *B.*

TABLE 2. Composition of the labial gland secretion of *B. lucorum* males.

Compound	Double bond position[a]	Relative proportions (%; N=26)		
		Mean	Range	S.D.
Aliphatic Esters				
Methyl tetradecenoate	n.d.	0.032	0-0.3	0.068
Ethyl dodecenoate	n.d.	0.032	0-0.2	0.083
Ethyl dodecanoate[b]	-	5.921	1.6-16.2	3.149
Ethyl tetradecenoate	7	0.282	0-1.0	0.272
Ethyl tetradecenoate[b]	9	52.880	15.1-87.4	20.879
Ethyl tetradecanoate[b]	-	1.834	0.2-5.3	1.464
Ethyl hexadecenoate	7	0.245	0.001-1.4	0.261
Ethyl hexadecenoate[b]	9	3.575	1.0-6.6	1.380
Ethyl hexadecenoate	11	0.227	0.04-0.6	0.174
Ethyl hexadecanoate	-	0.042	0-0.3	0.063
Ethyl octadecenoate[b]	9	1.821	0.3-4.3	0.990
Ethyl octadecatrienoate[b]	n.d.	0.209	0-1.8	0.376
Ethyl octadecanoate	-	0.083	0-0.3	0.067
Ethyl icosenoate	n.d.	0.022	0-0.2	0.034
Dodecyl acetate	-	0.022	0-0.5	0.096
Tetradecadienyl acetate	n.d.	0.127	0-0.6	0.125
Hexadecyl acetate[b]	-	0.526	0-4.6	1.005
Octadecadienyl acetate	9,?	0.048	0-0.4	0.082
Icosenyl acetate	n.d.	0.026	0-0.1	0.043
Icosyl acetate	-	0.043	0-0.4	0.079
Docosenyl acetate	n.d.	0.041	0-0.1	0.039
Hexadecyl dodecanoate	-	0.343	0.02-0.8	0.332
Octadecadienyl dodecanoate	n.d.	0.139	0.001-0.7	0.182
Hexadecyl tetradecenoate	n.d.	5.295	0.2-22.5	5.814
Tetradecenyl octadecatrienoate	n.d.	1.842	0.07-7.7	2.302
Aliphatic Aldehydes				
Tetradecanal	-	0.013	0-0.3	0.028
Hexadecenal	7	1.569	0.001-5.7	1.656
Hexadecanal	-	0.260	0-0.9	0.275
Octadecenal	9	0.524	0-1.8	0.606
Aliphatic Alcohols				
Hexadecenol	9	0.231	0-5.8	1.116
Hexadecanol[b]	-	3.591	0-14.8	4.143
Octadecadienol	9,12	0.829	0-5.0	1.453
Octadecatrienol	9,12,15	1.254	0-6.5	1.795
Octadecenol	11	0.110	0-1.7	0.330
Isoprenoids				
Farnesol	-	0.105	0-0.4	0.097
2,3-Dihydrofarnesol	-	0.054	0-0.2	0.061
Dihydrofarnesyl tetradecenoate	n.d.	0.152	0-1.2	0.241

Fatty Acids				
Dodecanoic acid	-	0.278	0-2.4	0.622
Tetradecenoic acid	n.d.	2.544	0-26.2	6.343
Hydrocarbons				
Henicosane[b]	-	0.360	0.001-1.1	0.245
Tricosene[b]	9	0.550	0.04-1.6	0.602
Tricosene	7	0.331	0-5.2	0.989
Tricosane[b]	-	5.428	0.8-9.5	2.481
Tetracosene	9	0.026	0-0.1	0.031
Tetracosane	-	0.125	0-0.4	0.106
Pentacosene[b]	9	1.859	0.1-3.8	1.024
Pentacosene	7	0.085	0-0.3	0.074
Pentacosane[b]	-	1.709	0.2-4.6	1.168
Hexacosene	9	0.048	0-0.2	0.046
Hexacosene	7	0.010	0-0.05	0.013
Heptacosene	9	0.885	0.05-3.3	0.803
Heptacosene	7	0.113	0.001-0.4	0.116
Heptacosane	-	0.137	0.004-0.7	0.169
Octacosene	9	0.016	0-0.1	0.026
Nonacosene	9	0.252	0.01-1.0	0.311
Nonacosene	7	0.009	0-0.07	0.017
Nonacosane	-	0.008	0-0.07	0.015
Hentriacontene	n.d.	0.020	0-0.1	0.030
Tritriacontatriene	n.d.	0.112	0-0.9	0.226
Tetratriacontadiene	n.d.	0.712	0.01-5.0	1.072

[a] *Z*-configuration in all cases as determined either by co-chromatography with standards or from infrared spectra

[b] reported by Bergström et al. (1973) in the blonde form (semiquantitative data), n.d. = not determined because of the low content of the component

lucorum (Bergström et al., 1973). The components of the marking pheromone of *B. magnus* have not been described in detail. Bertsch (1997a) mentions the two main components (9,12-octadecadienol and 9,12,15-octadecatrienol) of the secretion collected from artificially reared *B. magnus*, that originated from Scotland. Czech *B. lucorum* males produced these two compounds, too, but both in small amounts (0.8% and 1.3%, respectively).

B. lucorum is the most common species of the "*lucorum* species group" in the localities where we collected our specimens. This agrees with the data in the literature. *B. cryptarum* and *B. magnus* are rare in the Czech Republic as we have not found any individuals that belong to these species. Although the males we collected were slightly variable in colour, they all belonged to one "chemical form", similar to the Scandinavian blonde form of this species or more simply *B. lucorum*. It is apparent from our results and the literature that the composition of the labial gland secretion can be used to determine species in cases where morphological traits fail or are difficult to use.

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