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A Δ^9 Desaturase from *Bombus lucorum* Males: Investigation of the Biosynthetic Pathway of Marking Pheromones

Petra Matoušková,[a] Anna Luxová,[a] Jana Matoušková,[a] Pavel Jiroš,[a] Aleš Svatoš,[a, b] Irena Valterová,*[a] and Iva Pichová*[a]

The knowledge of the molecular basis of communication in bumblebee communities is limited. None of the enzymes that participate in pheromone production have been characterized. Here, we cloned the gene encoding the Δ^9 desaturase from cDNA prepared from the total RNA of the pheromone gland and fat bodies of Bombus lucorum *male. Functional expression of BlucNPVE desaturase in* Saccharomyces cerevisiae *and GC-MS analyses revealed its preference for C_{18} saturated fatty acids. This suggests that Δ^9 desaturase is involved in the desaturation of metabolic fatty acids stored in triacylglyceroles (TAGs), because oleic acid is the most abundant fatty acid bound in TAG in* B. lucorum *and it is present in low concentration in the pheromone blend. The incubation of pheromone precursors with a dissected labial gland as well as direct injection of labelled pheromone substrates into* B. lucorum *males revealed that esterification of pheromone products occurs in the labial gland. These results support both the biosynthesis of pheromones from common lipids and the de novo synthesis of unsaturated pheromones in the labial gland.*

Introduction

Communication in social insects is, to a high extent, based on the chemical signals produced by individuals of each cast. Life-important activities, such as foraging, caring for the brood, finding the way to food sources and back to the nest, as well as finding a partner and mating depend on pheromones that chemically mediate the necessary information.[1] In bumblebees, premating behaviour and the chemical nature of the signals involved—the male marking pheromones—have been studied extensively. Research in this field started in Sweden in the 1970s,[2,3,4] and was followed by many studies of Middle and West European bumblebee species.[5,6,7,8,9]

The marking pheromone is produced by the cephalic part of the male labial gland.[10] Each bumblebee species produces a specific blend of compounds (for a review see ref. [11]) that attracts conspecific females for mating.[12] The gland secretion contains mostly two types of compounds: 1) straight chain saturated and unsaturated hydrocarbons, alcohols, aldehydes, and esters and 2) terpenoids. The only paper that refers to the biosynthesis of the bumblebee labial gland components is highly theoretical and provides no experimental evidence.[13] Based on the analysis of compound patterns isolated from 22 bumblebee species, the authors suggest that these compounds are produced from saturated fatty acids by the action of specific glandular desaturases. Despite a relatively large number of papers discussing the biosynthesis of lepidopteran pheromones[14] or pheromones of beetles (see ref. [15] and references therein), there are no data available in the literature on the biosynthetic pathways of pheromone formation in bumblebees.

The white tailed bumblebee, *Bombus lucorum*, produces a labial gland secretion that consists of a mixture of sixty compounds.[16,6] The mixture is dominated by ethyl esters of fatty acids, chiefly ethyl (*Z*)-tetradec-9-enoate (53%). Medium abundant or minor components of the secretion were identified as ethyl dodecanoate (6%), ethyl tetradecanoate (2%), ethyl (*Z*)-hexadec-9-enoate (4%) and ethyl (*Z*)-octadec-9-enoate (2%). Among alcohols and aldehydes, hexadecan-1-ol (4%), (*Z*,*Z*,*Z*)-octadeca-9,12,15-trien-1-ol (1%) and (*Z*)-hexadec-7-enal (2%) were detected.[6]

There are two possible explanations for the presence of pheromone components in the labial gland. They are either biosynthesized from common lipids in the body followed by delivery into the labial gland, or they undergo de novo synthesis from acetate units in the labial gland itself.[17] The previous biosynthetic study by Luxová et al.[18] concluded that aliphatic pheromonal components might arise from pool lipids after their transport by haemolymph and modification of the carbon chain and functional groups in the labial gland. Application of deuterium-labelled palmitic acid to the head and abdomen of *B. lucorum* males gave rise to labelled ethyl [$^2H_{29}$]-(*Z*)-hexadec-9-enoate and ethyl [$^2H_{31}$]hexadecanoate in the labial gland. Furthermore, the labelled palmitic acid was built into triacylglycerols (TAGs) of the fat body. Thus, a hypothesis on the possi-

[a] *P. Matoušková, Dr. A. Luxová, J. Matoušková, Dr. P. Jiroš, Dr. A. Svatoš, Dr. I. Valterová, Dr. I. Pichová*
Institute of Organic Chemistry and Biochemistry
Academy of Sciences of the Czech Republic
Flemingovo n. 2, 166 10 Prague 6, (Czech Republic)
Fax: (+42) 02201-83556
Fax: (+42) 02201-83582
E-mail: irena.valterova@uochb.cas.cz
iva.pichova@uochb.cas.cz

[b] *Dr. A. Svatoš*
Max Planck Institute for Chemical Ecology
Hans-Knöll-Strasse 8, 07745, Jena (Germany)

ble connection of primary and secondary metabolism was created. However, no characterization of the putative enzymes involved in the biosynthesis of pheromones in bumblebees is available. Based on the composition of pheromone blends we can speculate that desaturases, lipolytic enzymes (esterases or lipases), and reductases might participate in the synthesis of pheromones in *B. lucorum*. Desaturases cloned and characterized to date come from Lepidoptera, Diptera and Ortoptera.[19] Rapidly evolving sequencing possibilities like expressed sequence tags (EST) and high-throughput cDNA (HTC) have revealed more sequences from other orders, such as Coleoptera (*Diaprepes*), Hemiptera (*Homalodisca*, *Lygus*) and Hymenoptera (*Apis*, *Bombus*, *Solenopsis*, *Lysiphlebus*). However, most are partial sequences, and none has been fully characterized.

Here, we focused on the further investigation of the biosynthetic pathway of pheromones in *B. lucorum* using two approaches. First, we directly applied deuterium-labelled substrates into different parts of the bumblebee body as well as the dissected labial gland, with the aim to gain information about the specificities of the enzymes involved in pheromone formation. Second, we isolated the gene that encodes the fatty acyl-CoA desaturase and characterized its catalytic properties in order to understand its role in the body. Our results represent the first characterization of desaturase in bumblebee spp.

Results and Discussion

In vitro incubation of putative pheromone precursors

To investigate the synthesis of the pheromone blend of *B. lucorum*, labelled fatty acids of different chain lengths and alcohols of different types were incubated in vitro with dissected labial glands. The major products of the incubation are summarized in Tables 1 and 2. Incubation of labial glands with labelled fatty acids led to formation of ethyl and methyl esters. Ethyl ester levels were about 1000-fold higher than methyl ester levels, which is in agreement with the native pheromonal composition (53% ethyl (*Z*)-tetradec-9-enoate vs. 0.03% methyl (*Z*)-tetradec-9-enoate[6]). The synthesis of ethyl and methyl ester products linearly increased over 13 and 16 days, respectively. No change in the concentration of these products was observed after this time period (Figure 1). The time course of ester formation was similar for all fatty acids used in this study. However, only saturated products were detected during the in vitro experiment, although the main pheromonal component in *B. lucorum* is an unsaturated aliphatic ester, the ethyl (*Z*)-tetradec-9-enoate.

Table 1. Products obtained from in vitro incubation of the labial gland of *B. lucorum* males with labelled acids.

Acid substrate	Main metabolite
$[^2H_{23}]$dodecanoic	ethyl $[^2H_{23}]$dodecanoate
$[^2H_{27}]$tetradecanoic	ethyl $[^2H_{27}]$tetradecanoate
$[^2H_{31}]$hexadecanoic	ethyl $[^2H_{31}]$hexadecanoate
$[^2H_{35}]$octadecanoic	ethyl $[^2H_{35}]$octadecanoate
$[^{13}C_{16}]$hexadecanoic	ethyl $[^{13}C_{16}]$hexadecanoate
1,2-$[^{13}C_2]$tetradecanoic	ethyl 1,2-$[^{13}C_2]$tetradecanoate

Table 2. Products obtained from in vitro incubation of the labial gland of *B. lucorum* males with different alcohols.

Alcohol substrate	Main metabolite
$[^2H_5]$ethanol	$[^2H_5]$ethyl (*Z*)-tetradec-9-enoate
$[^2H_4]$methanol	$[^2H_3]$methyl (*Z*)-tetradec-9-enoate
propan-1-ol	–[a]
propan-2-ol	–[a]
butan-1-ol	–[a]
decan-1-ol	–[a]
allyl alcohol	–[a]

[a] No product was obtained.

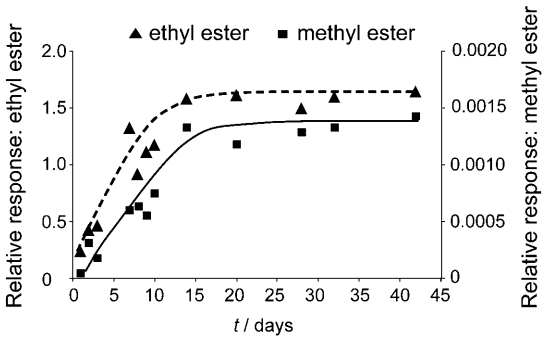

Figure 1. Time course for ester formation in vitro after incubation of the labial gland of a *B. lucorum* male with $[^{13}C_{16}]$hexadecanoic acid. The *y* axis represents the relative levels of methyl and ethyl ester formation as normalized to an internal standard (adamantane).

We further observed a preference for shorter substrates during the in vitro incubation of the *B. lucorum* labial gland with an equimolar mixture of deuterium-labelled fatty acids of different chain lengths (Figure 2). After 13 days incubation, the amount of ethyl $[^2H_{23}]$dodecanoate (d-12:Et) formed was highest, followed by ethyl $[^2H_{27}]$tetradecanoate (d-14:Et). The amount of ethyl ester formation from $[^2H_{31}]$hexadecanoic (d-16:Et) and $[^2H_{35}]$octadecanoic (d-18:Et) acids was significantly lower after the same incubation period. This result correlates with the composition of saturated ethyl esters in the pheromone gland (5.9% ethyl dodecanoate, 1.8% ethyl tetradecanoate, 0.04% ethyl hexadecanoate and 0.08% ethyl octadecanoate).

Incubation of the labial gland with labelled and unlabelled alcohols of different types and chain lengths also led to the formation of esters. For these experiments, deuterium-labelled methanol and ethanol were used to distinguish their metabolites from native pheromonal components. Other alcohols were not labelled as their esters are not present in the pheromones of *B. lucorum* males. Only corresponding products of ethanol and methanol were detected in the labial gland extracts; other tested alcohols remained unchanged (Table 2). The amount of $[^2H_5]$ethyl ester formed was about three times higher than that of $[^2H_3]$methyl ester. $[^2H_5]$Ethyl (*Z*)-tetradec-9-enoate dominated within the generated esters of different

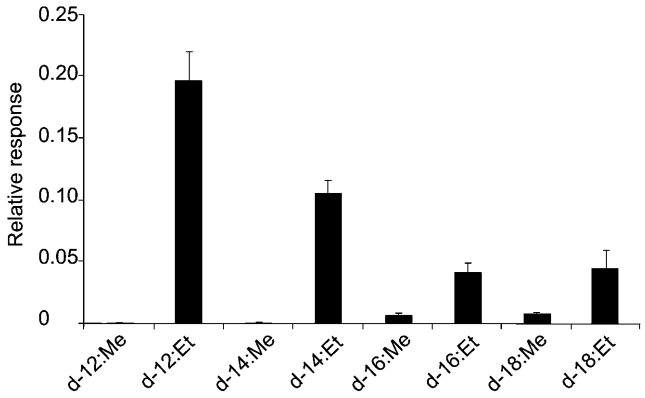

Figure 2. Products obtained from the in vitro incubation of the labial gland of a *B. lucorum* male with an equimolar mixture of deuterium-labelled fatty acids of different chain lengths (d-12:Me, methyl [$^2H_{23}$]dodecanoate; d-12:Et, ethyl [$^2H_{23}$]dodecanoate; d-14:Me, methyl [$^2H_{27}$]tetradecanoate; d-14:Et, ethyl [$^2H_{27}$]tetradecanoate; d-16:Me, methyl [$^2H_{31}$]hexadecanoate; d-16:Et, ethyl [$^2H_{31}$]hexadecanoate; d-18:Me, methyl [$^2H_{35}$]octadecanoate; d-18:Et, ethyl [$^2H_{35}$]octadecanoate). Data on the *y* axis represent levels relative to an internal standard (adamantane).

Table 3. Composition of free fatty acids found in the labial gland of *B. lucorum* males.

Fatty acid	%
dodecanoic	2.9
(*Z*)-tetradec-9-enoic	69.7
tetradecanoic	1.4
(*Z*)-hexadec-7-enoic	1.8
(*Z*)-hexadec-9-enoic	5.0
(*Z*)-hexadec-11-enoic	0.7
hexadecanoic	0.9
(*Z,Z*)-octadeca-9,12-dienoic	0.4
(*Z,Z,Z*)-octadeca-9,12,15-trienoic	4.8
(*Z*)-octadec-9-enoic	9.5
(*Z*)-octadec-11-enoic	1.2
(*Z*)-octadec-13-enoic	0.5
octadecanoic	0.5
(*Z*)-icos-9-enoic	0.3
icosanoic	0.1
docosanoic	0.1

lengths, and corresponds well to the main pheromonal component. In order to investigate the availability and concentrations of free fatty acids for pheromone synthesis in the labial gland, the homogenized gland was extracted with polar solvents. The fraction from a preparative TLC contained different free fatty acids (Table 3). The main free fatty acid found was (*Z*)-tetradec-9-enoic (69%), followed by (*Z*)-octadec-9-enoic (9.5%); this suggests that the availability of these acids in high concentrations can influence the level of newly synthesized esters.

To confirm the hypothesis about the synthesis of aliphatic pheromonal components from lipid pools (TAGs),[18] we incubated ^{2}H-labelled tripalmitine (deuterium atoms in all positions of the hexadecanoic acid) with the labial gland under the same conditions as described above. As expected, [^{2}H]tripalmitine was hydrolysed and free [$^2H_{31}$]palmitic acid was detected after two days incubation; this confirmed the presence of a hydrolyzing enzyme in the labial gland.

Cloning and functional expression of acyl-CoA desaturase of *Bombus lucorum*

To confirm the presence of desaturases, which participate together with other enzymes in the production of pheromones, we isolated RNA from the labial glands and fat bodies of *B. lucorum*, prepared cDNA from the isolated RNA, and screened the cDNA for the desaturase gene. Using hybrid primers we obtained an identical internal 450 bp fragment, which encoded a central part of Δ^9 desaturase. A BLAST search for this fragment showed a high correlation with fatty acyl-CoA desaturases, particularly with a predicted desaturase from *Apis mellifera*. The complete *B. lucorum* Δ^9 desaturase cDNA isolated from both labial gland and the fat body has a 1053 base long open reading frame (ORF) that encodes a 351 amino acid protein, and is flanked by a 290 base 5′ untranslated region (UTR) and 258 base 3′-UTR (BlucNPVE; GenBank accession No. AM709889). The primary structure of BlucNPVE contains all conserved sequences of fatty acyl-CoA desaturases: three catalytically essential histidine motifs[19] and four transmembrane helices that anchor the enzyme to the endoplasmic reticulum (Figure 3). The BlucNPVE desaturase of *B. lucorum* shares 73% sequence identity with the predicted desaturase of *Apis mellifera*, and 65, 62 and 58% identity with Δ^9 desaturases from *Acheta domesticus*,[20] *Musca domestica*[21] and *Drosophilla melanogaster*,[22] respectively.

Figure 3. Structure based alignment of acyl-CoA desaturase from *B. lucorum* (Bluc) and *Apis mellifera* (Apis). Identical amino acid residues are marked in black, similar residues are marked in grey. Amino acids of predicted transmembrane domains (Tm1–4) are underlined. Three conserved histidine boxes (H1–H3) and signature motif (SM) are framed.

The desaturase-deficient *ole1* strain of *S. cerevisiae* was used to test the activity of the BlucNPVE desaturase. *S. cerevisiae* were transformed with either pYES-DesBl or an empty pYES2.1 vector. As expected, transformants containing the empty vector did not grow without supplemental unsaturated fatty acids (UFAs). The GC chromatograms of methylated total lipid extracts of yeast cells expressing the desaturase showed two peaks with retention times that corresponded to *Z*9-16:Me and *Z*9-18:Me in a 1:5 ratio (Figure 4A). The *Z* stereospecifity of desaturase products was verified by gas-phase FTIR spectroscopy. IR spectra of the formed unsaturated esters did not contain the characteristic band of *E* double bond (970 cm^{-1}). The double-bond positions of unsaturated compounds that corresponded to the above detected GC peaks were identified by chemical ionization in ion-trap mass spectrometry by using acetonitrile as a reagent gas.[23] The mass spectrum (Figure 4C) showed the expected pseudomolecular peak of methyl octadecenoate (*m/z* 350, [*M*+54]), and two fragments that resulted from the cleavage at the Δ^9 double bond (*m/z* 208 and 252). Low amounts of *Z*9-C14:Me were found when yeast cells were supplemented with tetradecanoic acid. The yeast system appeared to be active in elongating C_{14} to C_{16} compounds, as we observed that *Z*11-16:acyl was synthesized from *Z*9-14:acyl in culture media supplemented with tetradecanoic acid (Figure 4B).

In vivo incubations of pheromone precursors

Although we successfully identified the Δ^9 desaturase gene in the labial gland, the specificity of this desaturase, which was tested with the functional-expression assays in yeast, did not correlate with the pheromone products of *B. lucorum*. To further study the metabolism of pheromone production, we performed in vivo experiments, in which an equimolar mixture of labelled saturated fatty acids of different chain lengths was injected into the head capsules (close to the labial gland) or abdomens (close to the fat body) of *B. lucorum* males. After two days incubation, we identified several labelled metabolites in the extract from the labial glands. We found mainly ethyl esters, alcohols, hydrocarbons and wax esters formed from labelled saturated fatty acids. We also detected the unsaturated labelled metabolites ethyl [$^2H_{25}$]-(*Z*)-tetradec-9-enoate, ethyl [$^2H_{29}$]-(*Z*)-hexadec-9-enoate and ethyl [$^2H_{33}$]-(*Z*)-octadec-9-enoate in the labial gland. However, the ratio of newly formed unsaturated products from labelled fatty acids was different to the ratio in the gland, where the predominant form was ethyl (*Z*)-tetradec-9-enoate. The distribution of labelled unsaturated fatty acids was in favour of hexadecenoic acid, followed by tetradecenoic acid and lower amounts of (*Z*)-octadec-9-enoic acid. When the labelled substrates were injected into the abdomen, their metabolites appeared in the labial gland in lower amounts than when applied directly to the head, as they could be exposed to other metabolic processes during transport (Figure 5).

To investigate whether there is a preference for fatty acids during the synthesis of TAGs in *B. lucorum*, we analysed the methanol/chloroform extracts of the fat bodies after applications to the head and abdomen of the bumblebees. After application to the head, we found that only low concentrations of [$^2H_{33}$]-(*Z*)-octadec-9-enoic acid was incorporated into fat bodies. But after direct application to the abdomen, we detected approximately eight times more of this labelled fatty acid in fat bodies. These results can be explained by the fact that after application to the head precursors must first be transported to the fat body by the haemolymph.

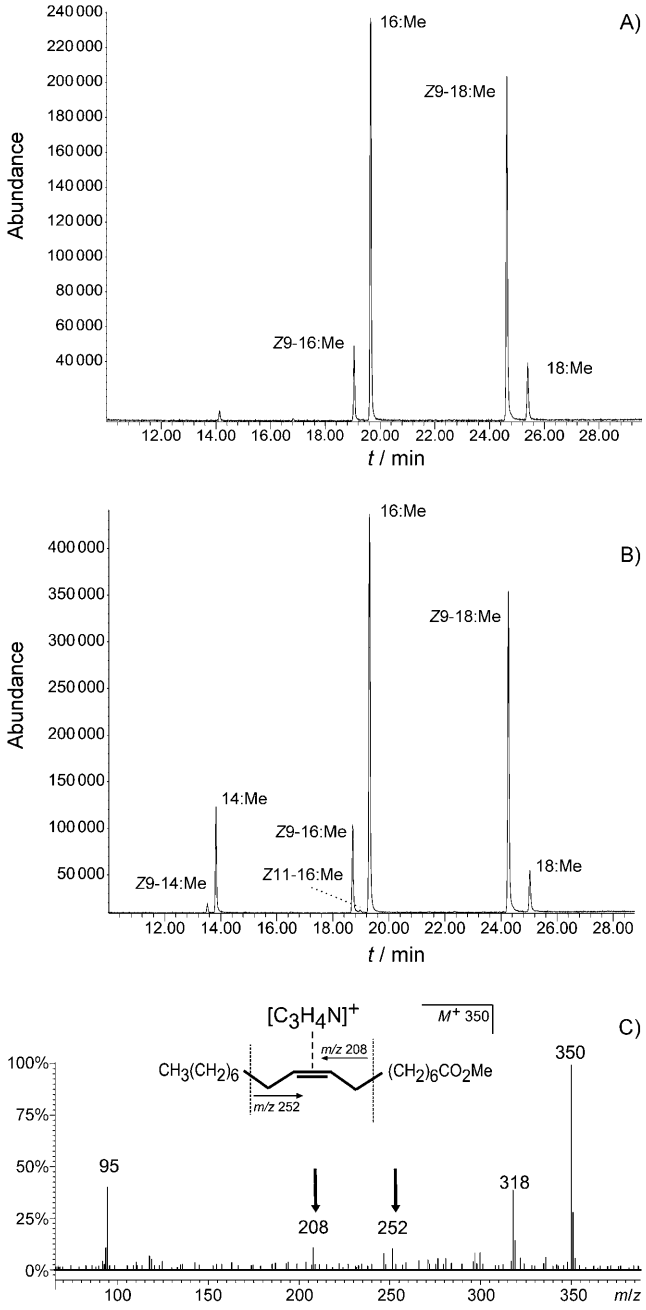

Figure 4. GC-MS analysis of the functional expression of BlucNPVE desaturase in *S. cerevisiae*. A) GC-MS total ion chromatogram of fatty acid methyl esters (FAME) obtained from *S. cerevisiae*. B) GC-MS total ion chromatogram of FAME obtained from *S. cerevisiae* cultured in the presence of myristic acid (14:0). C) Mass spectrum of *Z*9-18:Me; the arrows indicate two fragments that result from the cleavage at the Δ^9 double bond (*m/z* 208 and *m/z* 252).

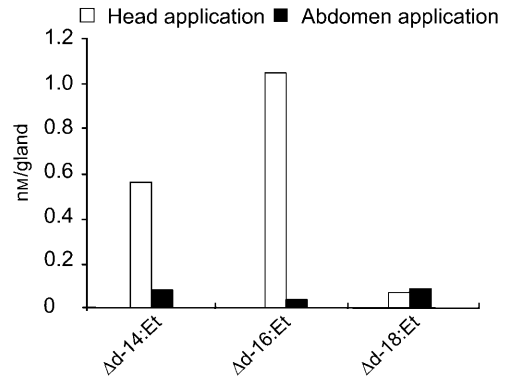

Figure 5. Unsaturated esters formed from fatty acids applied in vivo: comparison of head and abdomen applications. Data shown are the medians of three experiments; Δd-14:Et, ethyl (*Z*)-tetradec-9-enoate; Δd-16:Et, ethyl (*Z*)-hexadec-9-enoate; Δd-18:Et, ethyl (*Z*)-octadec-9-enoate.

Discussion

In contrast to moths, in which the biosynthetic pathway of pheromones is relatively well described, little is known about the biosynthesis of marking pheromones in bumblebees. In moths, sex pheromones are mostly released by the female to attract males for mating, while in bumblebees marking pheromone are released by males. The pheromone blend of *B. lucorum* consists of a large number of compounds and the main component, which represents about 53% of the blend, is ethyl (*Z*)-tetradec-9-enoate—an unsaturated aliphatic ester. In contrast, female moths produce pheromone blends that consist of a few (1–8) straight-chain C_{10}–C_{18} unsaturated aliphatic compounds derived from saturated C_{16} or C_{18} fatty acid intermediates in rather low concentrations. Interestingly, the concentration of pheromones in the labial gland of *B. lucorum* is much higher (some components reach milligram amounts; Valterová et al., unpublished results). It has not been elucidated whether the precursors of the pheromones (i.e., the fatty acids) are synthesized de novo in the labial gland (located in the head) and subsequently modified by limited chain-shortening and regio- and stereospecific desaturation reactions, or whether the pheromones are formed from fatty acid precursors that are released from pool TAGs in the lipid droplets of the fat body.

The results we obtained from in vitro as well as in vivo incubation experiments suggest that pheromones could be produced from common lipids present in the body. Furthermore, the composition of the free fatty acid fraction from the labial gland extracts corresponded well with products secreted from the labial gland. The highest level was confirmed for (*Z*)-tetradec-9-enoic acid (69.7%), which can serve as a precursor for the main pheromone product (Table 3). When we applied labelled alcohols, the main product formed was also [$^2H_5$]ethyl ester of (*Z*)-tetradec-9-enoic acid. Since all labelled fatty acids incubated with the isolated gland were metabolized into the corresponding ethyl esters and lower amounts of methyl esters, we postulate that the esterification step takes place in the labial gland. The lipidic enzyme that catalyzes this step displays preference for shorter fatty acid chains. This agrees well with the ratio of different saturated ethyl esters present in the gland.

Interestingly, in incubations of deuterium-labelled fatty acids we obtained only saturated ethyl or methyl ester derivatives; this suggests that desaturation does not proceed in the labial gland under these conditions. To exclude the isotopic effect of deuterium-labelled fatty acids on desaturase catalysis, we also tested ^{13}C-labelled acids of the same lengths. Again, desaturated products were not detected; this indicates that the desaturase was not active under these specific in vitro conditions. In moths, a neuromodulating agent present in the head ganglia is involved in the activation of different steps of the pheromone biosynthesis pathway.[24,25] In *B. lucorum*, similar neuromodulators that affect the activity of the specific desaturase might be present, but such agents have not yet been identified in Hymenoptera.

We were able to show the presence of Δ^9 BlucNPVE acyl-CoA desaturase in the labial gland and fat body of *B. lucorum* by cloning the gene from the isolated mRNA. This desaturase displayed a preference for stearic acid (C_{18}: saturated) as substrate since oleic acid (C_{18}: Δ^9) was preferentially formed during functional testing in transformed *S. cerevisiae*. Analysis of TAGs of fat bodies performed by Cvačka et al.[26] showed that oleic acid represents the most abundant fatty acid bound to TAG (39%). Palmitoleic acid (C_{16}: Δ^9, 1%) and myristoleic acid (C_{14}: Δ^9, 0.3%) are bound to TAG in low amounts. Therefore, we suggest that the Δ^9 BlucNPVE acyl-CoA desaturase is one of metabolic desaturases that participates in the desaturation of fatty acids stored in TAGs.

The direct application of labelled saturated fatty acids to the head capsules in *B. lucorum* (equimolar mixture of C_{12}, C_{14}, C_{16} and C_{18}-acids) yielded a mixture of corresponding unsaturated products. This experiment aimed to investigate the possible selectivity of the desaturase to certain chain lengths. The ratio of formed products did not correspond to the concentrations of unsaturated products in the gland, since the distribution of labelled products favoured ethyl [$^2H_{29}$]-hexadec-9-enoate, while in the native pheromonal blend tetradec-9-enoate dominates. However, the equimolar amounts of applied, labelled precursors differed from pheromone component ratios and this might have influenced the amounts of obtained products.

We can also hypothesize that the tetradecenoic acid is preferentially transported into the labial gland from the hemolymph, where it is stored as diacylglycerol (DAG). The relative amount of tetradecenoic acid in DAG is ten times higher than in TAGs of the fat body (4% vs. 0.3%). Since the dominant fatty acid bound in DAG is (*Z*)-hexadec-9-enoic acid (about 20%), the hypothetical lipase, which could participate in the hydrolysis of DAGs, should display a preference for tetradecenoic acid bound in DAG in lower amounts.

Conclusions

In conclusion, our results clearly indicate that the esterification of pheromone precursors proceeds in the labial gland of *B. lucorum*. The hypothetical enzyme (either lipase or esterase) that catalyzes this step shows a preference for shorter (C_{12}–C_{14})

fatty acid chains. The free fatty acids present in the labial gland could function as pheromone precursors for esterification, as their composition corresponds well with the final composition of the pheromones. The incubation data presented here support the hypothesis that precursors of aliphatic pheromonal components in *B. lucorum* can arise from lipid moieties and can be hydrolyzed from pool lipids by selective lipases. The BlucNPVE desaturase, which we identified in the pheromone gland and in the fat bodies of *B. lucorum*, is a metabolic desaturase rather than a pheromone-selective enzyme, because the myristic acid (the precursor for abundant pheromone component) serves only as its minor substrate. Thus, we have to consider the presence of another desaturase in the labial gland or fat bodies that is specific for the tetradecanoic substrate, which we have not obtained from our mRNA screening. Furthermore, we cannot exclude de novo biosynthesis of unsaturated pheromone products in *B. lucorum*. Complex screening of the enzymes that participate in pheromone synthesis including gene identification as well as proteomic analyses should be used next to elucidate the biosynthesis pathways of pheromone production in *B. lucorum* males.

Experimental Section

General materials: All chemicals were purchased from Sigma–Aldrich unless otherwise stated, at the highest available purity. Oligonucleotides were synthesized by Generi-Biotech (Czech Republic).

Isotopically labelled chemicals: The following compounds were used as substrates for in vitro incubations: deuterium- and ^{13}C-labelled fatty acids of different chain lengths, [$^2H_{23}$]dodecanoic, [$^2H_{27}$]tetradecanoic, [$^2H_{31}$]hexadecanoic, and [$^2H_{35}$]octadecanoic acids, separately and their equimolar mixture; [$^{13}C_{16}$]hexadecanoic acid, 1,2-[$^{13}C_2$]tetradecanoic acid (both Isotec, Miamisburg, USA) and labelled and unlabelled alcohols of different types and chain lengths [$^2H_5$]ethanol (Acros), [$^2H_4$]methanol and unlabelled propan-1-ol, propan-2-ol, octan-1-ol, and allyl alcohol. As a representative of TAGs, deuterium-labelled tripalmitine $(C_{15}{}^2H_{31}COO)_3C_3H_5$ (C/D/N Isotopes, Augsburg, Germany) was used.

Microorganisms, plasmids and growth conditions: *E. coli* DH5α was used for cloning and plasmid preparation. Cells were cultured in nutrient broth (Hymedia) medium supplemented with ampicillin (100 mg L^{-1}) at 37 °C, overnight.

E. coli TOP10 were used with the TOPO vector according to the manufacturer's protocol (Invitrogen).

Saccharomyces cerevisiae desaturase deficient *ole1* strain (DTY-11A; *MAT*α, *ole1*Δ*::LEU2*, *leu2-3*, *leu2-112*, *his3-11*, *his3-15*, *trp1-1*, *can1-100*, *ura3-1*, *ade2-1* (HIS^+))[27] was used for the expression of recombinant desaturase. Prior to transformation, the cells were grown (24 h, 30 °C, 220 rpm shaking) in YPGA medium (1% yeast autolysate, 2% peptone, 2% glucose and supplemented with 15 μg mL^{-1} of adenine) containing tergitol (1%) and oleic and palmitoelic acids (0.5 mM each). The transformed cells were grown (5 days, 30 °C) on a uracil-deficient YNB^{AHLT} plate (0.67% YNB supplemented with adenine, histidine, leucine and tryptophan 15 μg mL^{-1} each, 2% glucose, 2% agar) containing tergitol (1%) and oleic and palmitoelic acids (0.5 mM each). A single colony from the plate was inoculated into sterile YPGalA medium (15 mL, 1% yeast autolysate, 2% peptone, 2% galactose and supplemented with 15 μg mL^{-1} of adenine) and grown at 30 °C for 5 days with 220 rpm shaking in the presence or absence of myristic acid (0.5 mM, 14:0).

PCR2.1 TOPO vector (Invitrogen) was used for initial cloning of the PCR products. pYes2.1 (Invitrogen) was used for the functional expression of the desaturase gene in yeast under *GAL1* promoter.

Recombinant DNA technologies: Unless stated otherwise, standard DNA technologies were used.[28] The plasmid midi kit, and the QIAquick gel extraction kit (Qiagen) were used for plasmid DNA and DNA gel extractions, respectively. Restriction endonucleases, deoxynucleotide triphosphates and DNA ladders were obtained from New England BioLabs, Taq polymerase was from Top-Bio (Praha, Czech Republic). Pfu polymerase was purchased from Promega, T4 DNA ligase and reverse transcriptase were from Invitrogen.

DNA sequence analysis: Database searches for amino acid sequences of desaturases were performed by using SRS on the EBI website (http://srs.ebi.ac.uk/). Alignments were obtained by using multalin.[29] Six-frame translation of the sequences was performed by using ORF finder program, which is part of the NCBI server. The identification of obtained sequences was done by using BLAST (NCBI).

Total RNA isolation and cloning of desaturase gene: Male *B. lucorum* bumblebees were obtained from laboratory colonies as described by Šobotník et al.[30] Males (4 days old) were killed by freezing and kept deep frozen prior to dissection. Labial glands and fat body tissues were carefully dissected and stored at −80 °C for further use. Total RNA was isolated by using TRIreagent (Sigma) according to the manufacturer's procedure. First-strand cDNA synthesis by RT-PCR was performed by using Superscript III with an oligo(dT) primer by following the manufacturer's protocol. RT-PCR products were used as templates for the amplification of the central fragment of the gene encoding desaturase by using hybrid primers designed by CODEHOP.[31] The primers (nSF3 sense direction: 5′-GTAGTCCCAAGGGAAAACGtgrtgrtartt-3′, nSF5 antisense direction: 5′-CCGACACCGACGCTgayccncayaa-3′, where: y = C or T; n = A, G, C or T; r = A or G) consisted of a short 3′ degenerate core region and a longer 5′ consensus clamp region (lower and upper case letters, respectively) and were added to the final concentration of 25 pmol per 25 μL of reaction. Both primers were designed based on the sequence comparison of known insect desaturases. A standard polymerase chain reaction (25 μL) contained reaction buffer (1×), dNTPs (0.2 mM), nSF3 (1 μM), nSF5 (1 μM), Taq polymerase (5 units) and cDNA (1 μL). The reaction was kept for 5 min at 94 °C followed by 5 cycles of 94 °C for 30 s, 45 °C for 1 min, 72 °C for 1 min, and 25 cycles with 55 °C as the annealing temperature. A final 10 min extension at 72 °C was used to ensure all DNA was double stranded and with 3′-A overhangs. Purified PCR products (430 bp) were directly cloned into the TOPO vector and used to transform TOP10 cells. Colony PCR was used to screen positive (white) clones and plasmid DNA was purified and verified by sequencing. After the vector sequences had been excluded, the central region was obtained and compared with other central regions of desaturase genes by using BLASTx (NCBI) to confirm its identity. Two gene-specific primers were designed for RACE PCRs: H3 (5′-CTTCTTCGCGCATGTTGGTTG-3′) and D5 (5′-GTGCCAACCCTCACCTAAAG-3′). As a template for isolation of the 3′- and 5′ ends, double-stranded DNA was prepared by using two adaptor primers from a SMART kit for cDNA library preparation (Clontech) according to the manual. The 5′-RACE PCR was performed under the following conditions: 5 min 94 °C followed by 30 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 2 min, and a final 10 min extension at

72 °C by using SMART IV primer (from the kit) and GSP-D5. Similarly, the 3′-RACE PCR was performed by using a CDS III primer from the SMART kit and GSP-H3. PCR products were separated on a agarose gels (1%), and DNA fragments excised from the gel were purified and cloned into a linearized pCR2.1-TOPO vector for sequencing.

Accession number: The sequence for BlucNPVE desaturase from *B. lucorum* was deposited with GenBank with the access number AM709889.

Functional assay of desaturase in pYES2.1 expression system: Two primers, 3′-Sac/C (5′-CCCCG*GAGCTC*TTAATGATCTTTCTTCAAATTC-3′) and 5′Hind/C (5′-CCT*AAGCTT*AGAATGGCGCCGAATATAACG-3′; restriction sites SacI and HindIII are italicized) were designed to amplify the ORF of the BlucNPVE desaturase. PCR conditions were similar to those described above, except that an annealing temperature of 55 °C was used. The purified PCR products were double digested, ligated into a pYES2.1 expression vector (Invitrogen), and verified by sequencing. The vector pYES-DesBl and empty plasmid pYES2.1 were transformed by electroporation into the desaturase deficient *ole1* strain of *Saccharomyces cerevisiae* (DTY-11A) for functional expression.

Transesterification and FAME extraction: Yeast cells were harvested by centrifugation (5 min, 3000 *g*, 4 °C). The cell pellet was washed two times with water and extracted with of dichloromethane/methanol (1 mL, 2:1) at room temperature for 1 h. The solution was filtered through cotton wool, extracted further with chloroform, and the solvents were evaporated under argon. The residue was dissolved in KOH (100 μL, 0.5 M) in methanol (room temperature, 1 h). Then KH_2PO_4 (50 μL, 0.5 M) and Na_2HPO_4 (50 μL of 0.5 M) were added, and the mixture was neutralized with HCl (3 M). The hemolymph sample was transesterified and worked up in the same way. The resulting fatty acid methyl esters (FAME) were extracted three times with hexane (200 μL) and the combined extracts were analyzed by gas chromatography coupled to mass spectrometry (GC-MS). The double bond position in the products was determined by chemical ionisation in an ion-trap GC-MS (Varian Saturn 2000) by using acetonitrile as the reaction gas.[23]

In vitro incubation experiments: Cephalic parts of the labial glands were dissected, placed in 1.2 mL vials and rinsed with hexane (3×100 μL, 15 min shaking). Whole glands were extracted without homogenisation of the tissue. Phosphate buffer (pH 7.2, 10 μL) and a hexane solution of the substrate (1 mg mL^{-1}, 50 μL) were added to the dissected and rinsed glands. The glands were shaken in the heterogeneous system at room temperature for up to 30 days on a linear shaker (IKA, KS 130). Samples for analyses (1 μL) were taken from the vials at regular intervals and analysed directly by GC-MS. Adamantane was used as an internal standard (1 mg mL^{-1}). Three parallel incubations were performed, in order to allow statistical analysis of the results.

In vivo incubation experiments: An equimolar blend of [$^2H_{23}$]dodecanoic (6.5 mg, 29.1 μM), [$^2H_{27}$]tetradecanoic (7.2 mg, 28.2 μM), [$^2H_{31}$]hexadecanoic (8.1 mg, 28.2 μM) and [$^2H_{35}$]octadecanoic acid (10.1 mg; 31.6 μM) was dissolved in a mixture of dimethylsulfoxide, ethanol and water (7:2:1, 200 μL). The solution was injected in vivo into either the head capsule (0.5 μL) or abdomen (2 μL) of bumblebee males. Incubation was terminated after 2 days.

Thin layer chromatography: The tissue (labial gland or fat body) was homogenised and extracted with a mixture of chloroform/methanol (2:1, *v/v*, 2×100 μL) in a glass vial. The vial was shaken for 10 min, after which the contents were filtered and evaporated to dry in a stream of argon. The resultant residue was redissolved in hexane (100 μL). The extract was chromatographed on a silica gel plate (36×76 mm, Adsorbosil-Plus, Applied Science Lab.; elution with hexane:diethyl ether:acetic acid 80:20:1; visualisation by Rhodamine 6G in ethanol, 0.05%). The fraction containing the fatty acids was esterified with diazomethane to improve chromatographic properties and to enable accurate quantification. The triacylglycerol fraction was transformed to corresponding methyl esters by transesterification.[32]

Gas chromatography: The FAME mixture isolated from the yeast was analyzed by GC-MS (HP 6890N, Agilent Technologies, Palo Alto, CA, USA); the electron ionisation was at 70 eV (MS detector: 5975B MSD Agilent) with a DB-5MS capillary column (J&W Scientific, Folsom, CA, USA; 30 m×0.25 mm, film thickness 0.25 μm). Conditions for the analysis were as follows: carrier gas, He: 1 mL min^{-1}; split-less injection 1 μL; injector temperature 220 °C; temperature program from 40 °C for 2 min, then increase to 140 °C at 70 °C min^{-1}, then 140 °C to 240 °C at 3 °C min^{-1}, then 240 °C to 280 °C at 20 °C min^{-1}, then 280 °C for 5 min. Compound identification was performed by comparison of mass spectra against the NIST Library. The temperature program for chemical ionisation (determination of double-bond positions) started at 70 °C (2 min) followed by an increase of 10 °C min^{-1} to 320 °C.

Samples from the in vitro incubations were analyzed by using a gas chromatograph with a quadrupole mass detector (Fisons MD 800). A BPX5 column (30 m×0.22 mm, SGE) and helium gas (flow 0.55 mL min^{-1} at 50 °C) were used for separations. The temperature program started at 70 °C (2 min delay) after which the temperature of the oven was increased to 140 °C at the rate of 50 °C min^{-1}, then to 240 °C at the rate of 2 °C min^{-1}, and finally to 300 °C at the rate of 5 °C min^{-1}.

Labelled metabolites from in vivo experiments were identified and quantified by using a 2D GC with MS–TOF detection (Pegasus 4D). The arrangement consisted of a gas chromatograph (Agilent 6890) with a secondary oven and a time-of-flight mass detector with electron impact ionization mode. The temperature of the ion source was 200 °C. The nonpolar column in the first dimension was HP-5, 30 m×0.32 mm, film thickness 0.25 μm; the secondary polar microcolumn was BPX-50, 1 m×0.1 mm, film thickness 0.1 μm. The temperature program for the first column was 70 °C (1 min), 10 °C min^{-1} up to 300 °C (20 min) and for the second column 90 °C (1 min), 10 °C min^{-1} up to 320 °C (20 min). The flow of the carrier gas was 1 mL min^{-1}. The injector temperature was 250 °C and the split ratio was 1:10.

Deuterium-labelled metabolites were identified by their mass spectra and their characteristic retention behaviour. Their expected characteristic mass fragments were calculated on the basis of the same fragmentation principles of unlabelled analogues. Quantification of deuterated metabolites was calculated from calibration curves of methyl esters of labelled fatty acids. Different detector response for individual compounds was eliminated by the multiplication or division of peak areas by the counted relative response factors.

Gas chromatography-infrared spectroscopy (GC-FTIR): Double-bond positions were determined by using an Agilent 6850 gas chromatograph connected to an Equinox 55 FTIR spectrometer (Bruker Optics Inc., Ettlingen, Germany). A DB-5 column (30 m×0.32 mm, film thickness 0.25 μm, J&W Scientific) was used for the separations; injector temperature was 220 °C (split-less mode), carrier gas was He at a flow rate of 2.5 mL min^{-1}. The temperature

program: 50 °C (0 min), then 50 °C min^{-1} to 150 °C (0 min), then 1 °C min^{-1} to 240 °C (5 min). The MCT detector was used with FTIR resolution of 8 cm^{-1}; light pipe temperature was 200 °C. Methyl (*E*)-hexadec-9-enoate and methyl (*Z*)-hexadec-9-enoate (Applied Science Laboratories; 20 mg mL^{-1} in cyclohexane) were used as standards for IR spectra of unsaturated methyl esters.

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