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Chemistry of the Cephalic Labial Gland Secretions of Male *Bombus morrisoni* and *B. rufocinctus*, Two North American Bumblebee Males with Perching Behavior

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Abstract The labial gland secretions from males of the North American bumblebees *Bombus morrisoni* Cresson and *B. rufocinctus* Cresson were analyzed by gas chromatography/mass spectrometry. In both species, 3,7,11,15-tetramethyl-2,6,10,14-hexadecatetraenyl acetate was found as the major compound of a complex mixture of alkenols, acetates, hydrocarbons, and wax-type esters. In addition to a mixture of saturated and mono-unsaturated straight chain hydrocarbons, the labial gland of both species contained the isoprenoid hydrocarbons (6*E*, 10*E*)-3,7,15-trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene [β-springene], three isomers of 3,7,11,15-tetramethyl-hexadeca-1,3,6,10,14-pentaene [α-springene], and two further unidentified cyclic diterpenes. In *B. morrisoni*, 3,7,11-trimethyl-2,6,10-dodecatrien-1-ol and 3,7,11,15-tetramethyl-6,10,14-hexadecatrien-1-ol were detected as characteristic alcohols, as well as small amounts of 9-hexadecenol and hexadecanol. Furthermore, a large peak of hexadecyl dodecanoate and minor amounts of 9-hexadecenyl, 9-octadecenyl, and eicosenyl 9-tetradecenoate were found as typical esters in this species. In *B. rufocinctus*, 9-hexadecenol, hexadecanol, and 9-octadecenol were present in considerable amounts, with their acetates and 9-tetradecenoic, tetradecanoic, hexadecanoic, and 11-octadecenoic acids. The chemical composition of cephalic labial glands in male bumblebees with perching behavior is discussed.

Keywords Hymenoptera · *Bombus morrisoni* · *Bombus rufocinctus* · Perching · Cephalic labial gland · Geranylgeranyl acetate · Springene · Gas chromatography · Mass spectrometry

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Introduction

Bumblebee species can be classified into three main groups according to their male premating behavior (Schremmer 1972; Lloyd 1981; Bergman 1997; Hovorka et al. 1998): (1) species exhibiting *patrolling behavior*, in which males establish flight paths with scent marks; (2) species showing *perching behavior*, in which males wait individually at prominent places and dart passing queens or other moving objects; and (3) species showing *nest entrance-waiting behavior*, in which males, often in groups, wait for emerging queens directly at the nest entrance. Whereas the *patrolling behavior*, the most common type of premating behavior among bumblebee and cuckoo-bumblebee species has been investigated in many species, the *perching behavior* and the *nest entrance-waiting behavior* are less common, less investigated, and consequently not well understood.

Males with perching behavior are easily recognized by their large bulging eyes. Bumblebees with this type of premating strategy occur worldwide, e.g., *Bombus mendax*, *B. confusus*, and *B. vorticosus* in Europe; *B. asiaticus*, *B. rufofaciatus*, and *B. kashmirensis* in Asia; and *B. nevaden-*

sis, *B. griseocollis*, *B. morrisoni*, and *B. rufocinctus* in North America. In many of these species, scent marking of the perching site has been observed (Haas 1949; Alcock and Alcock 1983; O'Neill et al. 1991; Williams 1991; Kindl et al. 1999). Male labial gland secretions of only a few species with perching behavior have been investigated chemically (Hovorka et al. 1998; Bertsch et al. 2004; Rasmont et al. 2005). In this paper, we investigated the cephalic labial glands of males of the North American bumblebees *B. morrisoni* and *B. rufocinctus*.

Methods and Materials

Materials Males of *B. morrisoni* Cresson and *B. rufocinctus* Cresson were collected at 40°22′41″ N, 120°19′42″ W near Litchfield (about 30 km east of Susanville, CA, USA) where these species are abundant. Males of both species are low in numbers, shy, and fast in flight. Ten individuals of each species were caught when they were feeding at *Chrysothamnus* in late afternoon. Individual gland contents may be variable and change quantitatively and qualitatively (a) during a lifetime (see Sobotnik et al. 2008 for *B. terrestris*) and (b) during daytime (when secretions are used for scent marking in the morning, glands may be depleted later in the day; see Bergman 1997 for *B. lapidarius*). In our field collections, old males were recognized by their frayed wings and were discarded. The borders of the wings of all males used for gland preparation were smooth, indicating active males. Males were transported alive to the laboratory and fed with a honey solution for 2 days in flight cages so that they could refill their glands. Then, they were frozen after a short flight activity very early in the morning. The cephalic part of the labial glands was dissected from the head of frozen males and placed in vials (glands from five males per vial) containing 0.2-ml pentane. As the aim of this investigation was not to study the variability of gland content but to elucidate the complexity of a composition of bumblebee male labial glands, we pooled the glands of five males into a single vial. This method allowed us to detect even minor compounds present in the glands.

Gas Chromatography/Mass Spectrometry A Finnigan MAT TSQ700 gas chromatograph (GC)/tandem mass spectrometer (MS) was employed. GC was carried out on a Hewlett-Packard Ultra 1 column (50 m, 0.2 mm i.d., 0.11 μm film thickness) in a splitless mode with helium as carrier gas at an inlet pressure of 300 kPa. The split valve was opened for 1 min. Initial temperature of 120°C was held for 1 min, then increased at 8°C/min to 280°C, at 3°C/min to 310°C, and at 1°C/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 1,400 V. When using the positive ion chemical ionization (CI) 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 by retention times and molecular ions from CI spectra. To determine the position of double bonds, derivatization with dimethyl disulfide was used as described by Buser et al. (1983).

Results

The labial glands of both *B. morrisoni* and *B. rufocinctus* males contained a mixture of acyclic diterpenes (alcohols, acetates, and hydrocarbons), two cyclic terpenes, and various straight-chain fatty acid derivates (alcohols, esters, and both saturated and unsaturated hydrocarbons with C21–C31). Typical chromatograms of the male cephalic labial gland secretions of *B. rufocinctus* and *B. morrisoni* are shown in Fig. 1. The compounds are summarized in Table 1.

In labial glands of male *B. morrisoni*, the major compound was 3,7,11,15-tetramethyl-2,6,10,14-hexadecatetraenyl acetate (Fig. 1, 82% of total peak area, peak 20). In addition, considerable amounts of (6*E*, 10*E*)-3,7,15-trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene (peak 6), 3,7,11,15-tetramethyl-hexadeca-1,3,6,10,14-pentaene (peak 9), 3,7,11,15-tetramethyl-6,10,14-hexadecatrien-1-ol (peak 18), and hexadecyl dodecanoate (peak 30) were detected. Traces of 3,7,11-trimethyl-2,6,10-dodecatrien-1-ol (peak 3), two more isomers of 3,7,11,15-tetramethyl-hexadeca-1,3,6,10,14-pentaene (peak 7 and 11), two unidentified cyclic diterpenes (peak 13a and 14), 9-hexadecenyl 9-tetradecenoate (peak 31), 9-octadecenyl 9-tetradecenoate (peak 33), and eicosenyl 9-tetradecenoate (peak 34) complete the pattern of substances found. Small amounts of 9-hexadecenol (peak 4) and hexadecanol (peak 5) were identified. The peak area of hexadecyl dodecanoate (30) with about 4.5% of the total peak area shows that considerable amounts of hexadecanol may be used by this species to form esters.

In labial glands of male *B. rufocinctus*, the major compound was 3,7,11,15-tetramethyl-2,6,10,14-hexadecatetraenyl acetate (Fig. 1, 79% of total peak area, peak 20). Furthermore, considerable amounts of 9-hexadecenol (peak 4), (6*E*, 10*E*)-3,7,15-trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene (peak 6), and 3,7,11,15-tetramethyl-hexadeca-1,3,6,10,14-pentaene (peak 9) were detected. Minor amounts of two more isomers of 3,7,11,15-tetramethyl-hexadeca-1,3,6,10,14-pentaene (peak 7 and 11) and 3,7,11,15-tetramethyl-6,10,14-hexadecatrien-1-ol (peak 18)

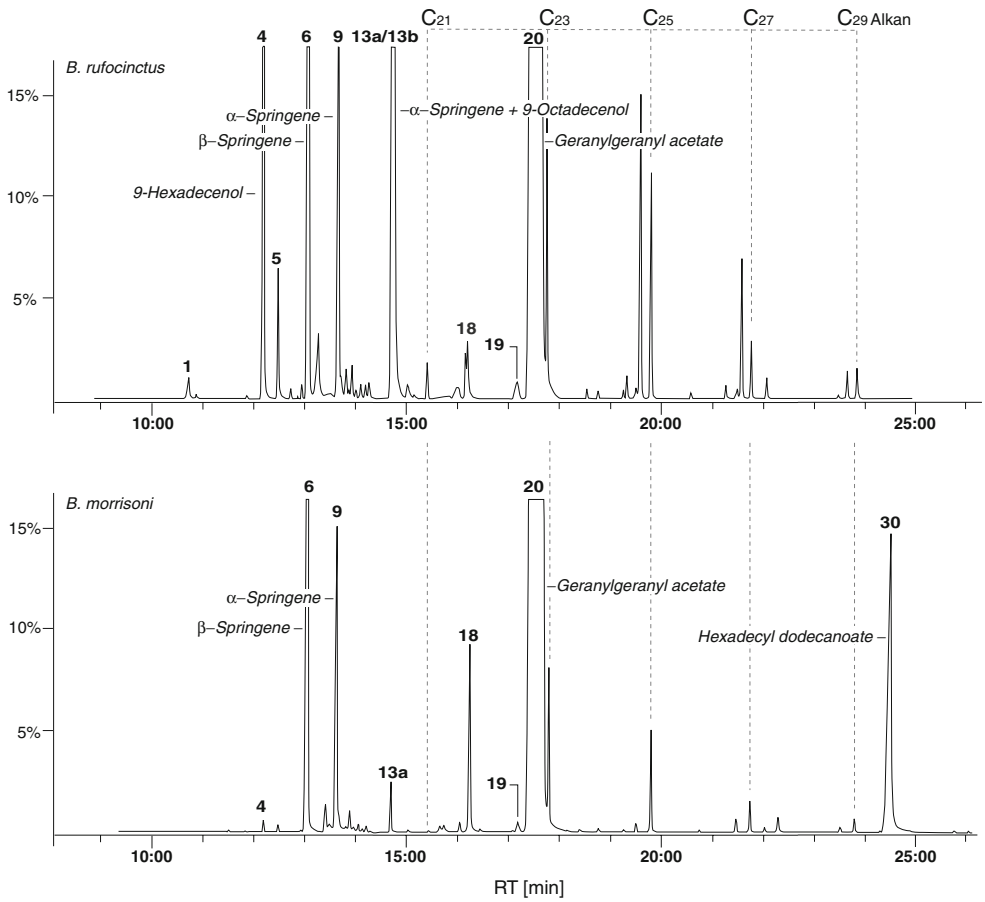

Fig. 1 Total ion chromatograms of the pentane extract of the cephalic labial gland from *B. morrisoni* and *B. rufocinctus*. *Numbers* correspond to the numbers in the peak list (Table 1). Some compounds listed in Table 1 were present in quantities too low to be visible in these chromatograms

were found. 9-Octadecenol (peak 13b) was also present in considerable amounts co-eluting with the unidentified cyclic diterpene detected in the GC of *B. morrisoni* (peak 13a). Small amounts of an isomer of this unidentified cyclic diterpene (peak 14), 9-tetradecenoic acid (peak 1), tetradecanoic acid (peak 2), hexadecanoic acid (peak 8), 9-octadecenoic acid (peak 17), 9-hexadecenyl acetate (peak 10), hexadecyl acetate (peak 12), and 9-octadecenyl acetate (peak 17) were detected.

Compared to the chemistry of labial glands of other male bumblebee species, the proportion of hydrocarbons and esters was low in the species studied here. Only few wax-type esters were found (Table 1). Typical GCs of the species studied here also contained four diterpenes, i.e., isomers of springene. Their molecular ion was *m*/*z*=272. Their mass spectra were identical with those of (6*E*, 10*E*)-7,11,15-trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene [β-springene] (peak 6) and α-springene (peaks 7, 9, 10). The relative retention times of (6*E*, 10*E*)-7,11,15-trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene [β-springene], (3*Z*,6*E*,10*E*)-3,7,11,15-tetramethyl-hexadeca-1,3,6,10,14-pentaene, and (3*E*,6*E*,10*E*)-3,7,11,15-tetramethyl-hexadeca-1,3,6,10,14-pentaene [the α-springenes] were as reported by Burger et al. (1981). As expected, the mass spectra of the three isomers of 3,7,11,15-tetramethyl-hexadeca-1,3,6,10,14-pentaene [α-springene] were nearly identical.

Peaks 13a and 14 in the secretions could not be identified. The mass spectra of both compounds exhibited molecular ions at *m*/*z*=272, indicative of a hydrocarbon of the molecular formula $C_{20}H_{32}$. The molecular mass was confirmed by CI (NH_3) and the pseudo molecular ion *m*/*z* 273 ($[M+H]^{+\cdot}$), whereas the pseudo molecular ion *m*/*z* 290 ($[M+NH_4]^+$) could not be detected. The mass spectrum of peak 13a was characterized by the following fragment ion *m*/*z* (%) values: 272 (19, M^+), 135 (100), 120 (11), 119 (10), 107 (90), 105 (36), 93 (43), 91 (33), 79 (11), 77 (13), 69 (46), 53 (13), 41 (33). The mass spectrum of peak 14 was nearly identical. The most intense fragment ions *m*/*z* 135 ($[C_{10}H_{15}]^+$) and *m*/*z* 107 ($[C_8H_{11}]^+$) were observed in cyclic and polycyclic hydrocarbons indicating peak 13a and peak 14 to be isomers of a cyclic terpene.

Discussion

The labial glands of most bumblebee males with the *patrolling* premating behavior contain a pattern of straight chain primary alcohols [C12–C26] (Bergström et al. 1981,

Table 1 Compounds of the cephalic labial glands of males of *B. rufocinctus* and *B. morrisoni* and structural evidence

No.	Compound	RT	RI	*B. rufocinctus* [% of total peak area]	*B. morrisoni* [% of total peak area]	$M^{+\bullet}$	Diagnostic mass spectral fragments (*m*/*z*)
1	9-Tetradecenoic acid	10:48		0.11	–	226	**55**, 69, 81, 166, 208, {117, 203, $M^{+\bullet}$ 320}
2	Tetradecanoic acid	10:50	1,720	0.67	–	228	60, **73**, 129
3	3,7,11-Trimethyldodecatrien-1-ol	12:00	1,744	Trace	Trace	222	**69**, 93, 107, 133, 161, 204
4	9-Hexadecenol	12:18	1,836	0.54	Trace	240	M-18=222, {145, 189, $M^{+\bullet}$ 348}
5	Hexadecanol	12:35	1,859	0.36	Trace	242	M-18=224
6	7,11,15-Trimethyl-3-methylene-1, 6,10,14-hexadecatetraene	13:13	1,906	3.79	6.67	272	41, 55, **69**, 133, 161, 187, 257
7	3,7,11,15-Tetramethyl-hexadeca-1, 3,6,10,14-pentaene	13:33	1,935	0.13	Trace	272	41, 55, **69**, 107, 119, 135,
8	Hexadecanoic acid	13:38	1,950	0.10	–	256	60, **73**, 129, 256
9	3,7,11,15-Tetramethyl-hexadeca-1, 3,6,10,14-pentaene	13:46	1,955	1.45	2.40	272	41, 55, **69**, 107, 119, 134, 191, 257
10	9-Hexadecenyl acetate	13:55	1,967	0.06	–	282	**43**, 55, 61, M-60=222
11	3,7,11,15-Tetramethyl-hexadeca-1, 3,6,10,14-pentaene	14:02	1,974	0.08	Trace	272	41, 55, **69**, 107, 119, 133, 187
12	Hexadecyl acetate	14:17	2,009	0.03	–	284	**43**, 55, 61, M-60=224
13 a	Cyclic terpene ? ($C_{20}H_{32}$)	14:48	2,039	~0.7	0.25	272	55, 69, 107, **135**
13 b	9-Octadecenol	14:52	2,045	~6.5	–	268	41, **55**, 67, 82 M-18=250, {145, 299, $M^{+\bullet}$ 444}
14	Cyclic terpene ? ($C_{20}H_{32}$)	15:07	2,068	0.04	Trace	272	55, 69, **107**, 135
15	Heneicosane	15:31	2,100	0.08	–	296	41, 43, **57**, 71, 85
16	9-Octadecenyl acetate	16:15	2,152	0.10	–	310	**43**, 55, 61, M-60=250
17	11-Octadecenoic acid	16:17	2,162	0.20	–	282	**55**, 69, 83, 125, 222, {145, 231, $M^{+\bullet}$ 376}
18	3,7,11,15-Tetramethyl-2,6,10, 14-hexadecatetraen-1-ol	16:20	2,169	0.10	1.17	290	41, 55, **69**, 81, 93, 95, 265, 271
19	3,7,11,15-Tetramethyl-6,10, 14-hexadecatrienyl acetate	17:17	2,250	0.15	0.02	334	41, 43, **69**, 81, 93, 265, 291, M-60=274
20	3,7,11,15-Tetramethyl-2,6,10, 14-hexadecatetraenyl acetate	17:45	2,278	78.77	82.18	332	41, 43, **69**, 81, 93, 263, 289, M-60=272
21	Tricosane	17:51	2,300	0.81	0.67	324	41, 43, **57**, 71, 85
22	3,7,11,15-Tetramethyl-2,6,10, 14-hexadecatetraenyl butyrate	19:21	2,445	0.02	–	360	**69**, 81, 93, M-88=272
23	?,?-Pentacosadiene	19:25	2,452	0.06	–	348	41, 43, **55**, 69, 83
24	7-Pentacosene	19:46	2,485	0.84	0.02	350	41, 43, **55**, 69, 83, {145, 299, $M^{+\bullet}$ 444}
25	Pentacosane	19:55	2,500	0.58	0.66	352	41, 43, **57**, 71, 85
26	7-Heptacosene	21:35	2,683	0.03	0.03	378	41, 43, **55**, 69, 83, {145, 327, $M^{+\bullet}$ 472}
27	Heptacosane	21:44	2,700	0.15	0.18	380	41, 43, **57**, 71, 85
28	3,7,11,15,19-Pentamethyl-2,6,10, 14,18-eicosapentaenyl acetate	22:02	2,729	0.05	–	400	**69**, 81, 93, M-60=340
29	Nonacosane	23:48	2,900	0.09	0.10	408	41, 43, **57**, 71, 85
30	Hexadecyl dodecanoate	24:39		–	4.51	424	[183, 201; 224][a]
31	9-Hexadecenyl 9-tetradecenoate	26:25		0.17	–	448	43, **55**, 69, [a][209, 227; 222]
32	Ergosta-5,24-dien-3β-ol	27:13		0.04	–	398	55, 69, **314**
33	9-Octadecenyl 9-tetradecenoate	28:53		1.15	–	476	[a][208, 227; 250]
34	Eicosenyl 9-tetradecenoate	31:30		0.65	–	504	[a][208, 227; 278]

RT retention time; *RI* retention index; $M^{+\bullet}$ molecular ion; ester: [a] [acylium ion of acid, protonated acid; alcohol M-18], DMDS adducts {xxx, xxx, $M^{+\bullet}$ xxx}

Valterová and Urbanová 1997). The corresponding acetates are often found only in minor amounts or as traces. Indeed, sometimes they are completely absent and occur only because of an aging process of the prepared glands. Our study and other previous studies show that the secretions of male bumblebees with the *perching* premating behavior differ from bumblebees with *patrolling* premating behavior in the occurrence of acetates as the leading components (Table 2).

In *B.* (*Cullumanobombus*) *rufocinctus*, 3,7,11,15-tetramethyl-2,6,10,14-hexadecatetraenyl acetate [geranylgeranyl acetate] and 9-octadecenol contributed 84% to the total peak area. In *B.* (*Separatobombus*) *morrisoni*, 3,7,11,15-tetramethyl-2,6,10,14-hexadecatetraenyl acetate [geranylgeranyl acetate] and 3,7,11,15-tetramethyl-2,6,10,14-hexadecatetraen-1-ol [geranylgeraniol] contributed 84% to the total peak area. In *B.* (*Separatobombus*) *griseocollis*, tetradecyl acetate and 3,7,11,15-tetramethyl-2,6,10,14-hexadecatetraenyl acetate [geranylgeranyl acetate] contributed 97% to the total peak area (Bertsch et al. 2004). In *B.* (*Confusibombus) confusus, (Z)*-9-octadecenyl acetate and 3,7,11,15-tetramethyl-6,10,14-hexadecatrien-1-ol [geranylcitronellol] contributed 77% to the total peak area (Hovorka et al. 1998), and in *B.* (*Sibiricobombus*) *vorticosus*, 3,7,11,15-tetramethyl-2,6,10,14-hexadecatetraenyl acetate [geranylgeranyl acetate] and 3,7,11,15-tetramethyl-2,6,10,14-hexadecatetraen-1-ol [geranylgeraniol] contributed 87% to the total peak area (Rasmont et al. 2005). In all labial gland secretions of males with perching behavior, two compounds dominate the marking secretions, with an acetate being the dominant compound.

A mixture of substances with high and lower volatility is characteristic of the scent 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 they could not detect 3,7,11,15-tetramethyl-2,6,10,14-hexadecatetraenyl acetate [geranylgeranyl acetate], also produced by the glands, in these samples. Kindl et al. (1999) showed that 3,7,11,15-tetramethyl-6,10,14-hexadecatrienyl acetate [geranylcitronellyl acetate], though present only in minor amounts in the labial glands of *B.* (*Confusibombus*) *confusus*, could be detected in headspace samples of the male-marked perch (dry flower head of *Centaurea stoebe*). O'Neill et al. (1991) studied marking behavior and labial glands of male *B. rufocinctus*. Their results suggest that males of this species mark leaves of *Symphoricarpus* and *Ribes* with at least three components of labial gland secretion. Even though these compounds were not identified, the retention times given for them indicate that they might be hexadecenol (O'Neill et al., Fig. 4, peak 12.31), octadecenol (peak 14.29), and 3,7,11,15-tetramethyl-2,6,10,14-hexadecatetraenyl acetate (peak 16.37). Less volatile compounds left on leaves may remain detectable until the next day, helping the bumblebees to find and reconstruct the activity, of the previous day. 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.

In labial gland secretions of *B.* (*Pyrobombus*) *pratorum*, two isomers of 3,7,11-trimethyl-2,6,10-dodecatriene [farnesene] were identified, the only isoprenoid hydrocarbons detected in bumblebee labial glands so far (Valterová et al. 1997). (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 [α- and β-springene, respectively] detected in the labial glands of *B.* (*Separatobombus*) *griseocollis* were newly identified isoprenoid hydrocarbons in bumble bee secretions (Bertsch et al. 2004). Meanwhile, these substances also have been detected in *B.* (*Sibiricobombus) vorticusus* (Rasmont et al. 2005), a male bumblebee with perching habit. (6*E*, 10*E*)-β-Springene has been isolated from a diversity of organisms, including the paracloacal gland of reptiles [the American alligator (Ibrahim

Table 2 Main compounds (% of peak area) of the cephalic labial glands of *Bombus* males with perching behavior

Compound	*B. rufocinctus*	B. morrisoni	*B. griseocollis*[a]	*B. confusus*[b]	B. vorticosus[c]	$M^{+\bullet}$
Tetradecyl acetate	–	–	82%	–	–	256
9-Octadecenol	5%	–	–	–	–	268
3,7,11,15-Tetramethyl-6,10, 14-hexadecatrien-1-ol	–	–	–	30%	–	292
3,7,11,15-Tetramethyl-2,6,10, 14-hexadecatetraen-1-ol	–	2%	–	–	13%	290
9-Octadecenyl acetate	–	–	–	47%	–	310
3,7,11,15-Tetramethyl-2,6,10, 14-hexadecatetraenyl acetate	79%	82%	15%	–	74%	332
Total	84%	84%	97%	77%	87%	

[a] (Bertsch et al. 2004), [b] (Hovorka et al. 1998), [c] (Rasmont et al. 2005)

et al. 1998) and the smooth-fronted caiman (Avery et al. 1993)], the dorsal secretions of mammals [collared peccary (Waterhouse et al. 1996), white-lipped peccary (Waterhouse et al. 2001), and springbok (Burger et al. 1978, 1981)], 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 *Habrocon hebetor* (Fukushima et al. 1990; Howard et al. 2003), and as a trace component in the stingless bee *Nannotrigona testaceicornis* (Cruz-Lopez et al. 2001)]. Additional isomers of α-springene described so far are 3,7,11,15-tetramethyl-hexadeca-1,4,6,10,14-pentaene (Waterhouse et al. 1996; *Tayassu tajacu*, Mammalia) and 2,6,11,15-tetramethyl hexadeca-2,6,8,10,14-pentaene (Burger et al. 1981; see Table 1; *Antidorcas marsupialis*, Mammalia, and Zhou et al. 2006; *Citrus grandis*, Spermatophyta).

Studies of the perching behavior of bumblebees have revealed that no activity of virgin females (gynes) is detected near perches that have been marked by males with labial glandular secretion. This effect of male labial glandular secretion in male–female interactions has been shown in *B.* (*Separatobombus*) *griseocollis* (Alcock and Alcock 1983), in *B.* (*Cullumanobombus*) *rufocinctus* (O'Neill et al. 1991), and in *B.* (*Confusibombus*) *confusus* (Kindl et al. 1999). The question whether male labial glandular secretions mark small territories or individual perches and thus, mediate male–male interactions, need to be addressed in future studies.

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