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RESEARCH ARTICLE

Monoterpenoid signals and their transcriptional responses to feeding and juvenile hormone regulation in bark beetle *Ips hauseri*

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ABSTRACT

Hauser's engraver beetle, *Ips hauseri*, is a serious pest in spruce forest ecosystems in Central Asia. Its monoterpenoid signal production, transcriptome responses and potential regulatory mechanisms remain poorly understood. The quality and quantity of volatile metabolites in hindgut extracts of *I. hauseri* were found to differ between males and females and among three groups: beetles that were newly emerged, those with a topical application of juvenile hormone III (JHIII) and those that had been feeding for 24 h. Feeding males definitively dominated monoterpenoid signal production in *I. hauseri*, which uses (4*S*)-(−)-ipsenol and (*S*)-(−)-*cis*-verbenol to implement reproductive segregation from *Ips typographus* and *Ips shangrila*. Feeding stimulation induced higher expression of most genes related to the biosynthesis of (4*S*)-(−)-ipsenol than JHIII induction, and showed a male-specific mode in *I. hauseri*. JHIII stimulated males to produce large amounts of (−)-verbenone and also upregulated the expression of several *CYP6* genes, to a greater extent in males than in females. The expression of genes involved in the metabolism of JHIII in females and males was also found to be upregulated. Our results indicate that a species-specific aggregation pheromone system for *I. hauseri*, consisting of (4*S*)-(−)-ipsenol and *S*-(−)-*cis*-verbenol, can be used to monitor population dynamics or mass trap killing. Our results also enable a better understanding of the bottom-up role of feeding behaviors in mediating population reproduction/aggregation and interspecific interactions.

KEY WORDS: Bark beetle, Bottom-up effects, Chemical communication, Species interactions, (4*S*)-(−)-Ipsenol, Transcriptome analysis

INTRODUCTION

Hauser's engraver beetle, *Ips hauseri* (Coleoptera: Curculionidae), mostly occurs in mountainous areas of Central Asia, where it causes severe damage to tree species in the genera *Picea*, *Pinus* and *Larix*, especially to Schrenk spruce (*Picea schrenkiana*), Siberian spruce (*Picea obovata*) and Scotch pine (*Pinus sylvestris*) (EPPO, 2005). The biological and ecological characteristics of *I. hauseri* have been well documented in the regions of its present distribution (Wen, 1992; EPPO, 2005; Vanhanen et al., 2008), where it usually develops one (at altitudes of 2200–3200 m) or two (at altitudes of 1200–1400 m) complete generations per year and prefers to attack the trunks and larger branches of weakened trees, mostly wind-felled trees or trees damaged by flooding. Numerous attacks from *I. hauseri* result in ecological impacts and economic consequences that are comparable to those of *Ips typographus* on other species of spruce.

Many conifer bark beetles exploit aggregation pheromones to coordinate mass attacks to overcome host defenses and achieve reproductive success (Blomquist et al., 2010; Keeling et al., 2016; Seybold et al., 2018). Male *Ips* bark beetles first colonize host trees and then construct nuptial chambers to prepare for mating. During this phase, *Ips* males biosynthesize and release aggregation pheromone (Birgersson and Bergström, 1989; Zhang et al., 2009; Seybold et al., 2018). The main aggregation pheromone compounds of *Ips* bark beetles are 2-methyl-3-butene-2-ol (MB), verbenol, *E*-myrcenol, ipsenol and ipsdienol (Schlyter et al., 1987, 1992; Ivarsson et al., 1993; Blomquist et al., 2010; Seybold et al., 2018). Two main biosynthetic pathways of these pheromone components have been developed: *de novo* biosynthesized pheromone components via the mevalonate (MVA) pathway, as in the case of the compounds MB, ipsenol and ipsdienol (Lanne et al., 1989; Ivarsson et al., 1993; Seybold et al., 1995); and those that are derived from an α-pinene transformation rather than *de novo* synthesis, such as the compounds verbenol and verbenone (Renwick et al., 1976; Fang et al., 2021). The molecular mechanisms underlying the *de novo* biosynthesis of these pheromones are studied in terms of the cloning, expression and functional identification of related genes in the MVA pathway, such as 3-hydroxy-3-methylglutaryl-CoA synthase (*HMGS*), 3-hydroxy-3-methylglutaryl-CoA reductase (*HMGR*) and geranyl diphosphate synthase (*GPPS*) (Martin et al., 2003; Blomquist et al., 2010; Sarabia et al., 2019). In addition, cytochrome P450 genes are also involved in the biosynthesis of aggregation pheromones in some bark beetles (Tittiger and Blomquist, 2017), such as *CYP9T* (which converts myrcene into ipsdienol) (Figueroa-Teran et al., 2012) and *CYP6* (which converts α-pinene into verbenol) (Chiu et al., 2019a,b). Likewise, some of these P450 genes are abundant in the antennae transcriptome of *Dendroctonus ponderosae*, suggesting specific or overlapping functions in olfaction and pheromone biosynthesis (Chiu et al., 2019c). The biosynthetic precursor of ipsenol could come from ipsdienol (Blomquist et al., 2010), when ipsdienol is oxidized to ipdienone and then reduced to ipsenone and finally to ipsenol (Fisher et al., 2021). Several genes and corresponding enzymes participate in this biosynthesis pathway, such as ipsdienol dehydrogenase (IDOLDH) (Figueroa-Teran et al., 2012) and ipsdienone reductase (Blomquist et al., 2010; Tittiger and Blomquist, 2017; Fisher et al., 2021). More importantly, the biosynthesis of these aggregation pheromones in *Ips* bark beetles is generally regulated by feeding and juvenile hormone III (JHIII) treatments (Tillman et al., 1998;

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Received 21 September 2020; Accepted 24 March 2021

Journal of Experimental Biology (2021) 224, jeb238030. doi:10.1242/jeb.238030

Blomquist et al., 2010), which can increase pheromone production via upregulated transcripts and enhanced enzyme activity for both HMGS and HMGR (Tillman et al., 2004). Nevertheless, feeding and JHIII show different regulation characteristics in different *Ips* species (Bearfield et al., 2009).

Feeding is usually a key driver for the bottom-up regulation of herbivore populations (Simpson et al., 2015), and it is important for the development of bark beetle populations, being involved in the production of pheromones that regulate the reproductive behaviors of bark beetles. Until now, little has been known about the composition of the reproductive signals of *I. hauseri* or its signaling regulation in relation to feeding behavior. In this study, we investigated volatile metabolites in the hindguts of *I. hauseri* in three groups of adults, namely, newly emerged adults, adults with topically applied JHIII and initial feeding adults. We also evaluated the electrophysiological and behavioral activities of volatile metabolites to confirm their potential roles as aggregation pheromones, and analyzed the differential responses of related genes in the MVA pathway in the three conditions. The elucidation of the monoterpenoid signals of *I. hauseri* and relative biosynthetic genes will advance our capacity to understand the feeding and reproductive strategies of *I. hauseri* and how to manage this economically important pest.

MATERIALS AND METHODS

Insect collection and treatments

Picea schrenkiana spruce logs (~50 cm long and ~10 cm in diameter) infested with immature adult *Ips hauseri* Reitter 1894 were shipped directly to a local insectarium in mid-July 2017 in Western Tien-Shan, China (43°11′10″N; 82°51′11″E; altitude, 1516 m). The emerging adults were collected every day and sampled in three treatments. Treatment 1 included newly emerged adult beetles. The hindgut glands of individuals of each sex were pulled out of the body directly with a fine forceps and extracted immediately with 20 µl high-performance liquid chromatography (HPLC)-grade hexane for 20 min in a glass tube. The samples were removed from the tubes, and the extracts were sealed and kept in a −20°C freezer until needed for chemical analysis (one hindgut extract per tube). Treatment 2 included beetles with topical application of JHIII. An aliquot of 1 µl JHIII (10 µg µl^{-1} in acetone) was topically applied to the ventral abdomens of newly emerged males and females, separately. We placed the JHIII-treated beetles individually into Petri dishes, where they were fed with smashed moist filter paper (5 g, Whatman) for 24 h, and then we extracted the hindgut glands following the procedure for treatment 1. Treatment 3 included individuals that had been feeding for 24 h. We inoculated the newly emerged male and female adults separately into uninfested *P. schrenkiana* logs and allowed them to feed on the host for 24 h. Then, we extracted the hindgut glands as in treatment 1. In addition, newly emerged males and females with topical application of 1 µl acetone were used as a negative control.

Chemical and electrophysiological analyses of hindgut extracts

GC and GC–MS analyses

The hindgut extracts (N=10) were analyzed using an HP 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a flame ionization detector (FID) and a CycloSil-B chiral capillary column [30 m×0.25 mm inner diameter (i.d.)×0.25 µm film thickness; J&W Scientific, Folsom, CA, USA]. The injector and detector temperatures were set to 200°C. The oven temperature was initially programmed to 50°C and held for 1 min, and was subsequently increased to 80°C at 6°C min^{-1}, then to 140°C at 2°C min^{-1}, and finally increased to 200°C at 20°C min^{-1}, where it was held for 2 min. Nitrogen was used as the carrier gas. Hindgut extracts were identified by comparing their retention times with those of synthetic standards. For further identification, all extracts (N=3) of each treatment were analyzed with a 7890 gas chromatograph,

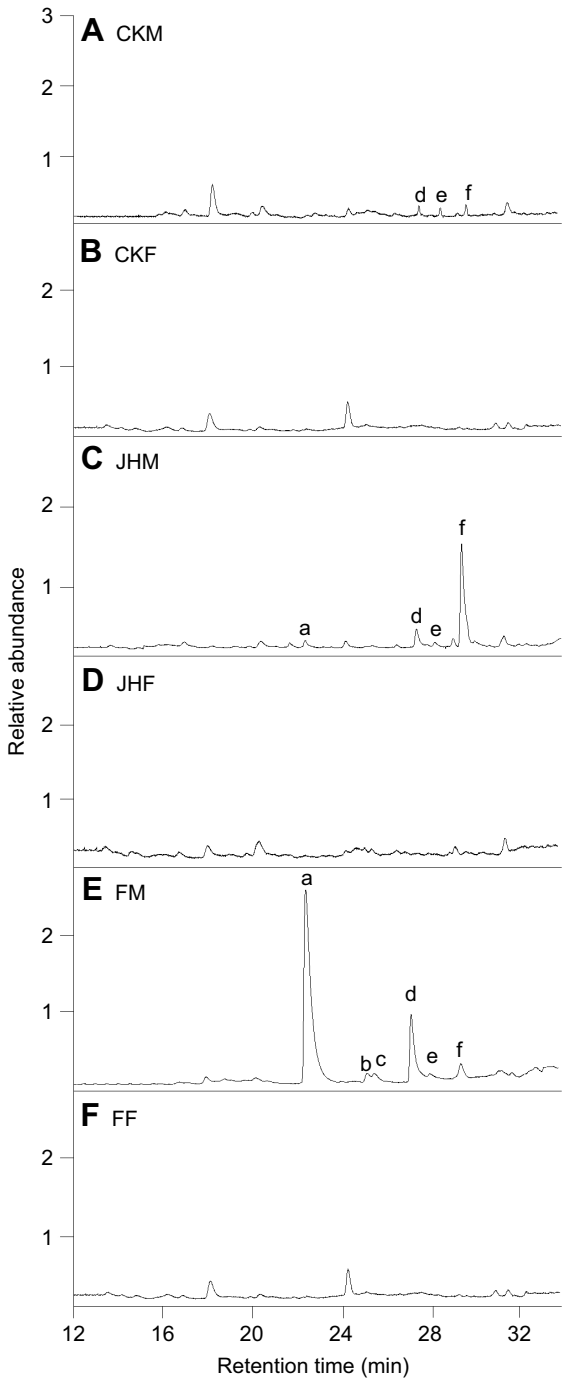

Fig. 1. Representative GC traces of volatile metabolites in hindgut extracts of male and female *Ips hauseri* under three conditions. (A–F) Analysis by gas chromatography (GC)–mass spectrometry (MS) on a CycloSil-B capillary column. Peak a, (4*S*)-(−)-ipsenol; peak b, (4*R*)-(−)-ipsdienol; peak c, (4*S*)-(+)-ipsdienol; peak d, *S*-(−)-*cis*-verbenol; peak e, (−)-*trans*-verbenol; peak f, (−)-verbenone. CKM, newly emerged male; CKF, newly emerged female; JHM, JHIII-treated male; JHF, JHIII-treated female; FM, feeding male; FF, feeding female.

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coupled with a 5975 mass spectrometer (Agilent Technologies; EI mode, 70 eV; mass range, 41–560 Da) on a CycloSil-B column (30 m×0.25 mm i.d.×0.25 µm), under the following conditions: injector, ion source and transfer line temperatures were held at 220°C, 200°C and 200°C, respectively; helium was used as the carrier gas at 1.0 ml min^{-1}; the scan time was 0.2 s; and the oven temperature program was the same as in the gas chromatography (GC) analyses. Compounds were identified by comparing their retention times and mass spectra with those of synthetic standards. Quantitative analyses via GC and GC–mass spectrometry (MS) were carried out with peak area comparisons in external standard heptyl acetate (5 ng/injection).

GC–EAD and EAG analyses

An aliquot of 1 µl male hindgut extract (a hindgut extract equivalent) as well as a synthetic mixture [60 ng (4*S*)-(−)-ipsenol, 60 ng (4*R*)-(+)-ipsenol, 60 ng (4*R*)-(−)-ipsdienol, 60 ng (4*S*)-(+)-ipsdienol, 60 ng *S*-(−)-*cis*-verbenol; 60 ng (−)-*trans*-verbenol, 4 ng (−)-verbenone] were injected splitlessly into an HP 6890 gas chromatograph equipped with a CycloSil-B column (30 m×0.25 mm i.d.×0.25 µm) and a 1:1 effluent splitter that allowed simultaneous recording of an FID and an electroantennogram (EAG) detector (EAD; Syntech, Kirchzarten, Germany) for the separated volatiles. Nitrogen was used as carrier gas at a constant flow mode (2.5 ml min^{-1}). The injector and detector temperatures were set at 200°C and 230°C, respectively. The oven temperature was set to 50°C for 1 min, increasing to 80°C at 10°C min^{-1} and then to 200°C at 3°C min^{-1}. EAG dose responses of males and females *I. hauseri* to (4*S*)-(−)-ipsenol and (*S*)-(−)-*cis*-verbenol were recorded using the same electrophysiological recording setup (Syntech) by applying them separately in 10 µl HPLC hexane onto a piece of filter paper (5×50 mm) in a Pasteur pipette and then tested at five doses (0.01–100 µg), from the lowest to the highest dose (N=15 for each sex, compound and dose). Each stimulus was followed by a ∼60 s purge period running humidified air (900 ml min^{-1}) over the antennae to ensure recovery of antennal receptors. Stimulus control (10 µl hexane) was done at the beginning and end of each preparation, and the mean response to the control was subtracted from each EAG measurement.

Field bioassays of the EAD-active volatile metabolites

A field trapping experiment was performed to evaluate the behavioral activity of key EAD-active volatiles from 2 to 15 June 2018, in a damaged *P. schrenkiana* stand in a valley of Western Tien-Shan that experienced a wind storm and flooding in 2017. Three sets of cross-barrier traps (Pherobio Technology Co., Ltd, Beijing, China) were deployed along the edge of a spruce forest stand with ∼10 m between traps within each set and >30 m between trap sets (blocks). Within the sets, three traps were baited with different blends of EAD-active volatile metabolites and the fourth trap was deployed with a blank lure. The basic lures consisted of 40 mg (4*S*)-(−)-ipsenol [97% purity; bubble cap dispenser, release rate ∼0.2 mg d^{-1}; Contech Enterprises, Victoria, Canada] and 40 mg (*S*)-(−)-*cis*-verbenol [95% purity; polyethylene dispenser, release rate ∼0.6 mg d^{-1}]. The (*S*)-(−)-*cis*-verbenol lures were formulated in the following way: 100 mg (*S*)-(−)-*cis*-verbenol was dissolved directly into 1 ml 1,3-butanediol solvent, and the solution was transferred onto 3 g cotton wool in a polyethylene bag (120 mm long×70 mm wide), sealed using a heat-sealing machine. Another field experiment was also conducted from 18 June to 6 July 2018, in the same location as the first field trial, to explore the interspecific interactions among pheromone signals of the closely related species *I. hauseri*, *I. typographus* and *Ips shangrila*. The lures containing 5 g MB (release rate ∼100 mg d^{-1}) were formulated as (*S*)-(−)-*cis*-verbenol lures. We purchased the (4*S*)-(+)-ipsdienol lures [97% purity, bubble cap lures, release rate ∼0.4 mg d^{-1}] from Contech Enterprises. Different lures were placed together or separately depending on the experimental purpose, such that the aggregation pheromone lures of *I. typographus* included MB and (*S*)-(−)-*cis*-verbenol, whereas those for *I. shangrila* included MB, (*S*)-(−)-*cis*-verbenol and (4*S*)-(+)-ipsdienol. All the dispensers were sealed separately in aluminium foil bags before being field tested. To minimize the positional effects, dispensers within each set were rotated after each observation. The numbers of *I. hauseri* captured in each trap were counted every 1–2 days, depending on the weather.

Transcriptome analyses

All living bark beetles from the three following treatments were dissected separately to collect the midgut tissues that were immediately frozen in liquid nitrogen for subsequent RNA extraction (Keeling et al., 2016; Nadeau et al., 2017): newly emerged males (CKM) and females (CKF) in treatment 1; JHIII-treated males (JHM) and females (JHF) in treatment 2; and feeding males (FM) and females (FF) in treatment 3. There were three biological replicates for each treatment of the six groups, and each replicate had 30 samples.

RNA extraction and sequencing

We extracted total RNA from the midguts using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) and removed genomic DNA using DNase I (TaKara Bio, Shiga, Japan). The integrity and purity of the total RNA was determined using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and quantified using a NanoDrop-2000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Wilmington, MA, USA). A high-quality RNA sample

Table 1. Amounts of volatile metabolites in hindgut extracts of adult *Ips hauseri* under three conditions

		Male hindgut extracts in different treatment conditions (ng per beetle, means±s.e.m., N=10 per condition)				
Peak	Volatile metabolite	CKM	JHM	FM	Statistical parameters	Female hindgut extract (N=10)
a	(4*S*)-(−)-ipsenol	–	0.25±0.03^{b}	95.16±7.63^{a}	t=57.31, d.f.=18, P<0.001	–
b	(4*R*)-(−)-ipsdienol	–	–	1.63±0.26	–	–
c	(4*S*)-(+)-ipsdienol	–	–	0.54±0.11	–	–
d	*S*-(−)-*cis*-verbenol	0.64±0.11^{b}	0.70±0.15^{b}	4.80±0.84^{a}	$F_{2,27}$=31.82, P<0.001	–
e	(−)-*trans*-verbenol	0.27±0.04^{b}	1.02±0.25^{a}	0.87±0.29^{b}	$F_{2,27}$=4.00, P=0.03	–
f	(−)-verbenone	0.96±0.34^{b}	25.79±6.18^{a}	2.12±0.47^{b}	$F_{2,27}$=45.99, P<0.001	–

Analysis by GC on a CycloSil-B capillary column. Different superscript lowercase letters indicate significantly different groups within each row among different conditions. For statistical analysis, t-test was used for peak a, and ANOVA for peaks d, e and f, at the level of α=0.05. –, compound not detected in hindgut extracts. CKM, newly emerged male; JHM, JHIII-treated male; FM, feeding male.

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(OD260/280=1.8–2.2, OD260/230≥2.0, RNA integrity number≥8.0, 28S:18S≥1.0, >2 µg) was used to construct a sequence library. RNA purification, reverse transcription, library construction and sequencing were performed at Majorbio Bio-pharm Biotechnology Co., Ltd (Shanghai, China). The RNA sequencing (RNA-seq) transcriptome libraries were prepared using a TruSeq™ RNA sample preparation kit (Illumina, San Diego, CA, USA). The synthesized cDNA was subjected to end repair, phosphorylation and A-base addition according to Illumina's library construction protocol. Libraries were size selected for cDNA target fragments of 200–300 bp on 2% Low-Range Ultra Agarose, followed by PCR amplification using Phusion DNA polymerase (New England Biolabs, Boston, MA, USA) for 15 PCR cycles. After being quantified by a TBS380 fluorometer (Turner BioSystems, Sunnyvale, CA, USA), two RNA-seq libraries were sequenced on an Illumina Hiseq Xten sequencer for 2×150 bp paired-end reads.

De novo assembly, annotation and differential expression analyses

The raw paired-end reads were trimmed and quality controlled using SeqPrep (https://github.com/jstjohn/SeqPrep) and Sickle (https://github.com/najoshi/sickle), according to the default parameters. Clean data from the samples were used to perform *de novo* assembly with Trinity (Grabherr et al., 2011). All of the assembled transcripts were searched against six databases using BLASTX (E-value<1.0×10^{-5}) to retrieve their functional annotations (https://www.blast2go.com/) (Conesa et al., 2005): the NR (https://www.ncbi.nlm.nih.gov/refseq/about/nonredundantproteins/), GO (http://geneontology.org/), COG (https://www.ncbi.nlm.nih.gov/research/cog-project/), KEGG (https://www.genome.jp/kegg/pathway.html), Swiss-Prot (https://www.uniprot.org/) and Pfam (http://pfam.xfam.org/) databases. According to the expression of transcripts in different treatments, principal component analyses were performed on the common and unique transcripts. RSEM (http://deweylab.biostat.wisc.edu/rsem/) was used to calculate the expression of the transcripts (Li and Dewey, 2011) and EdgeR (http://www.bioconductor.org/packages/2.12/bioc/html/edgeR.html) was used for differential expression analyses (Robinson et al., 2010). The distance calculation algorithm was used to cluster the expression patterns of differentially expressed genes (DEGs).

Chemicals

The chemicals 2-methyl-3-buten-2-ol (97% purity), (−)-verbenone (94% purity) and *S*-(−)-*cis*-verbenol (95% purity) were purchased from Acros Organics (Belgium, WI, USA), and (4*S*)-(−)-ipsenol (97% purity), (4*R*)-(+)-ipsenol (97% purity), (4*R*)-(−)-ipsdienol (97% purity), and (4*S*)-(+)-ipsdienol (97% purity) were purchased from Contech Enterprises. JHIII (95% purity) was purchased from Toronto Research Chemicals Inc. (Toronto, Canada). We synthesized (−)-*trans*-verbenol (95% purity) in our laboratory (Liu et al., 2020).

Statistical analyses

Log(X+1)-transformed data of the hindgut volatile metabolites were analyzed using one-way ANOVA, followed by a Tukey's HSD test at the level of α=0.05. In both field experiments, no bark beetles were caught in traps baited with blank controls, *S*-(−)-*cis*-verbenol or MB/*S*-(−)-*cis*-verbenol. These were excluded from analysis in order to avoid having treatments with zero mean and variance. The remaining trap catch data were converted into the proportion (P) of the total captured beetles within each set and observation, and were transformed by $\arcsin\sqrt{P}$ to meet the assumptions of normality and homoscedasticity, and then subjected to independent sample *t*-test (two-tailed) (SPSS 18.0 for Windows) at the level of α=0.05.

RESULTS

Volatile metabolites in hindgut extracts

We found significant differences in the volatile metabolic compositions of hindgut extracts between males and females and among the three treatments. No volatile metabolites of interest were

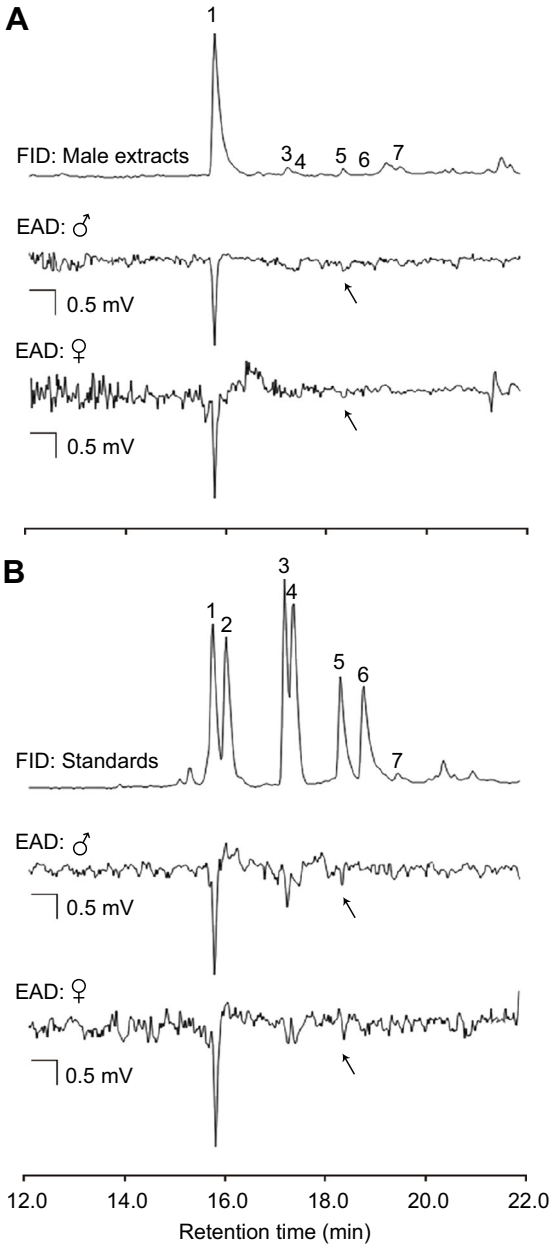

Fig. 2. GC–EAD responses of flying-period male and female *I. hauseri* to hindgut extracts of males after 24 h feeding and synthetic standards. (A) Responses to feeding male hindgut extracts. Peak 1, ~80 ng; peak 5, ~4 ng. (B) Responses to synthetic standards. Peaks 1–6, ~60 ng each; peak 7, ~4 ng. Peak 1, (4*S*)-(−)-ipsenol; peak 2, (4*R*)-(+)-ipsenol; peak 3, (4*R*)-(−)-ipsdienol; peak 4, (4*S*)-(+)-ipsdienol; peak 5, *S*-(−)-*cis*-verbenol; peak 6, (−)-*trans*-verbenol; peak 7, (−)-verbenone. FID, flame ionization detector; EAD, electroantennogram detector. The arrows indicate that the compound from male extract (A, peak 5) or synthetic compound (B, peak 5) showed weak EAD responses in the EAD traces.

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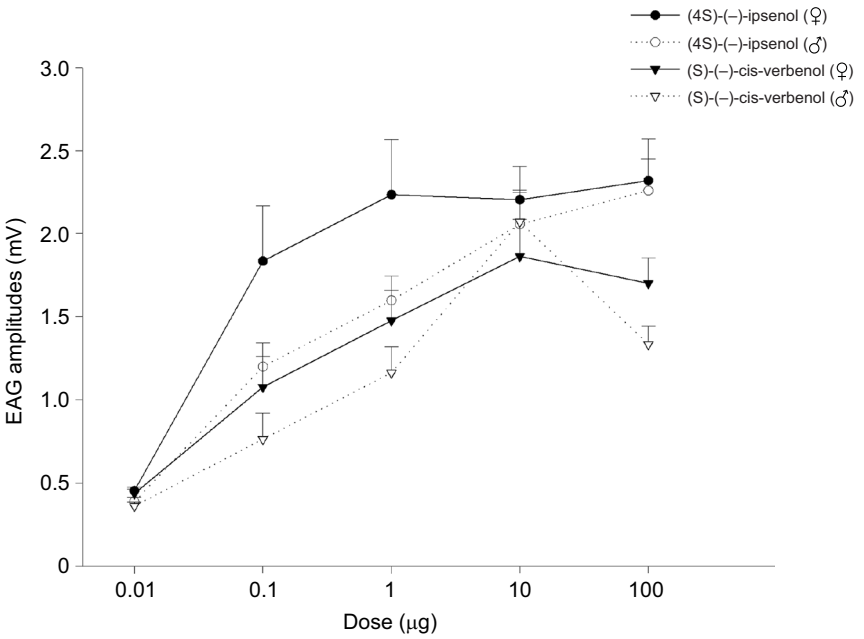

Fig. 3. EAG dose responses of antennae of male and female *I. hauseri* to synthetic (4*S*)-(−)-ipsenol and (*S*)-(−)-*cis*-verbenol. Data are means+s.e.m. (*N*=15). EAG, electroantennogram.

detected in extracts from females in any of the three treatment groups CKF, JHF and FF (Fig. 1B,D,F; Table 1). By contrast, we identified six volatile metabolites in males, and significant differences in quality and quantity were observed among the three treatment groups CKM, JHM and FM (Fig. 1A,C,E; Table 1).

Six metabolites were identified. Three were identified in CKM: *S*-(−)-*cis*-verbenol (Fig. 1A, peak d), (−)-*trans*-verbenol (Fig. 1A, peak e) and (−)-verbenone (Fig. 1A, peak f). Another one was found in JHM: (4*S*)-(−)-ipsenol (Fig. 1C, peak a). FM produced all four of these and another two: (4*R*)-(−)-ipsdienol (Fig. 1E, peak b) and (4*S*)-(+)-ipsdienol (Fig. 1E, peak c). Quantitatively, (4*S*)-(−)-ipsenol dominated the hindgut extracts of FM (Table 1, peak a, 95.16 ng per beetle), being found in amounts around 380 times greater than those in JHM, followed by *S*-(−)-*cis*-verbenol (Table 1, peak d, 4.80 ng per beetle), being found at around seven times the rate as in the other two male groups. Large amounts of (−)-verbenone (Table 1, peak f, 25.79 ng per beetle) were detected in JHM, which were only around 1/12 the amount in FM and 1/27 that in CKM.

Electrophysiological activity of volatile metabolites

Two of six volatile metabolites, (4*S*)-(−)-ipsenol (Fig. 2A, peak 1) and (*S*)-(−)-*cis*-verbenol (Fig. 2A, peak 5), were detected at a ∼20:1 ratio in post-feeding male extracts. We observed that (4*S*)-(−)-

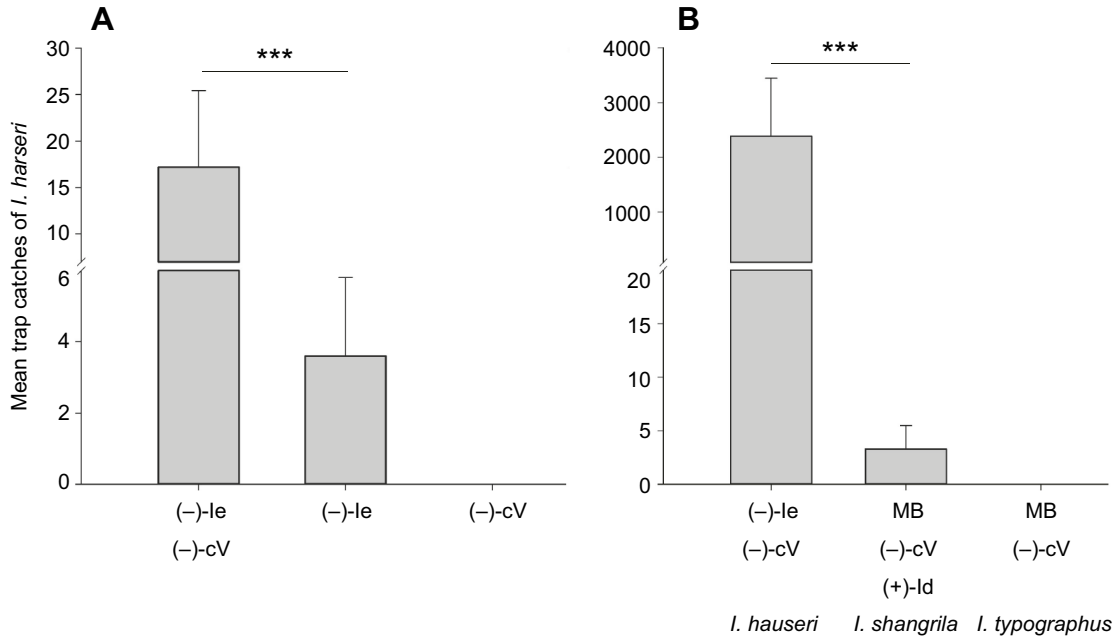

Fig. 4. Trap catches (number of beetles per trap per observation) of *I. hauseri* beetles baited with different combinations of semiochemicals. (A) Behavioral tests of EAD-active volatile metabolites in *I. hauseri* on 2–15 June 2018, *N*=6. (B) Behavioral tests of species-specific lures in *I. hauseri* on 18 June to 6 July 2018, *N*=6. (−)-Ie, (4*S*)-(−)-ipsenol; (−)-cV, (*S*)-(−)-*cis*-verbenol; (+)-Id, (4*S*)-(+)-ipsdienol; MB, 2-methyl-3-butene-2-ol. Data are means+s.e.m. ***P<0.001 (two-tailed t-test).

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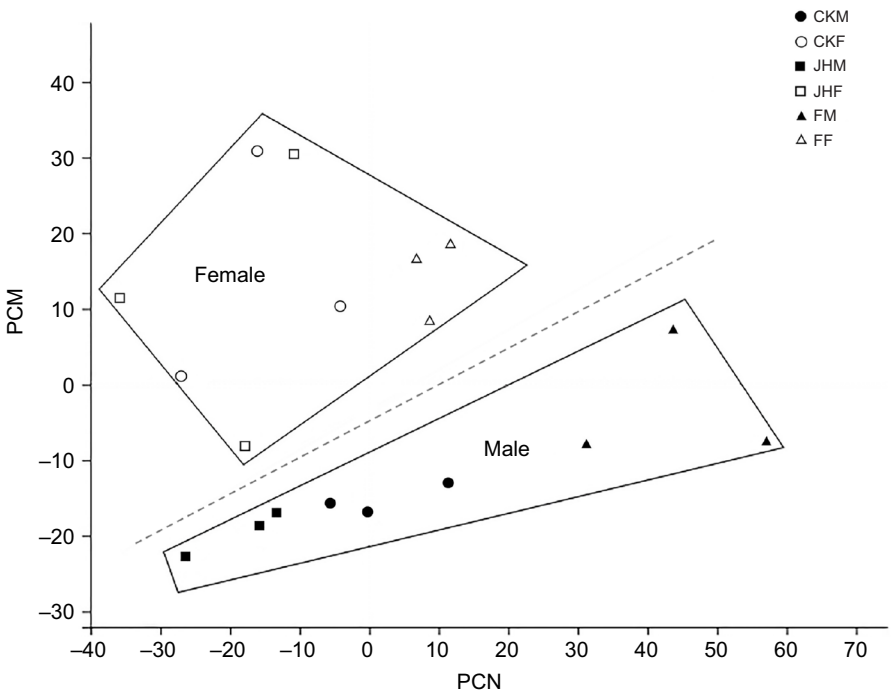

Fig. 5. Principal component analyses of differential gene expression between sexes and among conditions in *I. hauseri*. The graph shows relative coordinate points of the principal component after dimensional reduction analyses of samples. The closer together two sample points are, the higher the similarity between them. The abscissa represents the contribution of principal component N (PCN) to the differentiated samples in the two-dimensional graph, and the ordinate represents the contribution of principal component M (PCM) to the differentiated samples in the two-dimensional graph. CKM, newly emerged male; CKF, newly emerged female; JHM, JHIII-treated male; JHF, JHIII-treated female; FM, feeding male; FF, feeding female.

ipsenol consistently elicited very strong EAD responses, and (*S*)-(−)-*cis*-verbenol evoked very weak EAD activities for the antennae of both sexes (Fig. 2A). Synthetic (4*S*)-(−)-ipsenol and (*S*)-(−)-*cis*-verbenol retained the same elution times in the GC analyses, and the electrophysiological activities of the antennae of both sexes were the same as those from hindgut extracts (Fig. 2B), further confirming the identity of the two metabolites. We found that (−)-*trans*-verbenol (Fig. 2A, peak 6) and (−)-verbenone (Fig. 2A, peak 7) from hindgut extracts and their corresponding synthetics (Fig. 2B) did not activate any EAD response on the antennae of beetles of either sex. Trace amounts of (4*R*)-(−)-ipsdienol (Fig. 2A, peak 3) and (4*S*)-(+)-ipsdienol (Fig. 2A, peak 4) seemed to be able to evoke a very weak EAD reaction on the antennae of beetles of both sexes, and large amounts of corresponding synthetics confirmed their EAD activities.

We constructed dose–response curves with synthetic (4*S*)-(−)-ipsenol and (*S*)-(−)-*cis*-verbenol for male and female antennae, and these showed a dose-dependent response pattern (Fig. 3). Female antennae were more sensitive to (4*S*)-(−)-ipsenol than to (*S*)-(−)-*cis*-verbenol, producing 1.83±0.33 mV EAG amplitudes for (4*S*)-(−)-ipsenol and 1.07±0.18 mV for (*S*)-(−)-*cis*-verbenol at 0.1 µg stimulus loads, with a saturation level at 10 µg stimulus loads of the two synthetics. The male antennae showed a similar response pattern to (4*S*)-(−)-ipsenol and (*S*)-(−)-*cis*-verbenol in a dose range of 0.01–10 µg, and the EAG responses of (*S*)-(−)-*cis*-verbenol were always weaker than those of (4*S*)-(−)-ipsenol for male antennae.

Field bioassay of EAD-active volatile metabolites

We confirmed the bioactivity of (4*S*)-(−)-ipsenol and (*S*)-(−)-*cis*-verbenol in terms of the EAD responses they elicit in *I. hauseri* antennae by field trapping tests in a spruce forest stand. The lures that had both (4*S*)-(−)-ipsenol and (*S*)-(−)-*cis*-verbenol attracted significantly more *I. hauseri* beetles than the single lures, baited with only (4*S*)-(−)-ipsenol (t=−6.68, d.f.=10, P<0.001). Single lures baited with only (*S*)-(−)-*cis*-verbenol had no effect on *I. hauseri* beetles in the field (Fig. 4A).

To confirm whether the pheromone blends of *I. typographus* or *I. shangrila* had interspecific cross attraction for *I. hauseri*, we investigated and compared the bioactivity of their respective lures in field trapping tests (Fig. 4B). Lures with *I. typographus* blend formulated with MB and (*S*)-(−)-*cis*-verbenol had no effects on *I. hauseri*, and the lures with *I. shangrila* blend formulated with MB, (*S*)-(−)-*cis*-verbenol and (4*S*)-(+)-ipsdienol attracted only 20 *I. hauseri* beetles over the entire trapping period, a significantly lower rate than that of *I. hauseri* lures (t=102.7, d.f.=10, P<0.001).

Sequencing assembly and differential gene expression

To analyze the responses of related genes in the principal MVA pathway to monoterpenoid signals, we constructed RNA-seq libraries using 18 RNA samples from the midguts obtained from different treatments of *I. hauseri*. A total of 28,846 unigenes were generated, with an average length of 1233.1 bp and with 42.58% GC content (Table S1). All unigenes were aligned to six public databases: GO, KEGG, COG, NR, Swiss-Prot and Pfam (Fig. S1). The greatest similarity of unigenes between *I. hauseri* and another species in the NR database was found for *D. ponderosae* (45.64%) (Fig. S2). We assayed the levels of gene expression across different treatments of *I. hauseri* by using the DESeq functions for estimating size factors, with most variances being explained by sex and feeding treatment (Fig. 5). The numbers of upregulated genes were 676, 583 and 1556 for FF versus JHF, CKF versus JHF, and CKF versus FF, respectively, and 2942, 1470 and 2295 for FM versus JHM, CKM versus JHM, and CKM versus FM, respectively (Fig. S3). Venn diagram analyses showed that 810, 3369 and 6413 genes were independently expressed in CKM, JHM and FM, respectively, among which 6960 were shared (Fig. S4A). By comparison, 4672, 1206 and 967 genes were independently expressed in CKF, JHF and FF, respectively, among which 9175 were shared (Fig. S4B).

Journal of Experimental Biology (2021) 224, jeb238030. doi:10.1242/jeb.238030

Quantitative analyses of expression of key genes

We screened and analyzed 17 DEGs that might be involved in monoterpenoid signal production. All six treatments were clustered into two groups, with treated males as one group and treated females as another (Fig. 6), consistent with the PCA results (Fig. 5). *HMGS*, *FPPS*, *IDOLDH*, *MK* and *GPPS* were more highly expressed in FM than in CKM or JHM; however, *HMGR*, *CYP9T2* and *CYP6* were more highly expressed in JHM than in FM or CKM. The genes involved in the metabolism of JHIII featured different response modes (Fig. S5), in which most downstream genes were expressed at lower levels in FM and FF, such as the genes juvenile hormone epoxide hydrolase (*JHEH*) and juvenile hormone esterase (*JHE*).

DISCUSSION

In this study, we performed the first chemical analyses of volatile metabolites from *I. hauseri* in different conditions and clearly demonstrated that feeding males produce principal metabolic component (4*S*)-(−)-ipsenol and minor component (*S*)-(−)-*cis*-verbenol. These two metabolites function as an aggregation pheromone for *I. hauseri*, capable of monitoring outbreak dynamics or even mass trapping against beetles in spruce forests

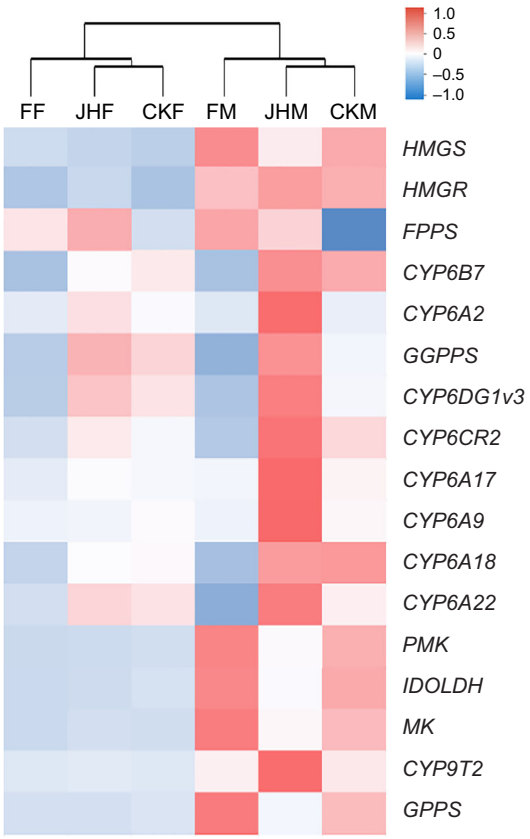

Fig. 6. Heatmap of differentially expressed genes associated with aggregation pheromone biosynthesis of *I. hauseri*. Each column represents a treatment condition (*N*=3), and each row represents a unigene. Different colors represent unigenes expressed in the sample at different levels, such that red represents a unigene expressed with a higher quantity in a sample and blue indicates lower expression; change trends are represented in the color bar in the top right-hand corner. A clustering tree graph is superimposed at the top of the heatmap. The closer the two sample branches are, the closer the expression patterns for all transcripts of the two samples, that is, the narrower the variation in unigene expression. CKM, newly emerged male; CKF, newly emerged female; JHM, JHIII-treated male; JHF, JHIII-treated female; FM, feeding male; FF, feeding female.

of Western Tien-Shan. Although trace amounts of (4*R*)-(−)-/(4*S*)-(+)-ipsdienol, (−)-*trans*-verbenol and (−)-verbenone were also detected in males, their biological activities for *I. hauseri* and ecological functions for interspecific interactions must be verified in future studies. None of the six metabolites were detected in females, which supports earlier findings that pheromone production in *Ips* bark beetles during attacks are dominated by males (Cognato, 2015).

In Northwest China, *I. shangrila*, *I. typographus* and *I. hauseri* are very important bark beetles for spruce forests, where their intermittent outbreaks greatly destroy the spruce forest ecosystem (Zhang et al., 2009; Cognato, 2015). Our results unequivocally support the conclusion that key chemical signals implement communication barriers between these three *Ips* bark beetles. Bakke (1976) reported that the binary blend of (4*S*)-(−)-ipsenol and (*S*)-(−)-*cis*-verbenol cannot be considered an aggregation pheromone of *I. typographus*, the pheromone components of which mainly consist of (*S*)-(−)-*cis*-verbenol and MB (Schlyter et al., 1987; Birgersson and Bergström, 1989). Bakke (1981) also reported that ipsenol exhibits an inhibiting effect on *I. typographus*. In addition, *I. shangrila* exploits the blend of MB, (*S*)-(−)-*cis*-verbenol and (4*S*)-(+)-ipsdienol to initiate mass attacks and to find mates (Zhang et al., 2009), which in our study showed a very weak effect on *I. hauseri* in field trapping tests. However, this ternary blend has been found to attract *I. typographus* beetles (Schlyter et al., 1992), but (4*S*)-(+)-ipsdienol is not an essential component for *I. typographus* (Schlyter et al., 1987) and *I. hauseri* in our study.

Feeding on phloem can greatly stimulate the corpora allata to synthesize and release JHIII, which induces the production of ipsdienol and its derivatives (Tillman et al., 1998). The principal pheromone component of *I. hauseri*, (4*S*)-(−)-ipsenol, is mainly biosynthesized *de novo* via the MVA pathway (Ivarsson et al., 1993; Seybold et al., 1995; Tillman et al., 1998; Gilg et al., 2005). Our results suggest the paramount importance of the feeding behaviors of male *I. hauseri* in triggering monoterpenoid signal production, and that these feeding behaviors have greater effects on monoterpenoid signal production than exogenous JHIII stimulation. Notably, topically applied JHIII cannot increase the production of (4*S*)-(−)-ipsenol, ipsdienol or verbenol for *I. hauseri*, supporting the functional divergence of exogenous JHIII in the pheromone production of *Ips pini* (Tillman et al., 1998; Martin et al., 2003; Gilg et al., 2005; Keeling et al., 2006), *Ips duplicatus* (Ivarsson and Birgersson, 1995), *Ips paraconfusus* and *Ips confusus* (Bearfield et al., 2009). Furthermore, we found that JHIII can stimulate large amounts of (−)-verbenone in male *I. hauseri* beetles, indicating that JHIII might be involved in the biosynthesis of verbenone from its host α-pinene precursor through intermediate verbenol (Renwick et al., 1976). Trace amounts of (*S*)-(−)-*cis*-verbenol, (−)-*trans*-verbenol and (−)-verbenone are also present in newly emerged male adults of *I. hauseri*, which might originate during the conversion of abundant precursors of monoterpenyl esters, as observed in *D. ponderosae* (Chiu et al., 2018). Thus, conclusive evidence regarding whether large amounts of verbenyl oleate and verbenyl palmitate are accumulated in the body of *I. hauseri* larvae remains to be found.

Further, key gene transcripts such as *GPPS* and *IDOLDH* are almost exclusively expressed and definitively dominated by *I. hauseri* males after 24 h feeding, with a similar result observed in *I. pini* (Figueroa-Teran et al., 2012). The *CYP9T2* gene is abundantly expressed in males, particularly in JHIII-treated males. Likewise, the protein products of these genes are key enzymes in the biosynthesis of (4*S*)-(−)-ipsenol: GPPS in *I. pini* and *I. confusus* has both GPPS activity and myrcene synthase activity (Gilg et al.,

2009), which catalyzes the formation of myrcene (Martin et al., 2003; Gilg et al., 2005); myrcene hydroxylase (CYP9T1/2) hydroxidizes myrcene to ipsdienol, mostly (4*R*)-(−)-antipode (Sandstrom et al., 2006, 2008); and IDOLDH oxidoreduces (4*R*)-(−)-ipsdienol to ipsenol via ipsdienone intermediate (Blomquist et al., 2010; Figueroa-Teran et al., 2012). The transcription levels of these three key genes are basically in accord with the observations that trace amounts of (4*S*)-(−)-ipsenol are detected in JHIII-treated *I. hauseri* males, whereas large amounts are produced in feeding males, results that are very similar to those of a previous report on *I. confusus* (Bearfield et al., 2009). Verbenol is a monoterpene alcohol from host α-pinene, which can be oxidized to verbenone (Birgersson and Bergström, 1989), and in which the cytochrome P450 (Blomquist et al., 2010; Tittiger and Blomquist, 2017) as well as endosymbiotic microorganisms resident in the alimentary canal (Leufvén et al., 1984; Hunt and Borden, 1990; Xu et al., 2015) play an important role. The *CYP6* genes are usually involved in detoxification of plant allelochemicals (Li et al., 2004; Chiu et al., 2019b; Nadeau et al., 2017); for example, *CYP6DE1* participates in converting α-pinene into verbenol in *D. ponderosae* (Chiu et al., 2019a). In our results, *CYP6* genes were highly expressed in JHIII-treated *I. hauseri* males, suggesting that they might be involved in the biosynthesis of (*S*)-(−)-*cis*-verbenol, (−)-*trans*-verbenol and (−)-verbenone, or in the metabolism of JHIII, to some extent.

Conclusion

A species-specific aggregation pheromone blend has been identified in *I. hauseri* consisting of (4*S*)-(−)-ipsenol and *S*-(−)-*cis*-verbenol, the origins of which represent two typical biosynthetic pathways in *Ips* bark beetles. Feeding on host phloem alone strongly induces pheromone production in male *I. hauseri*, whereas JHIII treatment has a very weak effect. However, feeding and JHIII treatments both significantly upregulate mRNA levels of key mevalonate pathway genes, meaning that (4*S*)-(−)-ipsenol is essential for the binary pheromone blend. These results advance our understanding of the biosynthesis of the aggregation pheromone in *Ips* bark beetles in relation to feeding and endocrine regulation, and enable the development of an effective control measure against this pest.

Acknowledgements

We acknowledge the constructive comments of two anonymous reviewers and the handling editor, Dr Julian Dow, as well as the help they gave in revising the manuscript. We also thank Dr Li Wen Song (Institute of Forest Protection, Jilin Provincial Academy of Forestry Sciences) for the field bioassay.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: Z.Z., X.B.K.; Methodology: Z.Z., X.B.K.; Formal analysis: J.X.F., H.C.D., F.L.; Investigation: J.X.F., H.C.D., X.S.; Writing - original draft: J.X.F., S.F.Z., X.B.K.; Writing - review & editing: P.J.Z., X.B.K.; Supervision: X.B.K.; Project administration: X.B.K.; Funding acquisition: X.B.K.

Funding

This project received funding from the Fundamental Research Funds of the Chinese Academy of Forestry (CAFYBB2017ZB002) and partial financial support from the National Key Research and Development Program of China (project no. 2017YFD0600103).

Data availability

All data for statistical analyses are publicly available from the figshare repository (doi:10.6084/m9.figshare.13888367). All quality-trimmed reads of transcriptome used in this study are available for download at the Short Read Archive (study accession number SRP306799; https://trace.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?study=SRP306799).

References

Bakke, A. (1976). Spruce bark beetle, *Ips typographus*: pheromone production and field response to synthetic pheromones. *Naturwissenschaften* **63**, 92. doi:10.1007/BF00622413

Bakke, A. (1981). Inhibition of the response in *Ips typographus* to the aggregation pheromone; field evaluation of verbenone and ipsenol. *Z. Ang. Ent.* **92**, 172-177. doi:10.1111/j.1439-0418.1981.tb01666.x

Bearfield, J. C., Henry, A. G., Tittiger, C., Blomquist, G. J. and Ginzel, M. D. (2009). Two regulatory mechanisms of monoterpenoid pheromone production in *Ips* spp. of bark beetles. *J. Chem. Ecol.* **35**, 689-697. doi:10.1007/s10886-009-9652-2

Birgersson, G. and Bergström, G. (1989). Volatiles released from individual spruce bark beetle entrance holes quantitative variations during the first week of attack. *J. Chem. Ecol.* **15**, 2465-2483. doi:10.1007/BF01020377

Blomquist, G. J., Figueroa-Teran, R., Aw, M., Song, M., Gorzalski, A., Abbott, N. L., Chang, E. and Tittiger, C. (2010). Pheromone production in bark beetles. *Insect Biochem. Mol. Biol.* **40**, 699-712. doi:10.1016/j.ibmb.2010.07.013

Chiu, C. C., Keeling, C. I. and Bohlmann, J. (2018). Monoterpenyl esters in juvenile mountain pine beetle and sex-specific release of the aggregation pheromone *trans*-verbenol. *Proc. Natl. Acad. Sci. USA* **115**, 3652-3657. doi:10.1073/pnas.1722380115

Chiu, C. C., Keeling, C. I. and Bohlmann, J. (2019a). The cytochrome P450 CYP6DE1 catalyzes the conversion of α-pinene into the mountain pine beetle aggregation pheromone *trans*-verbenol. *Sci. Rep.* **9**, 1477. doi:10.1038/s41598-018-38047-8

Chiu, C. C., Keeling, C. I., Henderson, H. M. and Bohlmann, J. (2019b). Functions of mountain pine beetle cytochromes P450 CYP6DJ1, CYP6BW1 and CYP6BW3 in the oxidation of pine monoterpenes and diterpene resin acids. *PLoS ONE* **14**, e0216753. doi:10.1371/journal.pone.0216753

Chiu, C. C., Keeling, C. I. and Bohlmann, J. (2019c). Cytochromes P450 preferentially expressed in antennae of the mountain pine beetle. *J. Chem. Ecol.* **45**, 178-186. doi:10.1007/s10886-018-0999-0

Cognato, A. I. (2015). Biology, systematics, and evolution of *Ips*. In *Bark Beetles: Biology and Ecology of Native and Invasive Species* (ed. F. E. Vega and R. W. Hofstetter), pp. 351-370. Pittsburgh, USA: Academic Press.

Conesa, A., Götz, S., García-Gómez, J. M., Terol, J., Talón, M. and Robles, M. (2005). Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* **21**, 3674-3676. doi:10.1093/bioinformatics/bti610

EPPO (2005). *Ips hauseri*. *EPPO Bull.* **35**, 450-452. doi:10.1111/j.1365-2338.2005.00881.x

Fang, J. X., Zhang, S. F., Liu, F., Cheng, B., Zhang, Z., Zhang, Q. H. and Kong, X. B. (2021). Functional investigation of monoterpenes for improved understanding of the relationship between hosts and bark beetles. *J. App. Entomol.* **145**, 303-311. doi:10.1111/jen.12850

Figueroa-Teran, R., Welch, W. H., Blomquist, G. J. and Tittiger, C. (2012). Ipsdienol dehydrogenase (IDOLDH): a novel oxidoreductase important for *Ips pini* pheromone production. *Insect Biochem. Mol. Biol.* **42**, 81-90. doi:10.1016/j.ibmb.2011.10.009

Fisher, K. E., Tillett, R. L., Fotoohi, M., Caldwell, C., Petereit, J., Schlauch, K., Tittiger, C., Blomquist, G. J. and MacLean, M. (2021). RNA-Seq used to identify ipsdienone reductase (IDONER): a novel monoterpene carbon-carbon double bond reductase central to Ips confusus pheromone production. *Insect Biochem. Mol. Biol.* **129**, 103513. doi:10.1016/j.ibmb.2020.103513

Gilg, A. B., Bearfield, J. C., Tittiger, C., Welch, W. H. and Blomquist, G. J. (2005). Isolation and functional expression of an animal geranyl diphosphate synthase and its role in bark beetle pheromone biosynthesis. *Proc. Natl. Acad. Sci. USA* **102**, 9760-9765. doi:10.1073/pnas.0503277102

Gilg, A. B., Tittiger, C. and Blomquist, G. J. (2009). Unique animal prenyltransferase with monoterpene synthase activity. *Naturwissenschaften* **96**, 731-735. doi:10.1007/s00114-009-0521-1

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q. et al. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat. Biotechnol.* **29**, 644-652. doi:10.1038/nbt.1883

Hunt, D. W. A. and Borden, J. H. (1990). Conversion of verbenols to verbenone by yeasts isolated from *Dendroctonus ponderosae* (Coleoptera: Scolytidae). *J. Chem. Ecol.* **16**, 1385-1397. doi:10.1007/BF01021034

Ivarsson, P. and Birgersson, G. (1995). Regulation and biosynthesis of pheromone components in the double spined bark beetle *Ips duplicatus* (Coleoptera: Scolytidae). *J. Insect Physiol.* **41**, 843-849. doi:10.1016/0022-1910(95)00052-V

Ivarsson, P., Schlyter, F. and Birgersson, G. (1993). Demonstration of *de novo* pheromone biosynthesis in *Ips duplicatus* (Coleoptera: Scolytidae): inhibition of ipsdienol and *E*-myrcenol production by compactin. *Insect Biochem. Mol. Biol.* **23**, 655-662. doi:10.1016/0965-1748(93)90039-U

Keeling, C. I., Bearfield, J. C., Young, S., Blomquist, G. J. and Tittiger, C. (2006). Effects of juvenile hormone on gene expression in the pheromone-producing midgut of the pine engraver beetle, *Ips pini*. *Insect Mol. Biol.* **15**, 207-216. doi:10.1111/j.1365-2583.2006.00629.x

Keeling, C. I., Li, M., Sandhu, H. K., Henderson, H., Yuen, M. M. S. and Bohlmann, J. (2016). Quantitative metabolome, proteome and transcriptome analysis of midgut and fat body tissues in the mountain pine beetle, *Dendroctonus ponderosae* Hopkins, and insights into pheromone biosynthesis. *Insect Biochem. Mol. Biol.* **70**, 170-183. doi:10.1016/j.ibmb.2016.01.002

Lanne, B. S., Ivarsson, P., Johnsson, P., Bergström, G. and Wassgren, A.-B. (1989). Biosynthesis of 2-methyl-3-buten-2-ol, a pheromone component of *Ips typographus* (Coleoptera: Scolytidae). *Insect Biochem.* **19**, 163-167. doi:10.1016/0020-1790(89)90087-5

Leufvén, A., Bergström, G. and Falsen, E. (1984). Interconversion of verbenols and verbenone by identified yeasts isolated from the spruce bark *beetleIps typographus*. *J. Chem. Ecol.* **10**, 1349-1361. doi:10.1007/BF00988116

Li, B. and Dewey, C. N. (2011). RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* **12**, 323. doi:10.1186/1471-2105-12-323

Li, X. C., Baudry, J., Berenbaum, M. R. and Schuler, M. A. (2004). Structural and functional divergence of insect CYP6B proteins: from specialist to generalist cytochrome P450. *Proc. Natl. Acad. Sci. USA* **101**, 2939-2944. doi:10.1073/pnas.0308691101

Liu, F., Fang, J.-X., Kong, X.-B., Zhang, S.-F. and Zhang, Z. (2020). Stereospecific synthesis of *S*-(–)-*trans*-verbenol and its antipode by inversion of sterically hindered alcohols. *J. Asian Nat. Prod. Res*. doi:10.1080/10286020.2020.1839433

Martin, D., Bohlmann, J., Gershenzon, J., Francke, W. and Seybold, S. J. (2003). A novel sex-specific and inducible monoterpene synthase activity associated with a pine bark beetle, the pine engraver, *Ips pini*. *Naturwissenschaften* **90**, 173-179. doi:10.1007/s00114-003-0410-y

Nadeau, J. A., Petereit, J., Tillett, R. L., Jung, K., Fotoohi, M., MacLean, M., Young, S., Schlauch, K., Blomquist, G. J. and Tittiger, C. (2017). Comparative transcriptomics of mountain pine beetle pheromone-biosynthetic tissues and functional analysis of CYP6DE3. *BMC Genomics* **18**, 311. doi:10.1186/s12864-017-3696-4

Renwick, J. A., Hughes, P. R. and Krull, I. S. (1976). Selective production of *cis*- and *trans*-verbenol from (–)- and (+)-α- by a bark beetle. *Science* **191**, 199-201. doi:10.1126/science.1246609

Robinson, M. D., McCarthy, D. J. and Smyth, G. K. (2010). edgeR: a bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**, 139-140. doi:10.1093/bioinformatics/btp616

Sandstrom, P., Welch, W. H., Blomquist, G. J. and Tittiger, C. (2006). Functional expression of a bark beetle cytochrome P450 that hydroxylates myrcene to ipsdienol. *Insect Biochem. Mol. Biol.* **36**, 835-845. doi:10.1016/j.ibmb.2006.08.004

Sandstrom, P., Ginzel, M. D., Bearfield, J. C., Welch, W. H., Blomquist, G. J. and Tittiger, C. (2008). Myrcene hydroxylases do not determine enantiomeric composition of pheromonal ipsdienol in *Ips* spp. *J. Chem. Ecol.* **34**, 1584-1592. doi:10.1007/s10886-008-9563-7

Sarabia, L. E., López, M. F., Obregón-Molina, G., Cano-Ramírez, C., Sánchez-Martínez, G. and Zúñiga, G. (2019). The differential expression of mevalonate pathway genes in the gut of the bark beetle *Dendroctonus rhizophagus* (Curculionidae: Scolytinae) is unrelated to the *de novo* synthesis of terpenoid pheromones. *Int. J. Mol. Sci.* **20**, 4011. doi:10.3390/ijms20164011

Schlyter, F., Birgersson, G., Byers, J. A., Löfqvist, J. and Bergström, G. (1987). Field response of spruce bark beetle, *Ips typographus*, to aggregation pheromone candidates. *J. Chem. Ecol.* **13**, 701-716. doi:10.1007/BF01020153

Schlyter, F., Birgersson, G., Byers, J. A. and Bakke, A. (1992). The aggregation pheromone of*Ips duplicatus* and its role in competitive interactions with*I. typographus* (Coleoptera: Scolytidae). *Chemoecology* **3**, 103-112. doi:10.1007/BF01370137

Seybold, S. J., Quilici, D. R., Tillman, J. A., Vanderwel, D., Wood, D. L. and Blomquist, G. J. (1995). *De novo* biosynthesis of the aggregation pheromone components ipsenol and ipsdienol by the pine bark beetles *Ips paraconfusus* Lanier and *Ips pini* (Say) (Coleoptera: Scolytidae). *Proc. Natl. Acad. Sci. USA* **92**, 8393-8397. doi:10.1073/pnas.92.18.8393

Seybold, S. J., Bentz, B. J., Fettig, C. J., Lundquist, J. E., Progar, R. A. and Gillette, N. E. (2018). Management of Western North American bark beetles with semiochemicals. *Annu. Rev. Entomol.* **63**, 407-432. doi:10.1146/annurev-ento-020117-043339

Simpson, S. J., Clissold, F. J., Lihoreau, M., Ponton, F., Wilder, S. M. and Raubenheimer, D. (2015). Recent advances in the integrative nutrition of arthropods. *Annu. Rev. Entomol.* **60**, 293-311. doi:10.1146/annurev-ento-010814-020917

Tillman, J. A., Holbrook, G. L., Dallara, P. L., Schal, C., Wood, D. L., Blomquist, G. J. and Seybold, S. J. (1998). Endocrine regulation of *de novo* aggregation pheromone biosynthesis in the pine engraver, *Ips pini* (Say) (Coleoptera: Scolytidae). *Insect Biochem. Mol. Biol.* **28**, 705-715. doi:10.1016/S0965-1748(97)00117-3

Tillman, J. A., Lu, F., Goddard, L. M., Donaldson, Z. R., Dwinell, S. C., Tittiger, C., Hall, G. M., Storer, A. J., Blomquist, G. J. and Seybold, S. J. (2004). Juvenile hormone regulates *de novo* isoprenoid aggregation pheromone biosynthesis in pine bark beetles, Ips spp., through transcriptional control of HMG-CoA reductase. *J. Chem. Ecol.* **30**, 2459-2494. doi:10.1007/s10886-004-7945-z

Tittiger, C. and Blomquist, G. J. (2017). Pheromone biosynthesis in bark beetles. *Curr. Opin. Insect Sci.* **24**, 68-74. doi:10.1016/j.cois.2017.09.005

Vanhanen, H., Veteli, T. and Niemelä, P. (2008). Potential distribution ranges in Europe for *Ips hauseri*, *Ips subelongatus* and *Scolytus morawitzi*, a climex analysis. *EPPO Bull.* **38**, 249-258. doi:10.1111/j.1365-2338.2008.01212.x

Wen, S. Y. (1992). *Ips hauseri* Reitter. In *Forest Insects of China* (ed. G. R. Xiao), pp. 622-623. Beijing, China: Forestry Publishing House.

Xu, L., Lou, Q., Cheng, C., Lu, M. and Sun, J. (2015). Gut-associated bacteria of *Dendroctonus valens* and their involvement in verbenone production. *Microb. Ecol.* **70**, 1012-1023. doi:10.1007/s00248-015-0625-4

Zhang, Q.-H., Song, L.-W., Ma, J.-H., Han, F.-Z. and Sun, J.-H. (2009). Aggregation pheromone of a newly described spruce bark beetle, *Ips shangrila* Cognato and Sun from China. *Chemoecology* **19**, 203-210. doi:10.1007/s00049-009-0026-6