Analysis of the Labial Gland Secretions of the Male Bumblebee *Bombus griseocollis* (Hymenoptera: Apidae)

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The labial gland secretions from males of the bumblebee *Bombus* (*Separatobombus*) *griseocollis* De Geer, a bumblebee exhibiting perching behaviour, were analysed by gas chromatography/mass spectrometry (GC/MS) in the electron impact and positive ion chemical ionization mode. The major compound of the complex mixture of alkenols, acetates, hydrocarbons, wax type esters and steroids is tetradecyl acetate, considerable amounts of hexadecyl, geranylinaloyl, geranylgeranyl, docosyl, tetracosenyl and hexacosenyl acetate were also found. 1,3-Tetradecanediol diacetate, detected as a minor component, has not yet been identified in male bumblebee labial gland secretions. Besides small amounts of primary alcohols (tetradecanol and hexadecanol) the tertiary alcohol geranylinalool (3,7,11,15-tetramethyl-hexadeca-1,6,10,14-tetraene-3-ol) was also present. The primary alcohols were also present as esters of butanoic, dodecanoic, tetradecanoic, and hexadecanoic acid. Besides the usual mixture of un- and mono-unsaturated straight chain hydrocarbons, the labial gland contains the isoprenoid hydrocarbons β -springene [(6*E*,10*E*)-7,11,15-trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene] and two isomers of α -springene [(3*Z*,6*E*,10*E*)- and (3*E*,6*E*,10*E*)-3,7,11,15-tetramethyl-hexadeca-1,3,6,10,14-pentaene]. The close relationship in chemical composition in male bumblebees with perching and flight pass behaviour is discussed.

Key words: *Bombus griseocollis*, Geranylinalool, Springene, 1,3-Tetradecanediol Diacetate

Introduction

According to their premating behaviour, male bumblebees can be classified into three main groups (Schremmer, 1972; Lloyd, 1981; Bergman, 1997; Hovorka *et al.*, 1998), as species exhibiting 1) *patrolling behaviour*, in which males establish flight paths with scent marks, 2) *perching behaviour*, in which males wait individually at prominent places, and dart passing queens or other moving objects, and 3) *nest entrance waiting behaviour*, in which males, often in groups, wait for emerging queens right at the nest entrance.

The *patrolling behaviour* is the most common type of premating behaviour among the bumblebee and cockoo-bumblebee species. Since Frank (1941) described this flight path activity of male bumblebees in detail, there was a growing interest in this peculiar behaviour of male bumblebees: a long-lasting, energy consuming daily flight activity (Bertsch, 1984) along fixed routes marked by scent produced in the cephalic part of the labial glands (Kullenberg *et al.*, 1973). The *perching behaviour*

and the *nest entrance waiting behaviour* are less common, less investigated and consequently not well understood.

Males of *Bombus griseocollis* belong to the group of large-eyed males exhibiting the perching behaviour. Bumblebees with this type of premating strategy occur world-wide, e.g. *Bombus mendax* and *B. confusus* in Europe, *B. asiaticus*, *B. rufofaciatus* and *B. kashmirensis* in Asia and *B. nevadensis* and *B. griseocollis* in North America. In these species, scent marking of their perching site was observed (Haas, 1949; Alcock and Alcock, 1983; Williams, 1991). Only *B. confusus* males were claimed to be entirely optically orientated in searching for mates without use of male pheromones (Schremmer, 1972). Therefore this species provided an example of a bumblebee species not using scent secretions in premating behaviour (Morse, 1982; Free, 1987). However Hovorka *et al.* (1998) found recently that *B. confusus* has a fully developed cephalic part of the labial gland. The secretion of this gland is used in scent marking

the perching sites (Kindl *et al.*, 1999). Only labial gland secretions from a few species of North American male bumblebees were investigated (Bergström *et al.*, 1996; Bertsch *et al.*, 2004) with no information on labial gland secretions of the subgenus *Separatobombus* (restricted to North America). Therefore we investigated the labial glands of *B. (Separatobombus) griseocollis*, a bumblebee with large-eyed males which scent mark perch sites in shady places in late summer (Alcock and Alcock, 1983).

Materials and Methods

Materials

Males of *B. (Separatobombus) griseocollis* De Geer were collected in late August 2003 in the area of Medford (Oregon, USA), where the species is abundant. The border of the wings of all males used for gland preparation was smooth, indicating young and active males. Old males can easily be rejected by their frayed wings. The males were then transported alive to the laboratory. In order to get $\pm$ filled glands, they were fed honey solution for a few days before they were frozen after a short flight activity early in the morning. The cephalic part of the labial glands was dissected from the head of males in frozen condition and placed in vials (glands from 5 males per vial) containing 0.2 ml pentane.

GC/MS

A Finnigan MAT TSQ700 gas chromatograph/tandem mass spectrometer was employed. Gas chromatography was carried out on a Hewlett Packard Ultra 1 column (50 m, 0.2 mm i.d., 0.11 μ m film thickness) in the splitless mode with helium as carrier gas at an inlet pressure of 300 kPa. Initial temperature of 120 °C was held for 1 min, then increased at 8°/min to 280 °C, at 3°/min to 310 °C and at 1°/min to 320 °C. This temperature was held for 10 min. Mass spectrometer conditions were: interface temperature, 300 °C; source temperature, 130 °C; electron energy, 70 eV; emission current, 0.2 mA; and electron multiplier, 1400 V. In the positive ion chemical ionisation mode ammonia CI gas pressure was 70 Pa.

Compounds were identified by comparing their mass spectra with those of the NIST '02 Library (National Institute of Standards and Technology, USA) and coinjection with commercially available standards.

Results

The labial glands contain a mixture of acyclic diterpenes (alcohols, acetates and hydrocarbons) and various straight-chain fatty acid derivatives (alcohols, esters, and both saturated and unsaturated hydrocarbons with 21 to 31 carbon atoms in chain length). A typical chromatogram for the labial

Fig. 1. Gas chromatogram of the labial gland secretion of *Bombus (Separatobombus) griseocollis* males up to 26 min retention time.

gland secretions of *B. (Separatobombus) griseocollis* is given in Fig. 1 and the compounds are summarised in Table I. The major compound was tetradecyl acetate (Fig. 1, peak 3) and considerable amounts of hexadecyl acetate (peak 10) and geranylgeranyl acetate (peak 15; two isomers: shoulder at 17:27 min, main peak at 17:36 min) were detected. Minor amounts of dodecyl acetate (peak 1), geranyllinaloyl acetate (peak 14), docosyl acetate (peak 20), tetracosenyl acetate (peak 28), and hexacosyl acetate (peak 34) complete the pattern of acetates found. Small amounts of tetradecanol

(peak 2), hexadecanol (peak 4) and geranyllinalool (peak 9) were also identified.

Besides the acetates 1,3-tetradecanediol diacetate was found (peak 11). In the positive CI-spectrum the ions $[M + H]^+$ (m/z 315), $[M + NH_4]^+$ (m/z 332), $[M + H - CH_3COOH]^+$ (m/z 255) and $[M + NH_4 - CH_3COOH]^+$ (m/z 272) were observed. Base peak in the EI-spectrum (Fig. 2) was $[CH_3CO]^+$ (m/z 59). The $[M]^+$ ion (m/z 314) could not be detected. Subsequent fragmentation of CH_3COOH resulted in fragments with m/z 254 and m/z 194. Characteristic for 1,3-diol diacetates

Table I. Compounds of the labial glands of *B. (Separatobombus) griseocollis* males and structural evidence.

Compound	No	RT ^a	M ^{+-b}	Diagnostic mass spectral fragments (m/z) ^c
Dodecyl acetate	1	8:53	228	43 , 55 , 61, M-60 = 168
Tetradecanol	2	9:50	214	41, 43, 55 , 69, 83 M-18 = 196
Tetradecyl acetate	3	12:06	256	43 , 55 , 61, M-60 = 196
Hexadecanol	4	12:37	242	41, 43, 55 , 69, 83, M-18 = 224
7,11,15-Trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene	5	13:08	272	41, 55, 69 , 81
(3Z,6E,10E)-3,7,11,15-Tetramethyl-hexadeca-1,3,6,10,14-pentaene	6	13:30	272	41, 55, 69 , 81
(3E,6E,10E)-3,7,11,15-Tetramethyl-hexadeca-1,3,6,10,14-pentaene	7	13:41	272	41, 55, 69 , 81
Tetradecyl butyrate	8	13:54	284	^c [71, 89; 196]
3,7,11,15-Tetramethyl-hexadeca-1,6,10,14-tetraene-3-ol	9	13:58	290	41, 55, 69 , 81
Hexadecyl acetate	10	14:11	284	43 , 55, 61, M-60 = 224
1,3-Tetradecanediol diacetate	11	14:37	314	43 , 55, 61, M-60 = 254, M-60-60 = 194
Heneicosane	12	15:26	296	43, 57 , 71, 85
Octadecenyl acetate	13	16:11	310	43 , 61, M-60 = 250
3,7,11,15-Tetramethyl-hexadeca-1,6,10,14-tetraen-3-yl acetate	14	17:09	332	41, 43, 69, 81, 93, 147, 161, 203
3,7,11,15-Tetramethyl-hexadeca-2,6,10,14-tetraenylacetate	15	17:36	332	41, 43, 69 , 81, 93, 263, 289, M-60 = 272
Tricosane	16	17:45	324	43, 57 , 71, 85
Tetradecyl octanoate	17	18:21	340	^c [127, 145; 196]
3,7,11,15-Tetramethyl-hexadeca-2,6,10,14-tetraenyl butyrate	18	19:16	360	69
Pentacosane	19	19:49	352	43, 57 , 71, 85
Docosyl acetate	20	20:43	368	43 , 55, 61, M-60 = 308
Hexacosane	21	20:47	366	43, 57 , 71, 85
Heptacosene	22	21:26	378	43, 55 , 69, 83
Heptacosene	23	21:33	378	43, 55 , 69, 83
Heptacosene	24	21:40	378	43, 55 , 69, 83
Heptacosane	25	21:46	380	43, 57 , 71, 85
3,7,11,15-Tetramethyl-hexadeca-2,6,10,14-tetraenyl hexanoate	26	22:04	388	43, 55, 69 , 81, 93
Tetradecyl dodecanoate	27	22:17	396	^c [183, 201; 196]
Tetracosenyl acetate	28	22:43	396	43 , 55, 61, M-60 = 336
Nonacosene	29	23:29	406	43, 55, 57 , 69, 83
Nonacosene	30	23:33	406	43, 55, 57 , 69, 83
Nonacosene	31	23:38	406	43, 55, 57 , 69, 83
Nonacosane	32	23:50	408	43, 57 , 71, 85
Tetradecyl tetradecanoate	33	24:23	424	^c [211, 229; 196]
Hexacosyl acetate	34	24:52	424	43 , M-60 = 364
Hentriacontane	35	26:05	436	43, 57 , 71, 85
Tetradecyl hexadecanoate	36	26:42	452	^c [209, 227; 196]
Ergosta-5,24-dien-3 β -ol	37	27:15	398	55 , 314
24-Ethyl-5-cholesten-3 β -ol (Sitosterol)	38	28:32	414	57 , 414
24-Ethylidene-5-cholesten-3 β -ol (Fucosterol)	39	28:41	412	55 , 314

^a Retention time.

^b Molecular Ion.

^c Ester (acylium ion of acid, protonated acid; alcohol M-18).

Fig. 2. EI-mass spectra of β -springene, α -springene and 1,3-tetradecanediol diacetate.

is an abundant α -cleavage peak containing both acetate units (m/z 159) as observed in 1,3-hexanediol diacetate (Ross *et al.*, 2001). Further fragmentation of $[C1-C3]^+$ results in the ions $[C1-C3-CH_2CO]^+$ (m/z 117) and $[C1-C3-CH_3COOH]^+$ (m/z 99).

Compared to most other investigated male bumblebee labial glands, the proportion of hydrocarbons and esters was low. Only few wax type esters were found in the chromatogram (see Table I). Besides the usual compounds octanoic, dodecanoic, tetradecanoic and hexadecanoic acid esters of tetradecanol, the identification of tetradecyl butyrate (peak 8) is unusual. In the mass spectrum of tetradecyl butyrate the ion of m/z 89 is the protonated butanoic (= butyric) acid, the ion of m/z 71 can be attributed to the acylium ion of this acid and the ion of m/z 196 corresponds to tetradecanol [M-18]. The characteristic MS fragment ions of the alcohol and the acid part of these esters (Pepe *et al.*, 1993) are good tools to detect small amounts of alcohols or acids, respectively, which are normally difficult to detect and identify in gas chromatograms.

Typical GCs also contain three diterpenes, isomers of springene. Their molecular ion has m/z 272. Their mass spectra (Fig. 2) are identical with those of β -springene [(6*E*,10*E*)-7,11,15-trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene, peak 5] and α -springene (peaks 6 and 7) (NIST'02 Library). The relative retention times of β -springene, (3*Z*,6*E*,10*E*)-3,7,11,15-tetramethyl-hexadeca-1,3,6,10,14-pentaene and α -springene [(3*E*,6*E*,10*E*)-3,7,11,15-tetramethyl-hexadeca-1,3,6,10,14-pentaene] are as reported by Burger *et al.*, 1981. As expected the mass spectra of (3*Z*,6*E*,10*E*)- and (3*E*,6*E*,10*E*)-3,7,11,15-tetramethyl-hexadeca-1,3,6,10,14-pentaene are nearly identical.

The tertiary alcohol geranyllinalool can easily be dehydrated resulting in the springene isomers. (3*E*,6*E*,10*E*)-3,7,11,15-tetramethyl-hexadeca-1,3,6,10,14-pentaene might also be formed from geranylgeraniol but dehydration of the primary alcohol is difficult. Besides the springenes, low amounts of geranyllinalool and geranyllinaloyl acetate could also be observed. Geranylgeraniol was not found in the chromatogram because esterification of geranylgeraniol is favoured, though the acetate, butyrate and hexanoate of geranylgeraniol were formed.

Discussion

Alcohols, acetates and wax esters

Of all the male bumblebees investigated, the secretions of *B. (Separatobombus) griseocollis* are most similar to the secretions of *B. (Alpinobombus) balteatus* (Svensson and Bergström, 1979), which also has tetradecyl acetate as main component and also contains esters of butanoic acid, currently the only evidence of butyrates in bumblebees. As *B. (Alpinobombus) balteatus* belongs to a different subgenus of *Bombus* and has males which scent mark a long lasting flight-path activity (Svensson, 1979), this is strong evidence that there is no fundamental difference in scent composition that defines premating strategy. The observations of Alcock and Alcock (1983) on *B. (Separatobombus) griseocollis* and Kindl *et al.* (1999) on *B. (Confusibombus) confusus* that no activity of virgin females (gynes) was detected in the vicinity of the perches and the scent marked surroundings of these perches make it very probable that, contrary to some theories, the scent marks are not signals for male-female interactions, but are used in male-male communication.

The labial glands of most bumblebee males with the patrolling premating behaviour contain a pattern of straight chain primary alcohols (C12–C26). The corresponding acetates are often only found in minor amounts or as traces, sometimes they are completely absent and occur only as a result of the ageing process of the prepared glands. The secretions of male bumblebees with the perching premating behaviour differ in the occurrence of acetates as the main components. In *B. (Confusibombus) confusus* Z-9-octadecenyl acetate is the main component, accompanied by geranylcitronellol (3,7,11,15-tetramethyl-6,10,14-hexadecatrien-1-ol) as a secondary major component, in *B. (Separatobombus) griseocollis* tetradecyl acetate is the main component with geranylgeranyl acetate (3,7,11,15-tetramethyl-2,6,10,14-hexadecatetraenyl acetate) as the secondary major compound. A mixture of substances with high and lower volatility is characteristic for the scent producing glands in male bumblebees. Bergman and Bergström (1997) detected the main component, farnesol, of the labial glands of *B. (Pyrobombus) pratorum* in headspace samples from marked leaves, but could not detect geranylgeranyl acetate, also produced by the glands, in the headspace samples. Kindl *et al.* (1999) could show that gera-

nylcitronellyl acetate, though present in the labial glands of *B. (Confusibombus) confusus* only in minor amounts, can be detected in headspace samples of the male-marked perch (dry flower head of *Centaurea stoebe*). It is likely, that these less volatile compounds remain detectable until the next day, helping the bumblebees to find and reconstruct the previous day's activity. Evaporation of alcohols and acetates is a first-order process (release rate is proportional to the amount of pheromone present) with long half-lives (Butler and McDonough, 1981; McDonough *et al.*, 1989) strongly depending on size, weight and polarity of the compound molecule.

Dialkenols and diesters

Until now only compounds with one functional group have been identified in labial gland secretions of male bumblebees; the detection of 1,3-tetradecanediol diacetate is unique. 1,16- and 1,15-hexadecanediol diacetate have been detected in the abdominal defense glands of the butterfly *Agraulis vanillae* (Nymphalidae: Heliconiinae) by Ross *et al.* (2001). In mammalian skin surface lipids (a mixture of lipids from the epidermis and the sebaceous glands) diester waxes have been identified consisting of 1,2-alkanediols in which both hydroxyl groups are esterified to unsubstituted fatty acids (Downing, 1976). 2,3-Alkanediol (uropygiol) and 2,3-alkanediol diesters have been identified in the uropygial glands of hens (Haahti and Fales, 1967), turkeys and pheasants (Jacob, 1976). The identification of 1,3-alkanediols is restricted to plant epidermis waxes (Vermeer *et al.*, 2003).

Isoprenoid hydrocarbons

In labial gland secretions of *B. (Pyrobombus) pratorum* two isomers of farnesene (3,7,11-trimethyl-2,6,10-dodecatrien) could be identified, the only isoprenoid hydrocarbons detected in bumblebee labial glands so far (Valterová and Urbanová, 1997). α - and β -springene [(3*E*,6*E*,10*E*)-3,7,11,15-tetramethyl-hexadeca-1,3,6,10,14-pentaene and 7,11,15-trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene, respectively] detected in the labial glands of *B. (Separatobombus) griseocollis* are newly identified isoprenoid hydrocarbons in bumblebee secretions. β -Springene has been previously isolated from a diversity of organisms, including the paracloacal gland of reptiles [the American alligator (Ibrahim *et al.*, 1998) and the smooth-fronted caiman (Avery *et al.*, 1993)], the dorsal secretions of mammals [collared peccary (Waterhouse *et al.*, 1996), the white-lipped peccary (Waterhouse *et al.*, 2001) and springbok (Burger *et al.*, 1978, 1981)] and the Dufour glands of insects [Australian ant *Nothomyrmecia macrops* (Billen *et al.*, 1988), the Old World army ant *Aenictus rotundatus* (Oldham *et al.*, 1994), the Ectoparasitoid *Bracon hebetor* (Fukushima *et al.*, 1990; Howard *et al.*, 2003) and, as a trace component, the stingless bee *Nannotrigona testaceicornis* (Cruz-Lopez *et al.*, 2001)].

The closely related compound geranylinalool (3,7,11,15-tetramethyl-hexadeca-1,6,10,14-tetraene-3-ol) also present in small amounts in the labial glands of *B. (Separatobombus) griseocollis* has been identified as component of the labial glands of the bumblebees species *B. (Megabombus) distinguendus* (Appelgren *et al.*, 1991), up to now this was the only indication of a tertiary alcohol in male bumblebee labial glands (Valterová and Urbanová, 1997).

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