ChemPubSoc Europe

DOI: 10.1002/cbic.201500415

CHEMBIOCHEM Full Papers

Regulation of Isoprenoid Pheromone Biosynthesis in Bumblebee Males

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Males of the closely related species *Bombus terrestris* and *Bombus lucorum* attract conspecific females by completely different marking pheromones. MP of *B. terrestris* and *B. lucorum* pheromones contain mainly isoprenoid (ISP) compounds and fatty acid derivatives, respectively. Here, we studied the regulation of ISP biosynthesis in both bumblebees. RNA-seq and qRT-PCR analyses indicated that acetoacetyl-CoA thiolase (AACT), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), and farnesyl diphosphate synthase (FPPS) transcripts are abundant in the *B. terrestris* labial gland. Maximal abundance of these transcripts correlated well with AACT enzymatic activity detected in the LG extracts. In contrast, transcript abundances of AACT, HMGR, and FPPS in *B. lucorum* were low, and AACT activity was not detected in LGs. These results suggest that transcriptional regulation plays a key role in the control of ISP biosynthetic gene expression and ISP pheromone biosynthesis in bumblebee males.

Introduction

Bumblebees (*Bombus* spp.) have a unique mate-finding strategy among insect species. Males patrol along regular routes and mark prominent objects, such as stones, fence posts, and leaves, with a highly species-specific mixture of compounds called marking pheromones (MP). These are synthesized in the cephalic part of the labial gland (LG), stored in numerous acini attached to the LG, and secreted from the gland at the base of the mandibles. Components of the blend attract conspecific females for mating.[1–4]

Male-produced compounds that elicit electroantennographic (EAG) responses from *B. terrestris* virgin queens include predominantly the isoprenoid (ISP) compounds (*E*)-2,3-dihydrofarnesol (DHF, 58%), geranylcitronellol (13%), and 2,3-dihydrofarnesal (0.6%), as wells as the fatty-acid-derived compounds ethyl dodecanoate (14.4%), octadeca-9,12,15-trienol (1.8%), and hexadecan-1-ol (1.6%). The blend contains a number of additional components that do not elicit EAG responses, such as icosenol (6.1%), tetradecenal, and tetradecanol.[2,5] In contrast, *B. lucorum* males primarily use fatty acid derivatives to attract females; the dominant EAG-active components of their pheromone blend are ethyl tetradecenoate (53%) and ethyl dodecanoate (6%). Isoprenoids present in *B. lucorum* MP in trace quantities (<1% of LG extract) do not elicit an EAG response from *B. lucorum* virgin queens.[4,6]

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Supporting information and ORCIDs from the authors for this article are available on the WWW under http://dx.doi.org/10.1002/cbic.201500415.

The *B. terrestris* pheromone composition changes over the course of the male lifespan; dihydrofarnesol exhibits the largest age-dependent quantitative changes. Unique compounds, such as dihydrofarnesyl dodecanoate, hecadecyl dodecanoate, and geranylcitronellyl dodecanoate, are only present in the LG of older *B. terrestris* males and do not elicit EAG responses in queen antennae.[5] In contrast, the MP composition and levels of EAG-active compounds remain relatively constant in *B. lucorum* adult males.[4]

Detailed information about pheromone biosynthesis, regulation, and evolution is available for moths[7–9] and dipterans.[10,11] In these insects, unsaturated fatty acid precursors can be modified by fatty acid desaturases that introduce a double bond in the fatty acid chain at a specific position and configuration, further by fatty acid reductases that are involved in the formation of alcohols, and by esterases that catalyze the production of fatty acyl esters. Differences in the activity or specificity of these enzymes are considered to be important features that contribute to diversification of fatty-acid-derived pheromone components.[12–16]

Pheromone biosynthesis in insects is controlled by distinct hormones, such as pheromone biosynthesis activating neuropeptide (PBAN), ecdysteroid, and juvenile hormone.[17] Hormonal regulation of pheromone biosynthesis in social hymenoptera remains poorly studied; however research on PBAN-regulated trail pheromone biosynthesis in fire ant *Solenopsis invicta*[18] and the identification of PBAN[19] and a putative gene homologue of PBAN-receptor in the honeybee *Apis mellifera*[20] indicate that PBAN might also play a role in the regulation of pheromone biosynthesis in social hymenoptera. It is thus a candidate hormone in MP regulation in bumblebees.

Recent analyses of fatty acid pheromone precursor biosynthesis in *B. terrestris* and *B. lucorum* indicated that pheromone

components are formed by two pathways: de novo biosynthesis from acetyl-CoA in LG, and modification of fatty acids released by lipases from lipidic precursor triacylglycerols stored in the fat body (FB) and transported to the LG.[15,21–24]

No information is available about the regulation of ISP pheromone biosynthesis in bumblebees. Biosynthesis of terpenoid aggregation pheromones in bark beetles and aphids has been described;[25–28] iridoids (monoterpenoids), used for protection against predation, have been studied in *Chrysomelina* species.[29,30] In general, different ISPs are synthetized from acetyl-CoA through the mevalonate pathway in many organisms, including humans. Reductive polymerization of acetyl-CoA leads to a great variety of compounds, including sterols (especially cholesterol), ubiquinone, dolichol, farnesol, geraniol, and diphosphate derivatives such as geranyl or farnesyl diphosphate. ISPs play roles in processes such as the prenylation of membrane proteins, electron transport, glycoprotein synthesis, apoptosis, signal transduction, and transcriptional and translational modifications.[31] Cells must control mevalonate synthesis and regulate the concentrations of multiple ISP compounds in different tissues. Unlike mammalian cells, insect cells do not synthesize cholesterol, and the main products of the mevalonate pathway are unique sesquiterpenoids such as juvenile hormone, which regulates development in several lifecycle stages and pheromone production in most insect species (reviewed by Belées et al.).[32]

In this study, we investigated age-dependent changes in the biosynthesis of ISP compounds in two bumblebee species that use diverse pheromone blends for sexual communication. RNA sequencing data analysis, qRT-PCR analysis of expression of genes involved in ISP biosynthesis, detection of enzyme activity, and quantification of the formation of ISP compounds in *B. terrestris* and *B. lucorum* demonstrated that biosynthesis of ISP compounds is regulated at the transcriptional level for the main ISP biosynthetic genes in LGs, where marking pheromones are synthesized and stored.

Results

2,3-Dihydrofarnesol and geranylcitronellol are abundantly synthesized in the *B. terrestris* LG

We quantified the formation of dihydrofarnesol and geranylcitronellol (GCOL) in the dissected LGs of two-day-old *B. terrestris* males incubated with deuterium-labeled acetate. The ratio of concentrations of deuterated DHF and GCOL in the LG was approximately 5 (Figure 1 A), which is in a good agreement with ratio of these compounds naturally present in the tissue (Figure 1 B). No deuterated ISPs were detected in *B. lucorum* under the same conditions. These results confirmed that *B. terrestris* ISP pheromone components (DHF and GCOL) are synthesized from acetate units in the male LG.

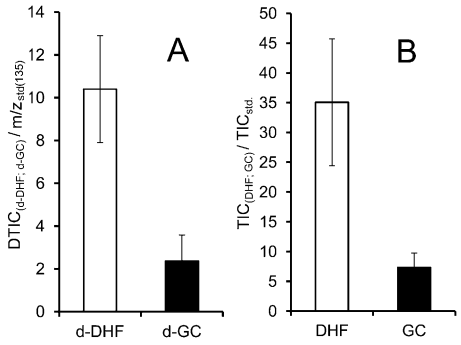

Figure 1. A) Relative concentrations of deuterium-labelled 2,3-dihydrofarnesol ([D]DHF) and geranylcitronellol ([D]GCOL) randomly containing deuterium atoms in *B. terrestris* LG hexane extracts. LGs from two-day-old males were incubated with [D_3]sodium acetate before extraction. B) Relative concentration of 2,3-dihydrofarnesol (DHF) and geranylcitronellol (GCOL) in LG hexane extracts from three-day-old males. All data are mean ± SE ($n = 5$ bumblebees).

Expression of genes encoding mevalonate and isoprenoid enzymes are specifically upregulated only in the LG of *B. terrestris*

To study genes involved in *B. terrestris* and *B. lucorum* ISP biosynthesis, we performed RNA sequencing of dissected LGs. In our *B. terrestris* LG transcriptome database, we identified complete sequences for mevalonate kinase (MK), phosphomevalonate kinase (PMK), diphosphomevalonate decarboxylase (DPMD), and geranylgeranyl diphosphate synthase (GGPPS), as well as partial sequences for acetoacetyl-CoA thiolase (AACT), HMG-CoA synthase (HMGS), HMG-CoA reductase (HMGR), isopentenyl diphosphate isomerase (IPPI), and farnesyl diphosphate synthase (FPPS). The complete sequences of AACT, HMGS, HMGR, IPPI, and FPPS were obtained by RACE PCR, and the sequences were deposited in GenBank (NCBI) under accession numbers JQ413981– JQ413989. The recent *B. terrestris* genome and transcriptome sequences[33] confirmed the LG and fat body transcriptome-derived sequences of ISP biosynthetic genes.

Analysis of RNA-seq data obtained from *B. lucorum* LG provided the complete sequences of AACT, HMGS, HMGR, MK, PMK, DPMD, and IPPI, along with almost-complete sequences of FPPS and GGPPS. The sequences were deposited in GenBank under accession numbers KP420143–KP420151. The identity of the ISP biosynthetic enzymes from both bumblebees is greater than 99%. The enzyme sequences from *B. lucorum* and *B. terrestris* display 71–85% identity with predicted ISP biosynthetic enzymes from *A. mellifera*, and also high sequence identity with other insect ISP biosynthetic enzymes (Table S1 in Supporting Information). BLAST searches of our *B. terrestris*[34] and *B. lucorum* transcriptome databases with various characterized insect short-chain isoprenyl diphosphate synthases, a class of prenyltransferases (including FPPS, GPPS, and GGPPS enzymes) as queries yielded two high-scoring hits. The first, a putative FPPS, shares high sequence similarity with human and insect FPPSs and also contains sequences encoding two conserved substrate-binding aspartate-rich motifs;[35] the second, a putative bumblebee GGPPS, shares high similarity with

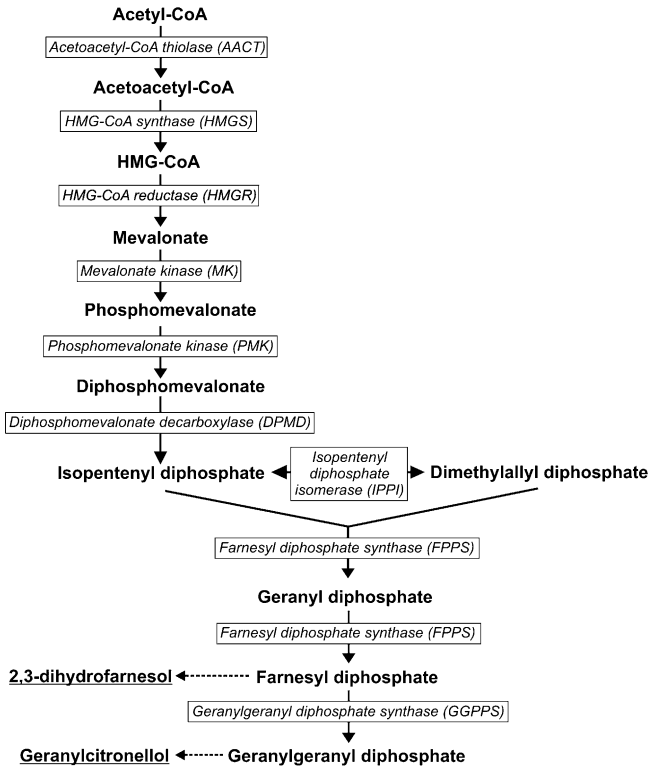

Figure 2. The isoprenoid biosynthetic pathway leading to the major terpenic compounds in the pheromone blend of *B. terrestris* and *B. lucorum*.

human and insect GGPPSs (Figure S1 in Supporting Information). BLAST searches of the *B. terrestris* genomic database (GenBank)[33] yielded similar results, with the exception that three predicted isoforms of FPPS differed only in the length of the N terminus (data not shown). Figure 2 summarizes the identified genes putatively involved in ISP biosynthesis in bumblebees.

The transcript abundances of ISP biosynthetic genes in *B. terrestris* (Table 1) and *B. lucorum* (Table 2) were assessed according to calculated reads per kb of transcript per per million mapped reads (RPKM) values. In *B. terrestris* LG, the FPPS, HMGS, IPPI, AACT, and HMGR transcripts were found to be among the most abundant in the whole transcriptome, thus suggesting contributions to the regulation of ISP biosynthesis. In *B. lucorum* LG, the abundances of all ISP biosynthetic gene transcripts were lower, and substantially so for HMGR, AACT, and FPPS, compared to *B. terrestris* LG.

Table 1. Transcript abundances of enzymes involved in isoprenoid biosynthesis in *B. terrestris*. RPKM values were calculated from RNA-seq data obtained from labial gland (LG) and fat bodies (FB).

Gene	Ranking in LG transcriptome[a]	LG RPKM	FB RPKM	Fold change LG/FB
AACT	15	3644	12	309
HMGS	4	8058	113	71
HMGR	31	2404	22	107
MK	381	452	40	11
PMK	680	210	15	14
DPMD	91	1515	55	28
IPPI	11	3813	33	114
FPPS	3	8226	64	129
GGPPS	1044	116	13	9

[a] According to RPKM.

Table 2. Transcript abundances of enzymes involved in isoprenoid biosynthesis in *B. lucorum*. RPKM values were calculated from RNA-seq data obtained from labial gland (LG) and fat bodies (FB).

Gene	Ranking in LG transcriptome[a]	LG RPKM	FB RPKM	Fold change LG/FB
AACT	489	200	13	15
HMGS	61	1737	20	87
HMGR	2392	36	25	1.4
MK	375	267	42	6
PMK	832	102	14	7
DPMD	136	758	31	24
IPPI	49	1987	46	43
FPPS	87	1175	62	19
GGPPS	8463	9	41	0.2

[a] According to RPKM.

To explore the tissue specificity of ISP biosynthesis, we also sequenced mRNA isolated from fat bodies (storage of food reserves, and intermediary metabolism). Comparison of fold changes calculated from RPKM values for individual transcripts in both tissues indicated higher expression of AACT, HMGR, IPPI, and FPPS in *B. terrestris* LG (Table 1). We also observed higher expression of ISP biosynthetic gene transcripts in *B. lucorum* LG compared to the fat body; however, the fold changes of AACT, IPPS, FPPS, and especially HMGR were low compared to *B. terrestris*. GGPPS was more abundantly expressed in the *B. lucorum* fat body (Table 2). These results indicate comparable ISP biosynthesis in *B. lucorum* LG and fat body, and suggest that the biosynthesis of ISPs in bumblebees is regulated at the transcriptional level.

Age-dependent changes in ISP biosynthetic gene expression in *B. terrestris* and *B. lucorum*

Age-dependent pheromone production differs between these two bumblebee species. Dihydrofarnesol, the main component in *B. terrestris* male MP, appears in the gland of one-day-old males, reaches a maximum on day 7, and then gradually decreases.[5] In contrast, the concentration of pheromone components in the LGs of *B. lucorum* males remains stable throughout the lifespan.[4] We analyzed the expression levels of ISP biosynthetic genes in LGs from *B. terrestris* males of different ages by qRT-PCR. The expression levels of MK, PMK, DPMD, and IPPI genes did not change significantly with age (Figure 3). However, we detected a significant difference between one-day-old and three-day-old males in the expression of AACT, HMGR, FPPS, and GGPPS. In older specimens, the expression of these genes slowly decreased. In contrast, the expression profiles of AACT and HMGR, which changed significantly during the *B. terrestris* lifecycle, remained constant in LGs from *B. lucorum* males of different ages (Figure 4).

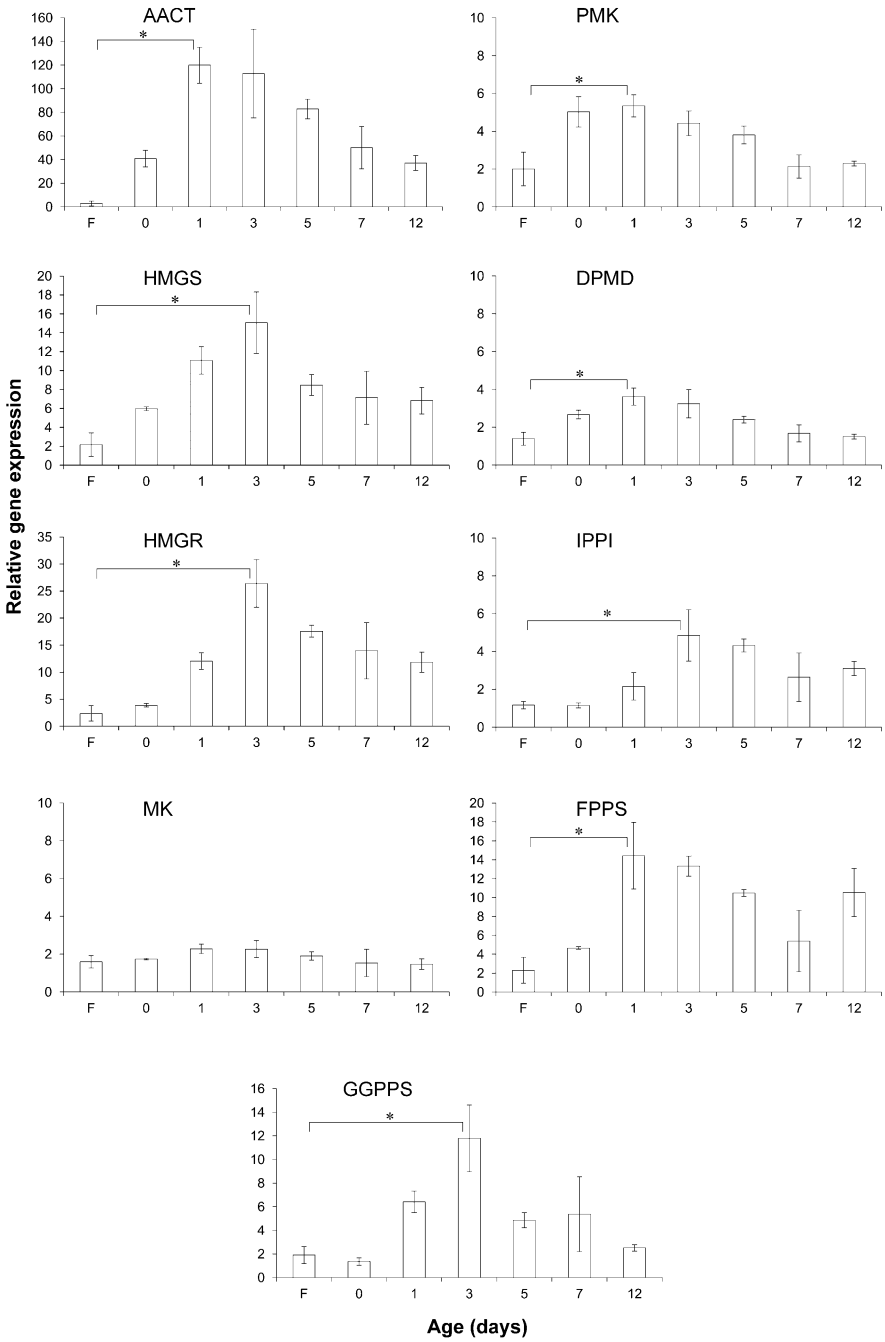

Figure 3. Relative expression of *B. terrestris* isoprenoid biosynthetic mRNA in LGs of pharate (F) and at 0, 1, 3, 5, 7, and 12 days. Data are mean ± SD ($n = 3$); *$p < 0.05$, determined by a two-tailed *t*-test.

Activity of acetoacetyl-CoA thiolase in the LG differs between *B. terrestris* and *B. lucorum*

We examined differences in ISP biosynthesis between bumblebee species by assaying enzyme activity in homogenate of dissected LGs from one-, two-, three-, four-, and five-day-old *B. terrestris* and *B. lucorum* males. We monitored AACT activity, as this gene exhibited high expression in the *B. terrestris* LG and is presumed to be one of the isoprenoid biosynthesis regulatory genes. We detected AACT activity only in young *B. terrestris* males: maximum in two-day-old males, moderately decreased in three-day-olds, and not detected in five-day-olds (Figure 5). This indicates robust biosynthesis of ISP pheromones in young *B. terrestris* males. The age-dependent activity correlates well with the expression of AACT mRNA obtained by qRT-PCR. In contrast, we did not detect AACT activity in identical amounts of *B. lucorum* LG homogenates (data not shown). AACT activity in LG lysate can depend on the sensitivity of the thiolase assay, and therefore we cannot exclude the presence of a low amount of active AACT either in LGs of older *B. terrestris* or in LGs of *B. lucorum*.

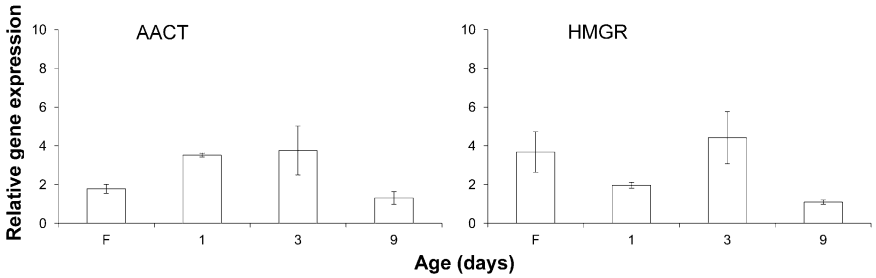

Figure 4. Relative expression of *B. lucorum* mRNA for AACT and HMGR in LGs from pharate (F) and at 1, 3, and 9 days. Data are mean ± SD ($n = 3$).

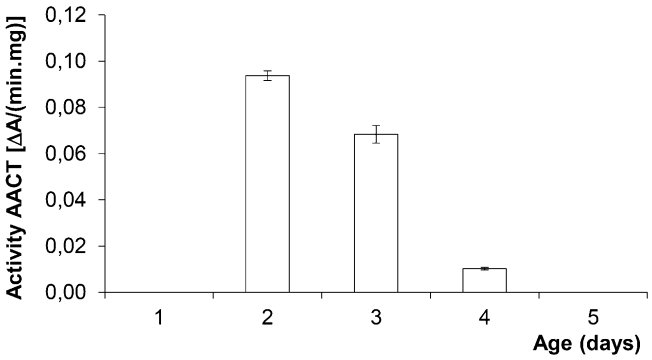

Figure 5. AACT activity in LGs from *B. terrestris* males at 1, 2, 3, 4, and 5 days.

Discussion

Two closely related bumblebee species, *B. terrestris* and *B. lucorum*, use completely different pheromone blends for sexual communication; these provide excellent models for investigating ISP biosynthesis regulation. The sequences of identified genes putatively involved in ISP biosynthesis were deduced either from RNA-seq data or from a combination of RNA-seq and RACE PCR, and were almost identical in the two species. The deduced protein sequences exhibited high similarity to predicted insect ISP biosynthetic enzymes (Table S1). Besides FPPS and GGPPS, in the examined databases we did not find a short-chain isoprenyl diphosphate synthase homologue that could be annotated as a geranyl diphosphate synthase. We therefore assume that the predicted bumblebee FPPSs produce farnesyldiphosphate by stepwise condensation of dimethylallyl diphosphate with two molecules of isopentenyl diphosphate, analogously to short-chain isoprenyl diphosphate synthases producing both geranyl diphosphate and farnesyl diphosphate in diverse insect orders.[36–40] Based on the predominance of sesquiterpenic and diterpenic MP compounds and the absence of monoterpenic compounds in the *B. terrestris* LG extracts,[3] we assume that the major product of the predicted bumblebee FPPSs is farnesyl diphosphate.

Quantitative detection of incorporated deuterium-labeled acetate into the final pheromone compounds DHF and GCOL indicated that biosynthesis of ISPs is localized in the LG of *B. terrestris*. The ISP biosynthetic pathway is also present in the fat body, but the levels of ISP biosynthetic gene transcripts were low (and similar in both species). Lipid and carbohydrate metabolism, protein synthesis, and amino acid metabolism take place in the fat body,[41] and ISPs synthesized here are likely used either for prenylation of membrane proteins or for another biological function, such as electron transport, glycoprotein synthesis, or transcription regulation.[32]

In contrast to the relatively stable expression of ISP genes in *B. lucorum* LG, we observed age-dependent changes in AACT, HMGR, HMGS, and FPPS gene expression in *B. terrestris* LG. Expression of these genes increased during the first 3 days after eclosion and slowly decreased in LGs of males older than 5 days. This correlates well with production of the main LG pheromone component (DHF), which rapidly increases up to 4–7 days and gradually decreases in older bumblebees.[5] AACT activity in the LG of *B. terrestris* males reached a maximum in three-day-old males. This is in agreement with our earlier observation (based on the morphology of the LG) that young *B. terrestris* males efficiently synthesize ISP pheromones and store them in the acinar lumen of secretory cells.[5] The volume of ISP pheromone components reaches a maximum in the acinar lumen of five-day-old *B. terrestris*. Males do not leave the nest before they are 4–5 days old, and then they mark their territories with pheromones that they produced earlier. Accumulation of up to 6 mg DHF in LGs of seven-day-old *B. terrestris* males[4] likely contributes to secretory cell apoptosis; this starts in five-day-old *B. terrestris* males.[5] Farnesol can act as an effective inducer of cell arrest and apoptosis in several carcinoma cell types (for a review see Joo et al.),[42] and in microorganisms such as *Candida albicans*,[43] *Aspergillus flavus*,[44] and *Penicillium expansum*.[45] We have recently identified candidate genes upregulated in the *B. terrestris* LG; these are presumably associated with LG secretory cell apoptosis,[34] but future experiments are needed to determine the effects of high concentrations of terpenoid compounds on age-dependent apoptosis observed in *B. terrestris* males.

Several enzymes have been recognized as important regulators in the biosynthesis of terpenoids in various organisms and tissues. HMGR, which catalyzes the reduction of 3-hydroxy-3-methyl-glutaryl-CoA to mevalonic acid, is likely the most highly regulated enzyme,[46] because its expression is modulated at the transcriptional, translational, and post-translational levels. In insects, the ISP biosynthetic pathway lacks the sterol branch that ends in the biosynthesis of cholesterol,[32] and the regulatory mechanism of this pathway might differ from that in vertebrates. HMGR has been suggested to serve as a regulatory enzyme in the ISP pathway of the bark beetle *Ips paraconfusus*,

and juvenile hormone was shown to increase HMGR transcript levels and ISP pheromone production in *I. paraconfusus* males.[47] Our data show that HMGR transcription is regulated in *B. lucorum* LG. Although HMGR is upregulated only 1.4-fold in *B. lucorum* LGs compared to fat bodies, the fold change of HMGR in LGs and fat bodies in *B. terrestris* reaches 107.

Another potential regulatory step is the condensation of two molecules of acetyl-CoA to acetoacetyl-CoA catalyzed by AACT. This enzyme has been reported to regulate ISP biosynthesis involved in abiotic stress adaptation for the plant *Medicago sativa*.[48] AACT from *Streptomyces* sp. strain CL190 has been described as a critical regulatory enzyme in the ISP pathway.[49] We observed the highest age-dependent upregulation of AACT transcript levels and activity in the *B. terrestris* LG. High expression of AACT could also guarantee sufficient production of intermediate acetoacetyl-CoA for ISP biosynthesis. We detected upregulated expression of FPPS, which catalyzes the synthesis of farnesyl diphosphate (the intermediate for DHF biosynthesis), in the *B. terrestris* LG. This enzyme is responsible for directing carbon flow away from the central ISP biosynthetic pathway, and therefore it could play a crucial role in the regulation of ISP metabolism.[50] The GGPPS transcript was upregulated only in *B. terrestris* LGs. In contrast, the GGPPS transcript was more abundant in the *B. lucorum* fat body, thus suggesting that this enzyme, which catalyzes the synthesis of geranylgeranyl diphosphate, contributes to downregulation of geranylcitronellol biosynthesis in *B. lucorum*. Preliminary analysis of the transcriptome of LGs from *Bombus lapidarius*, which uses only aliphatic pheromone compounds for sexual communication, showed downregulation of HMGR, FPPS, and GGPPS in the LG. This finding is highly similar to that observed in *B. lucorum* LG, thus suggesting transcriptional regulation of isoterpenoid biosynthesis as a general control mechanism in bumblebees.

Previous comparison of desaturase transcript abundances in LGs of both bumblebee species indicated post-transcriptional downregulation of Δ9-fatty acid desaturase in the *B. terrestris* LG,[15] thus indicating different regulation of the biosynthesis of fatty-acid-derived pheromone precursors. Hence, bumblebee species have evolved different mechanisms for regulating specific components of the pheromone blend.

Conclusions

We used next-generation sequencing and qRT-PCR to identify and quantify transcript abundances of genes from the ISP biosynthetic pathway in the LGs and the fat bodies of *B. terrestris* and *B. lucorum*. Our results indicate that the biosynthesis of ISPs is regulated at the transcriptional level for the key ISP biosynthetic genes HMGR, FPPS, and GGPPS in the LG, where biosynthesis proceeds from acetyl-CoA.

Further studies are needed to identify the detailed mechanisms of transcription regulation of ISP pheromones. We suggest that pheromone biosynthesis-activating neuropeptide, juvenile hormone, or 20-hydroxyecdysone (the three most frequent regulators of pheromone production in insects)[51] might be involved in this regulation.

Experimental Section

Insects: *B. terrestris* and *B. lucorum* males were obtained from laboratory colonies.[52] LGs and fat bodies were dissected from male bumblebees of different ages, and stored in TRIzol (ThermoFisher Scientific) at −80 °C. LGs from *B. terrestris* pharate imago and at 0, 1, 3, 5, 7, 10, and 12 days, and from *B. lucorum* pharate at 1, 3, and 9 days were used for qRT-PCR analysis. Fat bodies from *B. terrestris* pharate imago and at 1, 3, and 12 days were also used for qRT-PCR. The pooled LGs and fat bodies from *B. terrestris* males at 0, 1, 3, and 5 days; and *B. lucorum* males at 3 days were used for RNA-seq.[15]

RNA isolation: Total RNA was isolated from tissues in TRIzol according to the manufacturer's instructions. To eliminate DNA contamination, all samples were subjected to TURBO DNase (ThermoFisher Scientific) digestion for 1 h at 37 °C. RNA was further purified with the RNeasy MinElute Cleanup Kit or RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions (RNA Cleanup protocol). The RNA concentration was determined with a NanoDrop ND-1000 UV/Vis Spectrophotometer (Thermo Scientific), and RNA integrity was analyzed with a model 2100 Bioanalyzer (Agilent Technologies) with RNA Nano chips (Agilent Technologies).

RNA-seq data generation and gene sequence construction: RNA-seq was performed with dissected LGs and fat bodies from *B. lucorum* and *B. terrestris*. RNA-seq data generation, assembly, and annotation were as previously described.[15] Rapid amplification of cDNA ends (RACE) PCR was used for amplification of complete coding regions of AACT, HMGS, HMGR, IPPI, and FPPS from *B. terrestris*. A cDNA library (constructed in λTriplEx2 vector, prepared from LGs and fat bodies of a three-day-old male) was used as the template in RACE PCR reactions with gene-specific primers. The PCR products were separated on 1% agarose gels, excised from the gel, purified, and directly sequenced, or cloned into pCRII-TOPO vector (Invitrogen) and then sequenced.

Sequence analysis: BLAST searching of local databases (*B. terrestris*,[34] and *B. lucorum* contig sequences obtained in this study) was performed by BioEdit software (version 7.0.9.0).[53] The NCBI GenBank database was also searched by BLAST. Multiple alignments of amino acid sequences were performed with MultAlin.[54]

cDNA synthesis and quantitative real-time PCR (qRT-PCR): Single-stranded cDNA was obtained from total RNA (0.25 μg) with SuperScript III Reverse Transcriptase (Invitrogen) and random hexamer primers according to the manufacturer's procedure.

qRT-PCR experiments were performed with cDNA prepared from RNA isolated from three biological replicates of each age group by using a LightCycler 480 qRT-PCR System (Roche) with SYBR green fluorescent label (primers in the Supplementary Information). All samples were examined in two technical replicates. Data were exported from the LightCycler software into Microsoft Excel and analyzed by GenEx software (http://www.multid.se). Relative gene expression was normalized to the expression of phospholipase A2 (PLA2) and elongation factor 1α (EEF1A) genes, as previously described (analysis details in the Supporting Information).[55]

Preparation of crude enzyme mixture: LG tissue from 30 *B. terrestris* males (1, 2, 3, 4, and 5 days) was collected in ice-cold homogenization buffer (25 μL per organ; Tris·HCl (20 mM, pH 7.4) containing sucrose (0.25 M), EDTA (1 mM), benzamidine (0.1 mM), 2-mercaptoethanol (0.1%, *v/v*), leupeptin (10 mg L^{-1}, Sigma), and aprotinin (1 mg L^{-1}) Sigma). Tissue was homogenized in a Potter–Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged (20000 *g*, 20 min), then the floating fat cake was re-

moved, and the supernatant was transferred into Eppendorf tubes. The pellet was resuspended in homogenization buffer (25 μL per LG) and centrifuged (20000 *g*, 20 min). The supernatants were collected and centrifuged (20000 *g*, 30 min). All steps were carried out at 4 °C.

Thiolase assay: AACT activity was measured spectrophotometrically as previously described.[24] Briefly, the formation of acetoacetyl-CoA from acetyl-CoA in the presence of Mg^{2+} at pH 8.8 was monitored at 303 nm. The reaction was initiated by addition of enzyme solution to Tris·HCl (100 mM, pH 8.8) with $MgCl_2$ (25 mM) and acetyl-CoA (4 mM) at 30 °C. Specific activity is expressed as ΔA [$min^{-1} mg^{-1}$ protein]. The protein concentration of the crude extract was determined by a Bradford assay (BSA as the standard).[56]

In vitro incubation of LGs: Cephalic parts of LGs were dissected from two-day-old male bumblebees. The average LG weight in one experiment was 4.1 ± 0.1 mg (pooled from 5 LG). Incubation conditions and composition of the incubation solution (containing sodium acetate labeled with three deuterium atoms ([D_3]sodium acetate, Sigma–Aldrich)) were as previously described.[22] Briefly, incubations were carried out for 16 h in Zymo-Spin IIN filtration columns (Zymo Research, Irvine, CA) at 35 °C, and the reaction was terminated by separation of the tissue from the medium by centrifugation (2000 *g*, 20 s). Any remaining tissue on the column was washed with incubation medium without sodium acetate (3× 300 μL). Afterwards, LGs were removed from the filtration columns and placed into vials containing chloroform (400 μL). The tissue was then disrupted by freezing in liquid nitrogen. After thawing, the vials were sonicated in a water bath for 15 min. An internal standard (1 μL 1-bromodecane, 1.9 mg mL^{-1}) was added to a portion (50 μL) of the extract before analysis. The prepared samples were analyzed by comprehensive two-dimensional gas chromatography with mass detection (Pegasus III GC×GC-MS; LECO Corp., St. Joseph, MI): splitless injection, inlet temperature 250 °C, injection volume 1 μL, He column flow-rate 1 mL min^{-1}, modulation time 4 s (hot pulse 0.5 s), modulation temperature offset 30 °C; first dimension column: DB-5 (30 m×250 μm×0.25 μm; J&W Scientific/Agilent Technologies); 60 °C (1 min), increase at 4 °C min^{-1}, 320 °C (15 min); second dimension column: BPX-50 (1.86 m×100 μm× 0.1 μm; SGE, Victoria, Australia); 70 °C (1 min), increase at 4 °C min^{-1}, 330 °C (15 min). Biosynthesis products were identified by comparing retention times and mass spectra to those of non-deuterated analogues as previously described.[22]

Acknowledgements

This work was supported by Czech Science Foundation project No. 15-06569S, by LO 1302 project from the Ministry of Education of the Czech Republic, and by project RVO 61388963. We are grateful to Heiko Vogel (Max Planck Institute for Chemical Ecology, Jena, Germany) for RNA-seq data assembly and annotation and to Hillary Hoffman for proof-reading the manuscript.

Keywords: biosynthesis · *Bombus* spp. · gene expression · isoprenoids · pheromones · transcriptional regulation

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Manuscript received: August 15, 2015
Accepted article published: December 3, 2015
Final article published: December 22, 2015