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Biosynthetic studies on marking pheromones of bumblebee males

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Summary. The biosynthetic pathway of the formation of males' marking pheromones of two bumblebee species, *Bombus lucorum* and *B. lapidarius*, was studied for the first time. After applications of deuterium labelled palmitic acid ([$^2H_{31}$]-hexadecanoic acid) into either the head capsules or abdomens the corresponding deuterium labelled pheromone components were detected in the labial gland (LG) extracts of males of both species. In *B. lucorum*, ethyl [$^2H_{29}$]-hexadec-9-enoate and ethyl [$^2H_{31}$]-hexadecanoate were identified. No deuterium-labelled ethyl tetradecenoate, that would correspond to shortening of the carbon chain, was detected. In *B. lapidarius*, [$^2H_{31}$]-hexadecan-1-ol and [$^2H_{29}$]-hexadec-9-en-1-ol were found in the LG. Furthermore, the deuterium labelled precursor was incorporated into triacylglycerols (TAG) of the fat bodies of males. *In vitro* incubation of the labial glands of *B. lucorum* males with deuterium- and ^{13}C-labelled palmitic acid lead to the formation of saturated ethyl esters only.

Key words. *Bombus lucorum* – *Bombus lapidarius* – biosynthesis – bumblebees – male marking pheromone – labial gland – fat body

Introduction

The marking pheromone of the bumblebee male is produced by the cephalic part of the male's labial pheromone gland (LG) (Kullenberg *et al.* 1970). During the premating behaviour, patrolling males scent-mark their territories to attract conspecific females for mating (Bergman 1997). Each bumblebee species produces a specific blend of compounds (see review Valterová & Urbanová 1997 and references therein). The gland secretions contain mostly two different structural classes. The first class is comprised of straight chain saturated and unsaturated hydrocarbons, alcohols, aldehydes, and esters. The second class contains terpenoids, mostly acyclic mono-, sesqui-, and diterpenic alcohols and their esters.

Very little is known about the biosynthesis of bumblebee LG components, although the biosynthesis has been discussed (Lanne *et al.* 1987). Based on structural analysis of compounds isolated from 22 bumblebee species, it was suggested that these compounds are produced from saturated fatty acids (FA) by the action of specific glandular desaturases. Despite a relatively large number of papers dealing with the biosynthesis of lepidopteran pheromones (Jurenka & Roelofs 1993 and references therein), there are no experimental data available on the biosynthetic pathways of pheromone formation in bumblebees.

In the present study, we focused on two bumblebee species – *Bombus lucorum* (Linnaeus, 1758) and *B. lapidarius* (Linnaeus, 1761). Both produce predominantly long-chain aliphatic compounds found in the LG secretion. *B. lucorum* produces ethyl esters of fatty acids, chiefly ethyl (*Z*)-tetradec-9-enoate (**1**) (53%) (Bergström *et al.* 1973; Urbanová *et al.* 2001), while main components of the marking pheromone of *B. lapidarius* males are hexadecan-1-ol (**2**, 31%) and (*Z*)-hexadec-9-en-1-ol (**3**, 52%) (Calam 1969). There are two potential pathways which could deliver these compounds: biosynthesis from common lipids in the body or *de novo* from acetate units (Mann 1994). It was anticipated that the pathways could be delineated by applying deuterium-labelled fatty acids to bumblebees and investigating their metabolism.

Material and methods

Insects

The males of bumblebee species *Bombus lapidarius* were collected during summer seasons 1999-2000 at several localities of the Czech Republic (Table 1). Living insects were transported to the laboratory. The males of *Bombus lucorum* were obtained from the laboratory colonies established using a mated queen from the previous year (1999 and 2002).

Chemicals

A stock solution of [$^2H_{31}$]-hexadecanoic acid (**4**, Aldrich, 40 mg in solvents dimethylsulfoxide:ethanol:water 7:2:1, 1 ml) was used for *in vivo* applications. [$^{13}C_{16}$]-Hexadecanoic acid (Isotec Inc. Miamisburg, Ohio, USA) and [$^2H_{31}$]-hexadecanoic acid for *in vitro* incubations were used in concentrations 1 µg/µl. [$^2H_5$]-Ethanol (Acros Organics, Belgium) was dissolved in phosphate buffer (pH 7.2, 0.1 µl in 1 µl buffer). Hexane for extractions was purchased from Merck. The phosphate buffer was mixed from 0.2 M KH_2PO_4 and 0.2 M Na_2HPO_4 (7:18). Dimethyl disulfide was obtained from Aldrich.

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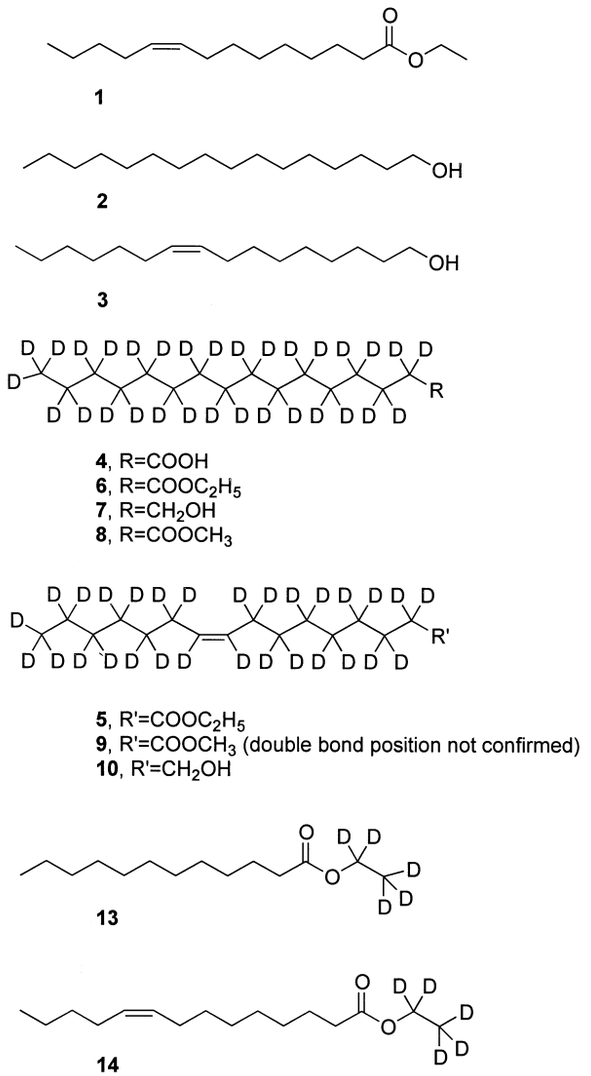

Fig. 1 Structural formulae

Applications, extraction

Solutions of the deuterium labelled [$^2H_{31}$]-hexadecanoic acid (1, 2 or 4 µl, *i.e.* 40, 80 or 160 µg of the acid **4**) were injected into the head capsules and into the abdomens of the bumblebee males. Labial glands and the peripheral fat bodies were dissected after 24 or 48 h of incubation. The dissected material was extracted with hexane (50 µl per gland or fat body). After shaking (15 minutes) and standing in the refrigerator (2 hours for glands, 24 h for fat bodies), the hexane extract was filtered and stored in a freezer prior to analysis. Each sample was analysed separately.

In vitro incubations were done with dissected labial glands. Phosphate buffer (5 µl) and a solution of the substrate (50 µg in 50 µl hexane) was added to the dissected gland in a vial and kept on a shaker at 24ºC in dark. The experiment with [$^2H_5$]-ethanol was done with 10 µl of the solution of the substrate in buffer (0.1 µl in 1 µl) to which hexane (50 µl) was added. The course of the formation of metabolites was followed by GC-MS in time intervals 1–13 days (for [$^2H_{31}$]-hexadecanoic acid, [$^2H_5$]-ethanol, and sodium [$^2H_3$]-acetate) and 1–42 days (for [$^{13}C_{16}$]-hexadecanoic acid), respectively. Control experiments without labial glands and without labelled substrates were done in parallel. For quantitative calculations, tricosane present in the labial gland secretion was used as internal standard.

Preparation of derivatives

Fractions of triacylglycerols (TAG) from the fat bodies extracts were obtained by preparative TLC (36 × 76 mm, Adsorbosil-Plus, Applied Science Lab., with 12% $CaSO_4$ added) by elution with light petroleum-diethylether (84:16). Detection was performed by spraying with Rhodamine 6G (Merck, 0.05% solution in ethanol) and UV light. After extraction, the TAG (R_F 0.54) were transesterified (Stránský & Jursík 1996) and the FAMEs analysed by GC-MS.

The positions of double bonds were determined from mass spectra of dimethyldisulfide (DMDS) adducts of unsaturated compounds. The DMDS adducts were prepared using a published procedure (Svatoš *et al.* 1999).

Chromatographic analyses

Material was analysed using a gas chromatograph with a splitless injector (200ºC) and a mass detector (Fisons MD 800) in electron impact ionisation mode. Two chromatographic columns with different separating properties were used, a DB-5 column (5% phenyl methyl silicone; 30 m × 0.25 mm, film thickness 0.25 µm, J & W Scientific) and a DB-WAX column (polyethylene glycol; 30 m × 0.25 mm, film thickness 0.25 µm, J & W Scientific). Helium (flow 0.55 ml/min at 50ºC) was used as carrier gas with both columns. A temperature gradient for the DB-5 column was applied: 70ºC (3 min), then 40ºC/min to 140ºC, then 2ºC/min to 240ºC and 5°C/min to 300ºC. The program for DB-WAX column was 50ºC (2 min), then 40ºC/min to 180ºC and 5ºC/min to 220ºC. For the identification of FA in fat bodies, the ECL values were calculated (Stránský *et al.* 1997).

Characterisation of the identified precursor, metabolites and their derivatives, by their mass spectra, m/z (%)

[$^2H_{31}$]-Hexadecanoic acid (**4**): $M^{+\cdot}$ 287 (22), 253 (3), 237 (14), 221 (5), 205 (12), 189 (9), 173 (12), 157 (9), 141 (36), 125 (14), 110 (16), 109 (15), 94 (26), 93 (24), 82 (36), 78 (72), 77 (84), 66 (75), 63 (71), 50 (100), 46 (98).

Ethyl [$^2H_{29}$]-(*Z*)-hexadec-9-enoate (**5**): $M^{+\cdot}$ 311 (1), 266 (22), 264 (21), 220 (19), 105 (10), 91 (97), 78 (95), 55 (75), 46 (100), 34 (27).

Ethyl [$^2H_{31}$]-hexadecanoate (**6**): $M^{+\cdot}$ 315 (11), 270 (10), 265 (12), 233 (6), 217 (5), 201 (6), 169 (22), 105 (62), 91 (100), 77 (18), 66 (23), 50 (36), 46 (27), 34 (9).

[$^2H_{31}$]-Hexadecan-1-ol (**7**): $M^{+\cdot}$ –HDO 254 (1), 253 (1), 178 (1), 150 (3), 142 (3), 108 (15), 96 (21), 94 (100), 78 (28), 62 (18), 50 (18), 46 (15), 34 (6).

Methyl [$^2H_{31}$]-hexadecanoate (**8**): $M^{+\cdot}$ 301 (12), 270 (5), 251 (10), 235 (2), 219 (3), 203 (4), 187 (6), 155 (6), 91 (41), 77 (100), 62 (20), 50 (24), 46 (20).

[$^2H_{29}$]-(*Z*)-Hexadec-9-en-1-ol (**10**): $M^{+\cdot}$–HDO 250 (1), 249 $M^{+\cdot}$–D_2O (11), 187 (3), 167 (4), 153 (8), 138 (10), 122 (29), 106 (72), 90 (83), 78 (38), 74 (78), 62 (100), 60 (73), 50 (34), 46 (92), 34 (24).

Ethyl [$^{13}C_{16}$]-hexadecanoate (**11**): $M^{+\cdot}$ 300 (4), 255 (4), 254 (5), 239 (1), 224 (2), 209 (3), 194 (2), 179 (1), 164 (10), 119 (5), 104 (60), 90 (100), 76 (24), 74 (23), 72 (24), 46 (63), 44 (63).

Methyl [$^{13}C_{16}$]-hexadecanoate (**12**): $M^{+\cdot}$ 286 (3), 255 (3), 240 (4), 210 (2), 195 (2), 180 (2), 150 (8), 135 (3), 105 (4), 104 (5), 90 (57), 76 (100), 59 (42), 46 (49), 44 (54).

[$^2H_5$]-Ethyl dodecanoate (**13**): $M^{+\cdot}$ 233 (5), 204 (3), 190 (8), 183 (11), 162 (10), 148 (6), 120 (6), 106 (49), 93 (100), 74 (27), 73 (23), 57 (25), 55 (47), 43 (45), 41 (49).

[$^2H_5$]-Ethyl tetradecanoate (**14**): $M^{+\cdot}$ 259 (8), 209 (15), 208 (26), 166 (24), 124 (12), 110 (12), 106 (76), 98 (33), 93 (100), 84 (53), 74 (40), 55 (15).

Estimation of the degree of incorporation and quantification

Quantification of components of the analysed samples was based on a calibration curve for ethyl hexadecanoate and hexadecanol. The ratio between native/deuterium-labelled compounds was

Table 1 Data regarding the bumblebee males

Species	Number of Males	Year of Collection	Region in the Czech Republic	Elevation [m]	Map Field Code
B. lucorum	12	1999[a]	South Moravia[a]	235	6765
B. lapidarius	2	1999	Czech Central Mountains	510	5548
B. lapidarius	1	1999	Central Bohemia	220	5852
B. lapidarius	11	2000[a]	South Moravia[a]	235	6765

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

Table 2 Analysis of FA composition of fat bodies (both free FA and TAG) compared with structure of LG components in *B. lucorum* and *B. lapidarius*. Relative % in native samples

Chain Length, Structural Type	*B. lapidarius*			*B. lucorum*		
	TAG[a]	Free FA[a]	LG	TAG[a]	Free FA[a]	LG[b]
14	4.4	3.4	0.3 (OH)	2.5	3.3	1.8
*Z*9-14	1.2	0	0	0.6	0	52.9 (1)
						2.5 (COOH)
16	23.1	20.2	31.0 (OH, **2**)	16.1	19.5	0.04
			0.1 (COOH)			3.6 (OH, **2**)
*Z*7-16	0	0	0	0.4	0	0.2
						1.6 (CHO)
*Z*9-16	40.9	36.3	52.0 (OH, **3**)	1.4	0	3.6
			2.9 (COOH)			
*Z*11-16	5.0	0	0	0.7	0	0.2
18	2.9	8.7	0	4.0	10.9	0.08
*Z*9-18	15.4	15.2	0	53.5	52.3	1.8
*Z*11-18	4.1	5.1	0	0	0	0
*Z*13-18	0	0	0	2.2	0	0

[a]Determined as methyl esters of fatty acids
[b]Ethyl esters of fatty acids if not stated otherwise (Urbanová *et al*. 2001)

determined from intensities of characteristic fragments in mass spectra. Fragments used for calculations were as follows: ethyl [$^2H_{29}$]-(*Z*)-hexadec-9-enoate (**5**), *m/z* 91, 105, 311; ethyl (*Z*)-hexadec-9-enoate, *m/z* 88, 101, 282; ethyl [$^2H_{31}$]-hexadecanoate (**6**), *m/z* 91, 105, 315; ethyl hexadecanoate, *m/z* 88, 101, 284; methyl [$^2H_{31}$]-hexadecanoate (**8**), *m/z* 77, 91, 301; methyl hexadecanoate, m/z 74, 87, 270; [$^2H_{29}$]-(*Z*)-hexadec-9-en-1-ol (**10**), *m/z* 46, 50, 62, 74, 78, 90, 106, 122, 138, 217, 249; (*Z*)-hexadec-9-en-1-ol (**3**), *m/z* 41, 43, 55, 67, 69, 81, 95, 109, 123, 194, 222; [$^2H_{31}$]-hexadecan-1-ol (**7**), *m/z* 46, 50, 62, 78, 94, 126, 142, 213; hexadecan-1-ol (**2**), *m/z* 41, 43, 55, 69, 83, 111, 125, 196.

The degree of incorporation in Tables 3 and 4 was calculated from the quantitative data based on the calibration and related to the applied dose of the precursor **4** in each particular case.

Results

Lanne and coworkers (Lanne *et al.* 1987) proposed that the long chain pheromone components originate from FA. There are two plausible sources of such FA: 1) *de novo* synthesis and desaturation in the LG; 2) sequestration of FA from TAG of fat bodies. However, the FA composition of TAG of fat bodies does not always agree with the composition of LG hexane extracts (Table 2). In *B. lapidarius* there is qualitative and quantitative correlation of long chain compounds found in LG with the corresponding tentative biosynthetic precursors in TAG. This contrasts with *B. lucorum* in which only marginal correlation can be deduced. The major constituent of the LG extract is ethyl (*Z*)-tetradec-9-enoate (**1**) but tetradec-9-enoic acid, the tentative biosynthetic precursor, was found in TAG of fat bodies in small amounts only. From this point a more detailed study using isotopically labelled FA should be performed.

After application of [$^2H_{31}$]-hexadecanoic acid (**4**) to *B. lucorum,* two deuterium labelled metabolites were detected in the LG of all treated males, ethyl [$^2H_{29}$]-hexadec-9-enoate (**5**) and ethyl [$^2H_{31}$]-hexadecanoate (**6**). Applications of the two higher doses (*i.e.* 80 and 160 µg of fatty acid, respectively) resulted in samples where the two labelled esters were unambiguously identified. Low dose (40 µg) applications lead to samples where the deuterated metabolites were still detectable on the basis of typical spectral fragments. Fragments $C_4H_5[^2H_3]O_2$ and $C_5H_5[^2H_4]O_2$ observed in mass spectra (MS) at *m/z* 91 and *m/z* 105, respectively, correspond to the key fragments in the native ethyl esters (*m/z* 88 and 101). Molecular ions *m/z* 315 and *m/z* 311 correspond to the ethyl esters **6** and **5**, respectively (Fig. 2). A loss of the ethoxy group was observed in the mass spectra of both metabolites (at *m/z* 270 in **5** and *m/z* 266 in **6**, respectively). The position of the carbon-carbon double bond in the metabolite **5** was deduced from the MS of a dimethyl disulfide (DMDS) adduct and shown to be located in position 9 (fragments *m/z* 159 and *m/z* 246). Traces of $\triangle^7$- and $\triangle^{11}$-isomers were also detected (fragments *m/z* 191 and 214 for $\triangle^7$; *m/z* 127 and 278 for $\triangle^{11}$).

In both injections (into head capsules and into abdomens of *B. lucorum* males) both deuterium labelled

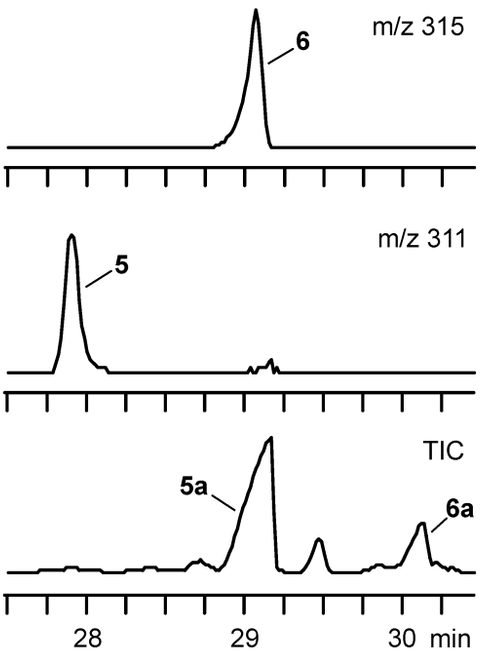

Fig. 2 Sections of reconstructed total ion chromatograms (TIC) and selective ion chromatograms plotted at *m/z* 311 and *m/z* 315, respectively, of hexane extracts of LG from acid **4** head-injected *B. lucorum* males measured on DB-5 capillary column (**5**, ethyl [$^2H_{29}$]-(*Z*)-hexadec-9-enoate; **5a**, ethyl (*Z*)-hexadec-9-enoate; **6**, ethyl [$^2H_{31}$]-hexadecanoate; **6a**, ethyl hexadecanoate)

esters **5** and **6** were detected in the labial glands (Table 3). While the ratio deuterium-labelled/native ethyl hexadecenoate was approximately 1/70 (head-injection, dose 80 μg) or 1/100 (abdomen-injection, dose 160 μg), the value varied between 1.4/1 (head-injection) and 1/4 (abdomen-injection) of the saturated ethyl hexadecanoate. These figures allowed the estimation of the rate of esterification *versus* desaturation (see Discussion).

No additional labelled pheromonal components other than ethyl esters **5** and **6** were found in the glands extracts of *B. lucorum* males. Alcohol **2** is produced by the non-treated males in relatively high proportion (4% of the secretion), however this was not detected in the treated males in the form of the corresponding labelled metabolite **7**. Furthermore, no shortening or elongation of the carbon chain, common in the similar experiments in Lepidoptera (Löfstedt *et al.* 1986), was observed. No trace of shortened and deuterium labelled main component **1** of the secretion was detected after the injection of [$^2H_{31}$]-hexadecanoic acid (Table 3). Approximately 2% of the deuterium labelled substrate was transformed to pheromonal components in the head-injection (dose 80 μg), which compares to only 0.4% in abdomen-injection (dose 160 μg).

In the hexane extracts of the males' fat bodies, small amounts (0.4% of the applied dose, 160 μg) of free [$^2H_{31}$]-hexadecanoic acid (**4**) was found in the abdomen-injection. After acid methanolysis of the TAG fraction isolated by TLC, [$^2H_{31}$]-methyl hexadecanoate (**8**) was detected in both types of injection (into head capsules and into abdomens; Table 3). Incorporation of the substrate **4** into TAG was roughly three times lower in head-injection (0.5%, dose 80 μg) compared to abdomen-injection (1.6%, dose 160 μg) (Table 3). Traces of methyl [$^2H_{29}$]-hexadecenoate (**9**) were also present, however, the double bond position could not be confirmed due to the small amounts. These results indicate that the deuterium-labelled fatty acid is transported by the haemolymph in the body and it is metabolised in different parts of the body, both to pheromonal components and to lipids.

The LG secretion of *B. lapidarius* male consists of 18 compounds, among which hexadecan-1-ol (**2**, 31%) and (*Z*)-hexadec-9-en-1-ol (**3**, 52%) are the main components of the secretion (Calam 1969 and our own results, Table 2). Injection of labelled acid **4** into the males' heads led to the formation of two metabolites – [$^2H_{31}$]-hexadecan-1-ol (**7**) and [$^2H_{29}$]-hexadecen-1-ol (**10**) (Fig. 3). The DMDS adduct of the unsaturated metabolite **10** showed that the carbon-carbon double bond was located in position C-9 (MS fragments *m/z* 159 and *m/z* 204). The alcohols **7** and **10** were found in the ratio deuterium-labelled/native 1/190 and 1/1380, respectively (dose 80 μg). These two compounds were not detected in the labial glands of those males where the precursor **4** was injected into the abdomens (Table 4). In no cases were labelled ethyl esters detected. Similarly to *B. lucorum*, no chain-shortening or elongation was observed in *B. lapidarius* and no trace of deuterium-labelled tetradecanol was detected in the labial gland secretions after [$^2H_{31}$]-hexadecanoic acid was applied. Approximately 0.4% of the applied substrate **4** was transformed to pheromone components in case of *B. lapidarius* (dose 80 μg).

The deuterated precursor was also, similarly to *B. lucorum*, built into fat bodies of males and labelled ester **8** was found after methanolysis of isolated TAG from the fat body of *B. lapidarius* males. However, no large differences in the degree of incorporation of the substrate **4** into TAG were found between the head-injection (2% incorporation, dose 80 μg, Table 4) and abdomen-injection (1% incorporation, dose 160 μg).

Applied acid **4** was detected in the LG in all cases of head-injections (both species, Tables 3 and 4). In both species, 18% of unmetabolised substrate was present in the labial gland extract. No trace of labelled unsaturated acid was detected even in *B. lapidarius*, where native hexadec-9-enoic acid is a secretion component (2.9% of the secretion) and the desaturation could be expected.

Incubation of dissected labial glands of *B. lucorum* males *in vitro* with deuterium- or ^{13}C-labelled palmitic acid lead to the formation of saturated ethyl esters (Table 5). The esters formed were clearly detectable after 24 h incubation and their content was increasing with the incubation time (13 days for [$^2H_{31}$]-hexadecanoic acid and 42 days for [$^{13}C_{16}$]-hexadecanoic acid). The amount of ethyl [$^{13}C_{16}$]-hexadecanoate reached its maximum in 14 days after which it was not increasing any longer (Fig. 4). At the end of incubation, the sample was methylated with diazomethane to evaluate the amounts of unreacted [$^{13}C_{16}$]-hexadecanoic acid. Approximately 80% of the starting acid was transformed to ethyl ester (estimated from the GC integration areas for methyl and ethyl esters). No traces of unsaturated ethyl esters were detected neither with [$^2H_{31}$]-hexadecanoic acid nor [$^{13}C_{16}$]-hexadecanoic acid. No esterification was noted in the control experiments.

Table 3 Observed metabolites of [$^2H_{31}$]-hexadecanoic acid (**4**) injected in *B. lucorum* males

	Deuterated compounds (incorporation in % of applied dose)[a] found in:		
Injection of **4** into	LG extract	Fat body hexane extract	TAG of fat body
Head (80 µg)	**4** (18), **5** (0.4), **6** (1.5)	–	**8** (0.5)
Abdomen (160 µg)	**5** (0.3), **6** (0.1)	**4** (0.4)	**8** (1.6), **9** (trace)

[a]Calculated from the calibration curve and from intensities of typical fragments in mass spectra (for details, see Material and methods)

Table 4 Observed metabolites of [$^2H_{31}$]-hexadecanoic acid (**4**) injected in *B. lapidarius* males

	Deuterated compounds (incorporation in % of applied dose)[a] found in:		
Injection of **4** into	LG extract	Fat body hexane extract	TAG of fat body
Head (80 µg)	**4** (18), **7** (0.3), **10** (0.1)	–	**8** (2.2)
Abdomen (160 µg)	–	**4** (0.7)	**8** (0.8)

[a]Calculated from the calibration curve and from intensities of typical fragments in mass spectra (for details, see Material and methods)

Deuterium-labelled ethanol used in the incubation experiments gave two products, [$^2H_5$]-ethyl dodecanoate and [$^2H_5$]-ethyl tetradecenoate (Table 5). The two corresponding acids are present in the labial gland secretion it their free acid forms (dodecanoic acid 0.3% and tetradecenoic acid 2.5% of the secretion). Formation of these two esters and absence of any other deuterium-labelled esters in the incubation mixture indicate a presence of an ethyl esterase that operates in favour of the synthesis of ethyl esters and not of transesterification.

Discussion

In *B. lucorum* a conclusion can be made on the rate of enzymatic reactions leading to the pheromonal components. As the application of acid **4** initially gave ethyl esters, it seems that formation of esters is the fastest reaction of all reactions that could be expected. The saturated ester **6** was clearly detectable despite the small proportion of the native ethyl hexadecanoate in the labial gland secretion (0.04% of the secretion). Moreover, the labelled/native ratio for ethyl hexadecanoate was in favour of the labelled metabolite (dose 80 µg). The concentration of the deuterium-labelled substrate **4** after head-injection must have exceeded the concentration of the native substrate, palmitic acid.

The amount of the unsaturated ester **5** formed in *B. lucorum* was lower than expected (native ethyl hexadecenoate is a medium-abundant pheromonal component, 3.6% of the secretion). This observation indicates that the desaturation in position C-9 was slower than the formation of ethyl ester (for quantitative estimation, see Table 3). It could be a result of primary isotopic effects (Abad *et al.* 2000) caused by deuterium atoms in the perdeuterated carbon chain of the precursor **4**. The *in vitro* incubation experiments confirmed a quick formation of ethyl esters, but the absence of unsaturated metabolites after a long-time incubation indicates that the desaturation enzymes may be located at some other place in the male's body. To eliminate the alternative that the deuterium primary-isotopic effect is

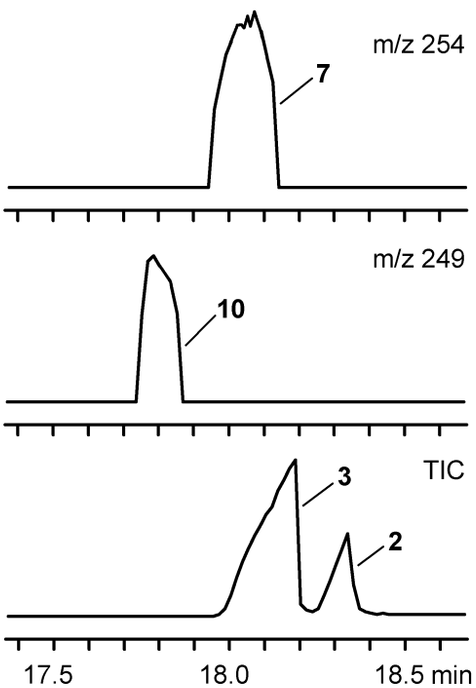

Fig. 3 Sections of reconstructed total ion chromatograms (TIC) and selective ion chromatograms plotted at *m/z* 249 and *m/z* 254, respectively, of hexane extracts of LG from acid **4** head-injected *B. lapidarius* males measured on DB-5 capillary column (**2**, hexadecan-1-ol; **3**, (*Z*)-hexadec-9-en-1-ol; **7**, [$^2H_{31}$]-hexadecan-1-ol; **10**, [$^2H_{29}$]-(*Z*)-hexadec-9-en-1-ol)

Table 5 Observed metabolites in incubations of different substrates with labial glands of *B. lucorum* males *in vitro*

Substrate	Metabolite found	Characteristic MS fragments
[$^2H_{31}$]-Hexadecanoic acid	Ethyl [$^2H_{31}$]-hexadecanoate (**6**)	91, 105, 315
[$^{13}C_{16}$]-Hexadecanoic acid	Ethyl [$^{13}C_{16}$]-hexadecanoate (**11**)	90, 104, 300
[$^2H_5$]-Ethanol	[$^2H_5$]-Ethyl dodecanoate (**13**)	93, 106, 233
	[$^2H_5$]-Ethyl tetradecenoate (**14**)	93, 106, 259

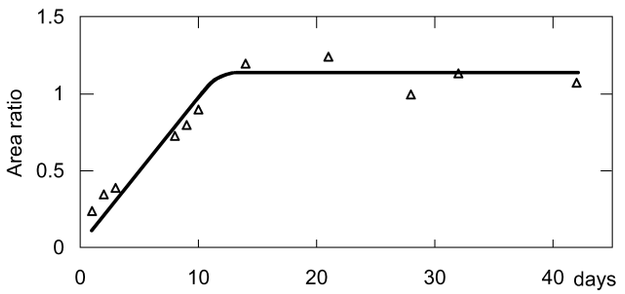

Fig. 4 Time course of the formation of ethyl [$^{13}C_{16}$]-hexadecanoate *in vitro*. Y-axis shows the ratio of integrated areas for ethyl [$^{13}C_{16}$]-hexadecanoate/tricosane (mean values from 2 samples)

hampering the desaturation, we repeated the incubations with [$^{13}C_{16}$]-hexadecanoic acid. In this case the ^{13}C labels in the backbone of the acid will have only limited effect on the rate of the hydrogen atoms abstraction during the desaturation step. But again with this substrate, no trace of unsaturated ethyl ester has been found, however, fast formation of ethyl [$^{13}C_{16}$]-hexadecanoate was observed instead. To exclude a possibility that the desaturase is inactive under *in vitro* conditions, incubations of the bumblebee fat body tissue will be performed under similar conditions. However, for lepidopteraen desaturases no decrease in the desaturation activity *in vitro* was noted (Fabrias *et al.* 1995) and a similar stability for a presumable labial gland desaturase can also be expected.

The incubation of deuterium-labelled ethanol confirms the presence of an ethyl esterase in the labial gland. A putative esterase seems to tolerate harsh conditions and operates *in vitro* for more then 3 weeks at room temperature. The esterification has stopped at 80% conversion, however, we do not know if because of depleting of ethanol source or reaching the equilibrium point. It seems that the esterase uses both free FA and ethanol as substrates. It esterifies both acids with short (C-12, C-14, C-16) chain lengths and with unsaturation in^9 position. Further experiments aimed to determine the substrate specificity of this esterase are necessary and are in progress in our laboratory.

Absence of esters in the LG secretion of *B. lapidarius* clearly indicates lack of an ethyl esterase. The concentration of acid **4** metabolites in *B. lapidarius* LG was lower than that of *B. lucorum*, despite the same dose and conditions of application (Table 4). Approximately 0.4% of the substrate was transformed to pheromonal components in the head-injection (dose 80 μg), while no metabolites were found after abdomen-injection. In this case, the reduction of the carboxylic group of the precursor **4** to a primary hydroxyl group is slower than formation of ethyl esters in *B. lucorum.* Putative biosynthetic intermediates, as are monoenic esters or aldehydes, were not observed (as in experiments on sex pheromones of moth, Tumlinson *et al.* 1996) and the succession of reduction and/or desaturation steps in *B. lapidarius* could not be determined.

Presence of acid **4** in TAGs in case of head-injections in both species indicates its transport by the haemolymph into the fat bodies; the contamination of the fat body with **4** in our experiments was excluded. Additionally, in abdominal injections no trace of the applied precursor **4** was found in the labial gland. This result may indicate that the precursor is built into the fats for possible later use in metabolism. The degree of incorporation of the substrate **4** into different parts of a bumblebee body leads to the conclusion that a significant portion of the applied substrate is present in some transport form. The FA precursors are likely to be transported in the form of diacylglycerols associated with fat bodies (Arrese *et al.* 2001).

Conclusion

Our *in vivo* incubation experiments provide conclusive proof of the presence of species-specific esterases, desaturases and FA reductases in male bumblebees. The *in vitro* incubation experiments indicate that different steps of the pheromone biosynthesis take place in different parts of the body. By comparison of our findings on bumblebee males with the biosynthetic pathways of periodic production of sex pheromones in female moth (Jurenka & Roelofs 1993), one can see that marking pheromones are produced in much larger quantities. We are proposing that putative ethyl specific esterase is essential for formation of male marking pheromone in *B. lucorum*. Further research on the localization of *de novo* FA synthesis, their transport and distribution in the bumblebee bodies is currently in progress.

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