

# Two Regulatory Mechanisms of Monoterpenoid Pheromone Production in *Ips* spp. Of Bark Beetles

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**Abstract** Bark beetles use aggregation pheromones to coordinate host colonization and mating. These monoterpenoid chemical signals are produced *de novo* in midgut cells via the mevalonate pathway, and pheromone production is induced when an adult beetle feeds on phloem of a host tree. In *Ips pini*, juvenile hormone (JH) III influences key regulatory enzymes along the mevalonate pathway that leads to pheromone production. In fact, topically applied JH III is sufficient to stimulate pheromone production in unfed males. In this study, we explore the influence of feeding and JH III treatment on pheromone production in male *Ips confusus*, the pinyon *Ips*. We also characterize the influence of feeding and JH III treatment on transcript levels and activity of three key enzymes involved in pheromone biosynthesis: 3-hydroxy-3-methylglutaryl-CoA (HMG) synthase (HMGS), HMG-CoA reductase (HMGR) and geranyl diphosphate synthase (GPPS). We also extend the current understanding of the regulation of pheromone biosynthesis in *I. pini*, by measuring the influence of feeding and JHIII treatment on enzymatic activity of HMGS and GPPS. Feeding on host phloem alone strongly

induces pheromone production in male *I. confusus*, while JH III treatment has no effect. However, feeding and JH III both significantly up-regulate mRNA levels of key mevalonate pathway genes. Feeding up-regulates these genes to a maximum at 32 h, whereas with JH III-treatment, they are up-regulated at 4, 8, and 16 h, but return near to non-treatment levels at 32 h. Feeding, but not JH III treatment, also increases the activity of all three enzymes in *I. confusus*, while both feeding or treatment with JH III increase HMGS and GPPS activity in *I. pini*. Our data suggest that pheromone production in *Ips* is not uniformly controlled by JH III and feeding may stimulate the release of some other regulatory factor, perhaps a brain hormone, required for pheromone production.

**Keywords** *Ips confusus* · *Ips pini* · Pheromones · Juvenile hormone · Mevalonate pathway

## Introduction

In *Ips* species, production of aggregation pheromones is induced when a pioneering adult beetle feeds on host phloem while attacking a tree. If a sufficient number of beetles respond to these chemical signals, host defenses can be overcome and the tree colonized. For example, male *I. confusus* produce a pheromone of monoterpenoid alcohols ipsenol, ipsdienol, and *cis*-verbenol (Birch et al. 1977). In *I. pini*, ipsenol and ipsdienol are produced *de novo* in midgut tissues via the mevalonate pathway (Seybold et al. 1995b; Ivarsson et al. 1998; Hall et al. 2002a). It is clear that in *I. pini* and other *Ips* species juvenile hormone (JH) III influences key regulatory enzymes along the mevalonate pathway that leads to pheromone production (see Fig. 1; Byers and Birgersson 1990; Ivarsson et al. 1993; Seybold et

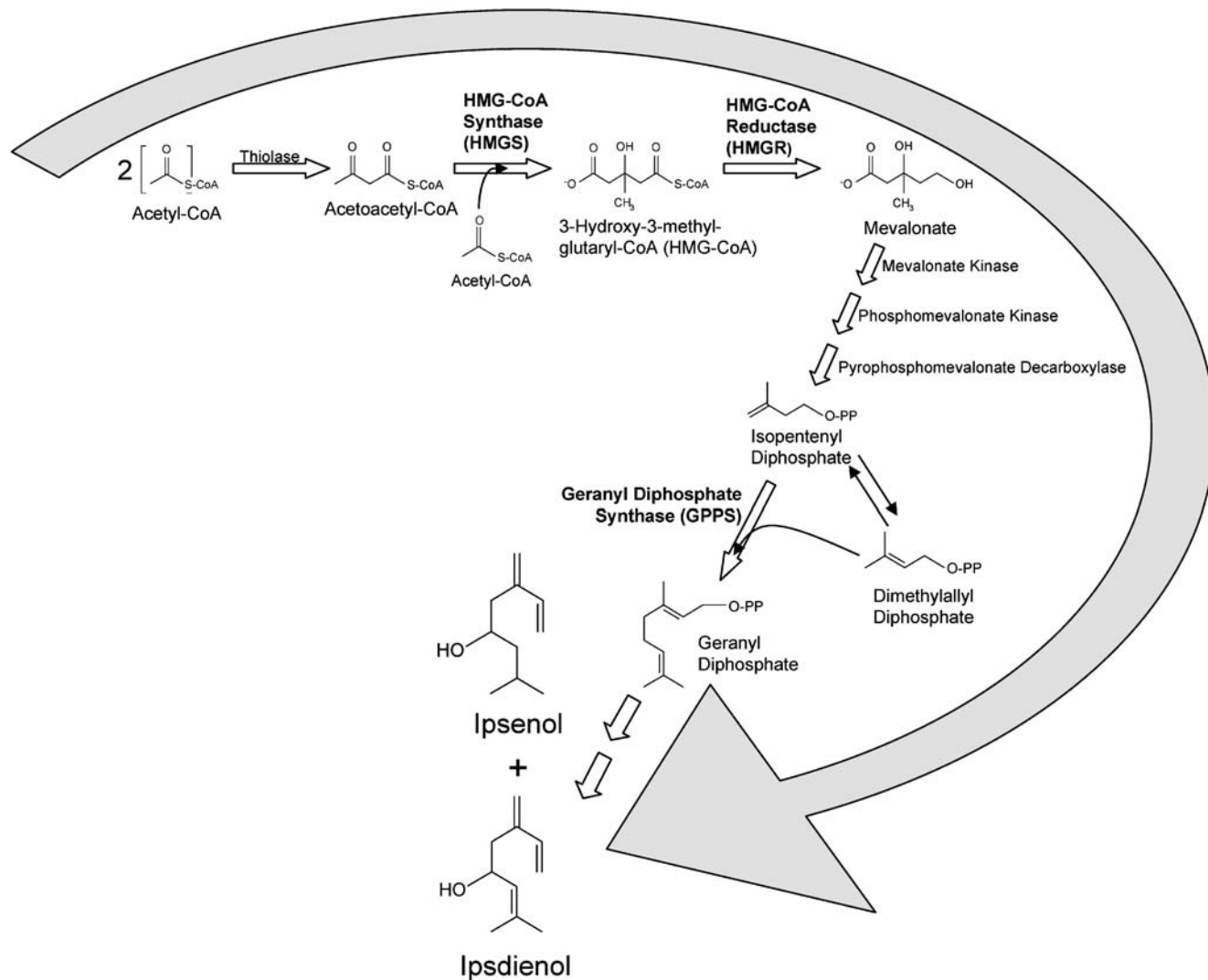
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**Fig. 1** Mevalonate pathway genes and associated enzymes involved in biosynthesis and regulation of pheromone production in *Ips* spp

al. 1995a; Seybold et al. 1995b; Tillman et al. 1998). In male *I. paraconfusus* and *I. pini*, JH III regulates mRNA levels of 3-hydroxy-3-methylglutaryl-CoA (HMG) reductase (HMGR) (Tittiger et al. 1999), the first committed and a highly regulated step in the mevalonate pathway. Moreover, feeding and JH III treatment strongly up-regulate levels of HMGR transcript in the anterior midgut tissues of *I. pini* (Keeling et al. 2004). From these studies, it appears that in *I. pini* feeding on host phloem stimulates JH III synthesis by the *corpus allatum*, which, in turn, increases the expression level and enzyme activity of HMGR in the mevalonate pathway resulting in high levels of pheromone production (Tillman et al. 1998). In addition, JH III also upregulates transcription of other mevalonate pathway genes, including HMG-CoA synthase (HMGS) and geranyl diphosphate synthase (GPPS), in male *I. pini* (Gilg et al. 2005; Bearfield et al. 2006; Keeling et al. 2006).

Although JH III strongly regulates HMGR activity in *I. pini*, it has no influence on enzymatic activity in other *Ips* species. In assays measuring the conversion of  $^{14}\text{C}$ -HMG-CoA to  $^{14}\text{C}$ -mevalonolactone by fed and JH III treated males in several species, JH III treatment caused large increases in HMGR activity in *I. pini* but had no effect in *I. paraconfusus* (Tillman et al. 2004). However, feeding strongly increased the enzymatic activity of HMGR in both species; suggesting that members of the *grandicollis* subgeneric group (e.g., *I. paraconfusus*) are quite different from *I. pini* with regard to the regulation of pheromone production by juvenile hormone. Interestingly, *Ips confusus* is closely related to *I. paraconfusus* and falls into the same *grandicollis* group based on multiple gene analysis (Cognato and Vogler 2001) and mitochondrial cytochrome oxidase I DNA sequences alone (Cognato and Sperling 2000).

In this study, we use biochemical and molecular approaches to test the hypothesis that JH III does not exclusively regulate pheromone production in *Ips confusus*, a member of the *grandicollis* subgeneric group. We also evaluate for the first time the effect of JH III treatment and feeding on the transcriptional regulation of key mevalonate pathway genes in male *I. confusus*.

## Materials and Methods

**Insects** Immature *I. confusus* were obtained from infested *Pinus monophylla* bolts collected from the Bureau of Land Management (BLM) land east of Carson City, Nevada, USA. We also collected bolts of *Pinus jeffreyi* infested with immature *I. pini* from the University of Nevada Whittell Forest, NV and from within the Lake Tahoe Basin Management Unit, South Lake Tahoe, CA. The insects were reared to adults in a greenhouse according to Browne (1972) and emerged adults were collected daily. Adult beetles were separated by sex according to Lanier and Cameron (1969) and stored for up to two weeks at 4°C in moist paper towels until used in experiments.

**Influence of JH and Feeding on the Production of Key Pheromone Components** We assessed the influence of JH III on the production of key pheromone components, ipsenol and isdienol, by collecting headspace volatiles of adult beetles on an absorbent. Individual male beetles ( $N=30$ ) were topically treated with either 10 µg of racemic JH III (Sigma-Aldrich, St. Louis, MO) dissolved in 0.5 µl of acetone or acetone alone (control) applied to the abdominal venter. Each group of beetles was then placed in a modified glass septum-inlet adaptor (29/32 joint, Sigma-Aldrich) and an absorbent filter containing 100 mg of 50/80 mesh Super Q<sup>®</sup> (Alltech Associates, Deerfield, IL) was attached to one end of the chamber with a 3 cm section of Tygon<sup>®</sup> tubing. Humidified and charcoal-purified air was pulled through the apparatus at a rate of ~500 ml/min. The beetles were aerated in the dark for 20 hr and then the Super Q<sup>®</sup> filters were eluted with 1 ml of pentane: ether (1:1) spiked with *n*-octanol (20 µg), as an internal standard. The resulting extracts were analyzed by gas chromatography (GC) using a Hewlett-Packard 5890 Series II GC equipped with an HP-INNOWax (J&W Scientific) capillary column (30 m × 0.25 mm × 0.25 µm film thickness) in splitless mode with a constant column flow of 1.5 ml He/min. The oven temperature was ramped from 60 to 150°C at 5°C/min, then 150 to 240°C at 10°C/min with a 5 min hold at 240°C. The identity of ipsenol and ipsdienol present in the samples was confirmed by comparing their retention times to those of authentic standards.

To determine the influence of feeding on the production of ipsenol and ipsdienol by male *I. confusus*, we treated 30

male beetles with 0.5 µl of acetone each and then placed them into individual 4 mm holes drilled through the bark of a small bolt of pinyon pine (~10 cm diameter and 15 cm long). The infested bolt was then placed in an inert oven cooking bag supported by a cylindrical wire cage (25 cm height, 20 cm diameter) and the beetles were allowed to feed. Humidified and charcoal-purified air was drawn out of the bag (~1 l/min) and volatile compounds were collected for 20 hr on an absorbent filter made of 100 mg of 50/80 mesh Super Q<sup>®</sup>. As a control, we also collected volatiles from an uninfested bolt of pinyon pine into which 30 holes had been drilled. The filters were then eluted and the resulting extract analyzed by GC as described above. The amount of ipsenol and ipsdienol produced by beetles in each treatment was compared by one-way ANOVA, followed by Fisher's least significant difference (LSD) test (STATSOFT 2005; Sokal and Rohlf 1995). We sampled volatiles three times for each treatment.

**Gene Expression Analysis by Real-time PCR** Male *I. confusus* were either treated with JH III (10 µg in 0.5 µl acetone), fed on *P. monophylla* phloem, or treated with 0.5 µl of acetone alone (control), or left untreated. All insects were kept at room temperature throughout the experiment. After 4, 8, 16, and 32 hr, the anterior midguts were excised from the beetles in water, purged of their contents, frozen immediately in liquid nitrogen and stored at -80°C. Each treatment was replicated six times for each time point with five midguts were used per replicate per time point.

Total RNA was prepared from the midgut tissue using the RNeasy Mini plant kit (Qiagen). A portion of the RNA was reverse-transcribed into first strand cDNA template using Superscript III RNase H- reverse transcriptase and random hexamer primers (Invitrogen). *Ips confusus cytoplasmic actin* was chosen as the endogenous control gene because it is unresponsive to JH III treatment or feeding in *I. pini* midguts (Keeling et al. 2004, 2006). After cloning *cytoplasmic actin* from *I. confusus*, semi-quantitative RT-PCR was used to confirm its lack of response to JH III or feeding. Moreover, further studies using quantitative real time (qRT-PCR) also confirmed that *I. confusus cytoplasmic actin* is a suitable normalizing gene (Sandstrom et al. 2008). Fragments of *I. confusus* HMGR, HMGS, and GPPS were isolated using degenerate primers based on previously reported sequences, and then sequenced to design specific real-time primers. All gene-specific primers were chosen only after first being identified with the Primer Express software program (Applied Biosystems, version 2.0) and then analyzed using Vector NTI Advance software (Invitrogen, version 9.1) to decrease the possibility of primer dimer formation. PCR amplifications were performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) following the manufacturer's protocol. Each

reaction was prepared in a separate well containing cDNA template, 300 nM of both forward and reverse primers and SYBR Green PCR Master Mix (Applied Biosystems). Relative gene expression was determined using the  $\Delta\Delta\text{CT}$  method (Livak and Schmittgen 2001).

*Influence of JH III Treatment and Feeding on Enzyme Activity of HMGS, HMGR, and GPPS* We assessed the enzymatic activity of HMGS, HMGR and GPPS in groups of male *I. confusus* treated with 0.5  $\mu\text{l}$  of acetone and allowed to feed in a bolt of pinyon pine for 4, 8 and 16 hr. Beetles were placed in holes predrilled into the phloem and strips of aluminum screen were secured over the holes to prevent the insects from leaving the log. Males were also treated with JH III (10  $\mu\text{g}$  JH III in 0.5  $\mu\text{l}$  of acetone) and incubated in the dark at room temperature for 4, 8 and 16 hr. The same number of beetles treated with 0.5  $\mu\text{l}$  of acetone and incubated in the dark served as controls for each time point. We also measured the enzymatic activity of HMGS and GPPS in male *I. pini* treated as described above. The enzymatic activity of HMGR in response to feeding and JH III treatment has recently been reported for *I. pini* (Tillman et al. 2004) and was not further studied here.

*HMG-CoA Synthase Activity* Midguts of  $\sim 30$  individual beetles for each treatment were dissected, stored in liquid nitrogen and homogenized with a mortar and pestle in 20 ml of homogenization buffer (0.25 M sucrose, 0.1 mM EDTA, 2.0 mM Hepes, and 100 mM of PMSF, pH 7.2). Microsomes were separated by differential centrifugation and enzyme activity assayed as described by Clinkenbeard et al. (1975) and revised by Scharnagl et al. (1995). In this assay, 100  $\mu\text{g}$  of microsomal protein was combined with 120  $\mu\text{M}$  acetoacetyl-CoA in 200  $\mu\text{l}$  of assay buffer (0.1 M Tris-HCl (pH 8.0), 0.1 mM EDTA and 20 mM  $\text{MgCl}_2$ ). The reaction mixture was then incubated for 2 min in a 30°C water bath and 400  $\mu\text{M}$  total acetyl-CoA ( $\sim 55,500$  dpm/reaction) was added. The reaction then remained in the water bath and after 9 min (linear range) was stopped by pipetting a 50  $\mu\text{l}$  aliquot of the mixture into scintillation vials containing 200  $\mu\text{l}$  of 6 N HCl. The tubes were then placed in a 90°C sand bath for 2 hr. Under these conditions, CoA thioesters are hydrolyzed and non-reacted [ $^{14}\text{C}$ ] acetate is evaporated, and only [ $^{14}\text{C}$ ] HMG acid, from enzymically formed HMG-CoA, remains. The amount of labeled HMG acid formed during the reaction by HMG-CoA synthase was then determined using a TRI-CARB 2900 TR liquid scintillation analyzer. Samples were run in triplicate and, as an appropriate control, we also measured the amount of radiolabeled HMG acid formed in reactions that contained no enzyme protein.

*HMG-CoA Reductase Activity* HMG-CoA reductase activity was assayed using a protocol modified from Casals et al.

(1996) and Tillman et al. (2004). In this assay, midguts were dissected from  $\sim 90$  adults for each treatment, placed in liquid nitrogen, and homogenized in 500  $\mu\text{l}$  of assay buffer (100 mM sucrose, 40 mM  $\text{K}_2\text{PHO}_4$ , 30 mM EDTA, 50 mM KCl, 10 mM DTT, pH 7.4). Microsomes were then isolated by serial centrifugation (HMGR protein is located in the endoplasmic reticulum). The tissue homogenate was first centrifuged at 7740 $\times g$  for 5 min at 4°C to remove large debris, and the resultant supernatant was further centrifuged at 12,100 $\times g$  for 20 min to remove the mitochondria. To pellet the microsomal fraction, the supernatant from the 12,100  $\times g$  spin was ultracentrifuged at 165,000 $\times g$  for 69 min at  $\sim -5^\circ\text{C}$ . The resulting pellet was then resuspended in 20  $\mu\text{l}$  of assay buffer (50 mM  $\text{K}_2\text{PHO}_4$ , 5 mM EDTA, 200 mM KCl, 5 mM DTT, pH 7.4) and total protein concentration was determined by the Bradford method (1976) using bovine serum albumin (1 mg/ml) as an external standard.

3-Hydroxy-3-methylglutaryl-CoA reductase activity was determined by measuring the conversion of [ $3\text{-}^{14}\text{C}$ ] HMG-CoA to [ $3\text{-}^{14}\text{C}$ ] mevalonolactone. Specifically, the reaction mix contained 175  $\mu\text{M}$  total HMG-CoA (1:1 labeled: unlabeled;  $\sim 44,000$  dpm), 10 mM NADPH,  $\sim 100$   $\mu\text{g}$  of microsomal protein in 200  $\mu\text{l}$  of assay buffer ( $N=3$  assay samples from one preparation of microsomes). The mixture was incubated for 10 min in a 37°C water bath with shaking and then the reaction was terminated with the addition of 20  $\mu\text{l}$  of 5 M HCl. The samples were then incubated for another 30 min in the 37°C water bath to catalyze the cyclization of linear mevalonate to mevalonolactone (MLL). Following the incubation, the reaction mixture was extracted three times with 5 ml aliquots of diethylether which were combined over  $\text{Na}_2\text{SO}_4$ . The combined ether extracts were then concentrated to  $\sim 200$   $\mu\text{l}$  and the [ $^{14}\text{C}$ ] mevalonolactone was separated from the unreacted [ $^{14}\text{C}$ ] HMG-CoA by silica paper chromatography using a solvent development system of 1:1 hexane:acetone. A MLL standard was also run along with the radiolabeled reaction products. The migration ( $R_f$  value) of the radiolabeled compounds along the silica plate were determined using a BioScan Imaging Scanner System 200-IBM (Bioscan, Inc., Washington, D.C.). The position of the [ $^{14}\text{C}$ ]mevalonolactone on the silica plate was determined by comparing its  $R_f$  value to that of the MLL standard. The portion of silica containing the labeled MLL was then scraped from the plate and the radioactivity was quantified using a Packard TRI-CARB 2900 TR (Perkin Elmer, Germany) liquid scintillation analyzer (LSA).

*GPPS Activity* Prenyltransferase activity was measured as per Gilg et al. (2005) with modifications. A standard acid-lability assay was used to measure the incorporation of [ $1\text{-}^{14}\text{C}$ ] isopentenyl diphosphate (IPP) into polyprenyl

products. Specifically, ~ 100 µg of soluble beetle tissue extract (~30 beetles per treatment) was combined with allylic substrate (50 µM DMAPP) and 10 µM [ $1-^{14}\text{C}$ ] total IPP (55 µCi/µmol) in 200 µl of assay buffer (20 mM Hepes (pH 7.2), 2.5 mM  $\text{MgCl}_2$ , 5 mM KF, 1 mM DTT, 10% glycerol, and protease inhibitor). The reaction mixture was then incubated for 1 h at 32°C. The resulting acid was hydrolyzed by adding 10 µl of 5 N HCl and reincubating the mixture for 30 min at 32°C. After the incubation period, the reaction products were extracted with 300 µl  $\text{ddH}_2\text{O}$  and 1 ml hexane and the radioactivity of 500 µl of the organic/hexane phase radioactivity measured using a Packard TRI-CARB 2900 TR (Perkin Elmer, Germany) liquid scintillation analyzer (LSA).

We also used reverse phase-high performance liquid chromatography (HPLC) to confirm that isoprenyl diphosphate was the product of the reaction. The reaction was carried out as described above, but terminated by boiling the samples for 5 min and then spinning them at 16,000×g for 5 min. Clear homogenates (injection volume 200 µl: 100 µl of reaction sample mixed with 75 µl of buffer and 25 µl of authentic mixed standards (DMAPP, GPP, FPP, GGPP; 1:1:0.5:0.5) were directly loaded onto a Discovery 250×4.6  $\text{C}_{18}$ , 5 µm, silica column (Supelco) using a HP 1050 series HPLC. Phosphorylated short-chain isoprenyls were cleanly separated using a solvent system of 25 mM  $\text{NH}_4\text{HCO}_3$  pH 7.0: acetonitrile as described by Zhang and Poulter (1993). Samples were eluted at a flow rate 1 ml/min by using a linear gradient program: 100% 25 mM  $\text{NH}_4\text{HCO}_3$  for 5 min and 0–100% acetonitrile for 30 min. Eluent was monitored by UV (214 nm). One milliliter fractions were collected by using an Amersham Pharmacia LKB-FRAC-100 Fraction Collector (Amersham Pharmacia Biotech), and fractions corresponding to IPP/DMAPP, GPP, FPP, and GGPP peaks (retention times 4, 16, 20, and 25 min, respectively) were assayed for radioactivity by using a TRI-CARB 2900 TR LSA.

**Statistical Analysis of Enzyme Activity** The activity of HMGS, HMGR, and GPPS in response to feeding and JH III treatment was expressed as a fold increase in enzymatic activity compared to acetone-treated controls (see Schiefelbein et al. 2008). Data are reported as means±S.E. and enzymatic activity over time by treatment was compared using a nested analysis of variance (nested ANOVA, Dunnett's method; STATSOFT 2005).

## Results

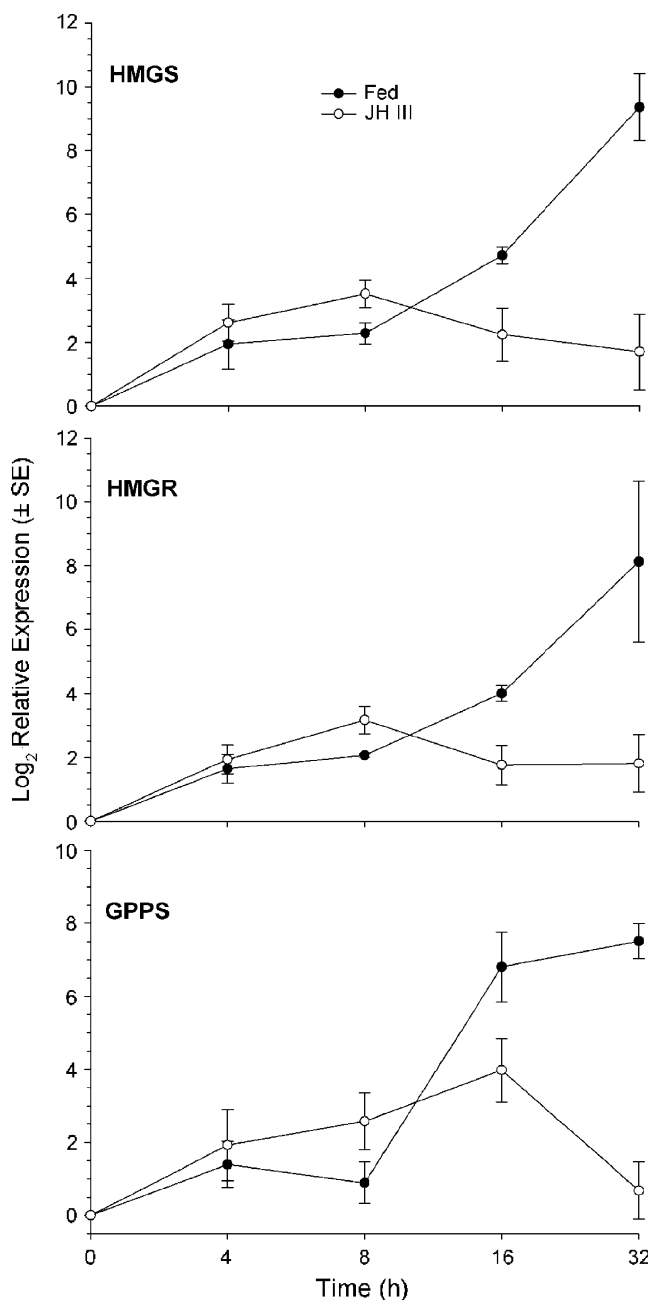
**Influence of Feeding and JH on the Production of Key Pheromone Components** GC analysis of volatiles collected from fed and JH III-treated male *I. confusus* revealed that

ipsenol and ipsdienol are produced only in response to feeding (one-way ANOVA,  $F_{4, 10}=45.6$ ,  $P<0.0001$ ). In response to feeding for 20 h, males released  $563.4\pm 31.1$  ng of ipsenol (mean±SE) and  $194.3\pm 65.2$  ng of ipsdienol; these data suggest that JH III alone is not sufficient to stimulate pheromone production in male *I. confusus*.

**Expression Analysis** Partial cDNA clones were obtained for *I. confusus* HMGR (IcHMGR; accession no. FJ536869), HMGS (IcHMGS; accession no. FJ536868), GPPS (IcGPPS; accession no. FJ536870), and *cytoplasmic actin* (IcActin; accession no. FJ536867) using *I. pini* gene-specific primers and degenerate primers in conjunction with homology-based PCR techniques. The effect of a 10 µg dose of JH III on the mRNA levels of the three mevalonate pathway genes in the anterior midgut of male beetles was determined at multiple time points using quantitative real-time PCR. In male *I. confusus*, feeding and JH III treatment were both sufficient to raise transcript levels for the three genes examined (Fig. 2). For male beetles, each gene demonstrated slightly different transcript values at each time point, but the expression patterns over time were very similar. In general, the mRNA levels following JH III-treatment showed a modest increase and peaked at either 8 or 16 hr and gradually decreased thereafter, reaching a level at or below the 4 hr time point at 32 hr post-treatment (Fig. 2). In comparison, transcript levels following feeding demonstrated a similar pattern as JH III treatment at the first two time points, but the induction was not as pronounced. Whereas the induction of the mevalonate pathway genes begins to decline at the latter time points (16 and 32 hr) with JH III treatment, feeding causes a marked increase in mRNA levels, reaching their highest values at 32 hr post treatment.

**HMG-CoA Synthase Activity** In both male *I. confusus* and *I. pini* there was a significant increase in enzyme activity over time by treatment (ANOVA, *I. confusus*,  $F_{6, 18}=168.6$ ,  $P<0.001$ ; *I. pini*,  $F_{6, 18}=22.9$ ,  $P<0.001$ ; Fig. 3). In male *I. pini*, both JH III and feeding caused significant increases in HMGS activity over the acetone-control at the 16 hr time point ( $2.32\pm 0.89$  and  $4.23\pm 0.32$  fold increases, respectively; ANOVA, Dunnett's method,  $P<0.001$ ). However, only feeding alone caused a significant increase in enzymatic activity over the acetone-treated control in male *I. confusus* ( $4.85\pm 0.25$  fold increase; ANOVA, Dunnett's method,  $P<0.001$ ).

**HMG-CoA Reductase Activity** There was a significant increase in enzymatic activity of HMGR with time by treatment (ANOVA,  $F_{6, 17}=3.65$ ,  $P<0.05$ ; Fig. 4), and feeding caused a significant increase in enzyme activity over the control at the 8 and 16 hr time points (ANOVA,



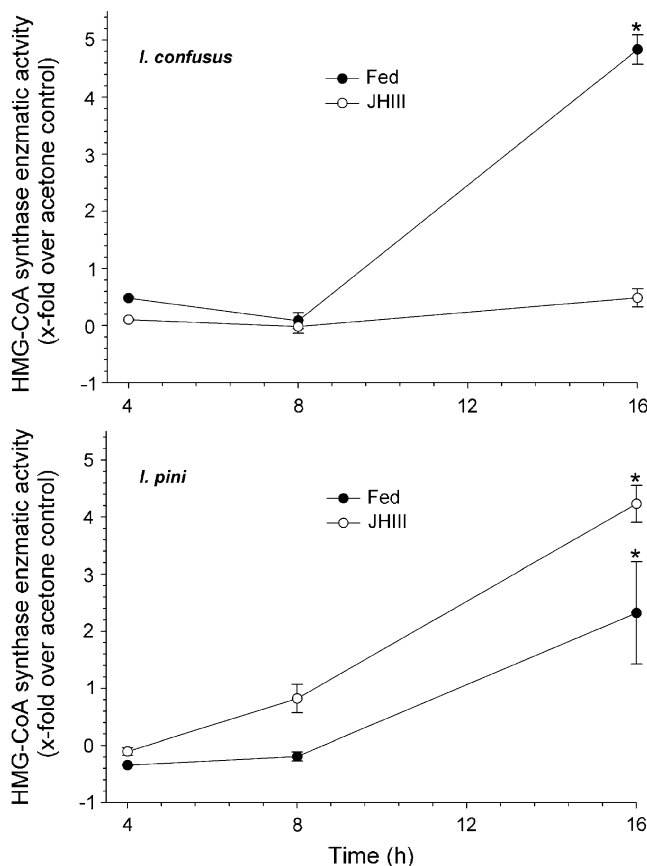
**Fig. 2** Effect of JH-III treatment and feeding on the relative expression of *HMGS* (top), *HMGR* (middle), and *GPPS* (bottom) in midguts of male *I. confusus* as measured by qRT-PCR. Expression ( $\text{Log}_2$ ) is relative to either acetone-treated males for JH-III treatment or unfed controls for fed males

Dunnett's method,  $P < 0.001$  and  $P < 0.05$ , respectively). In fact, *HMGR* activity was increased by  $1.32 \pm 0.29$  fold over the control at 8 h and  $0.86 \pm 0.41$  fold at 16 hr. Treatment with JH III, however, caused no significant increase in activity over the acetone treated controls at all time points (Fig. 4).

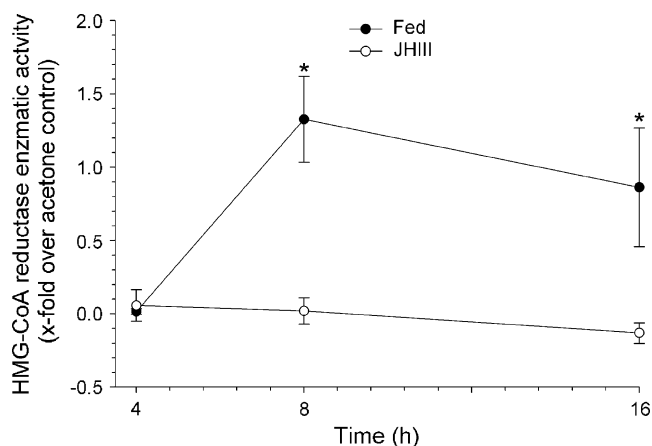
**GPPS Activity** GPPS activity increased with time by treatment for both *I. confusus* and *I. pini* (ANOVA, *I. confusus*,  $F_{6, 18} = 8.85$ ,  $P < 0.001$ ; *I. pini*,  $F_{6, 18} = 15.1$ ,  $P < 0.001$ ; Fig. 5). For *I. pini*, both JH III-treatment and feeding caused a significant increase in activity over the acetone-treated control at 16 hr ( $1.28 \pm 0.32$  and  $0.987 \pm 0.18$  fold increases, respectively; ANOVA, Dunnett's method,  $P < 0.001$ ). Interestingly, feeding alone caused an increase in GPPS activity at 8 and 16 hr post treatment in male *I. confusus* ( $0.44 \pm 0.13$  and  $1.16 \pm 0.09$  fold increases, respectively; ANOVA, Dunnett's method,  $P < 0.05$ ). Moreover, it was confirmed by HPLC analysis that GPP was the only short-chain isoprenyl diphosphate product of these assays.

## Discussion

The present results support the hypothesis that in *I. confusus*, a member of the *I. grandicollis* subgeneric group, JH III does not exclusively regulate pheromone production.



**Fig. 3** Effect of feeding and JH III treatment on the enzymatic activity of HMG-CoA synthase in male *I. confusus* (top) and *I. pini* (bottom). An asterisk denotes a significant difference in enzyme activity relative to acetone-treated controls (Dunnett's method,  $P < 0.05$ )

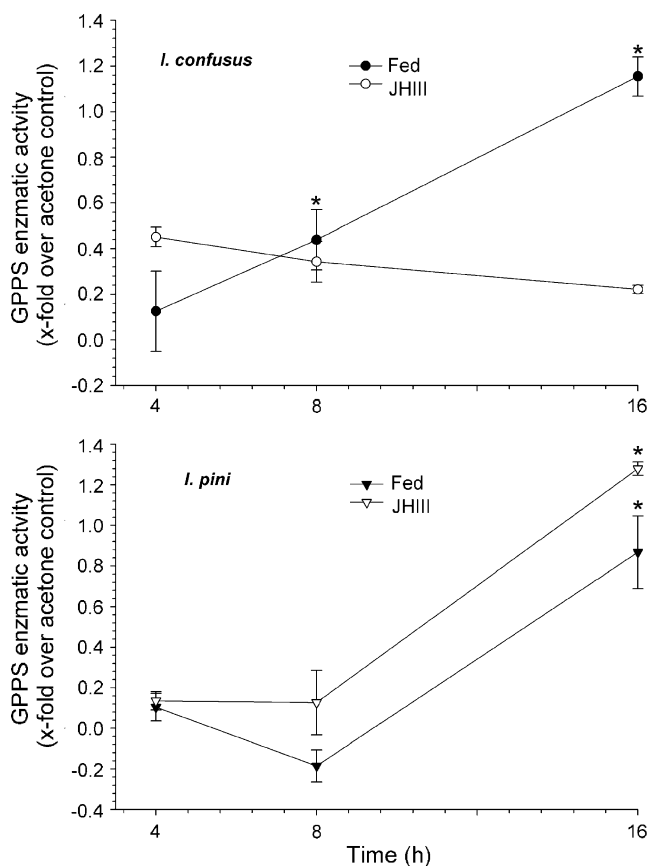


**Fig. 4** Effect of feeding and JH III treatment on the enzymatic activity of HMG-CoA reductase activity in male *I. confusus*. An asterisk denotes a significant difference in enzyme activity relative to acetone-treated controls (Dunnnett's method,  $P < 0.05$ )

Here, we show that feeding, but not JH III treatment, stimulates pheromone production in male *I. confusus*. Previous work by Borden et al. (1969) demonstrated that gut extracts of male *I. paraconfusus* (then referred to as *I. confusus*) become highly attractive to female beetles after topical application of synthetic JH (Borden et al. 1969). However, large non-physiological doses (50–100  $\mu\text{g}$ ) of hormone were required to attain a strong response, and females were not attracted in bioassays to midguts treated with 10  $\mu\text{g}$  of JH, the amount used in our study (Borden et al. 1969). Moreover, females were attracted to as little as 0.02 male equivalents of extract from males which had fed on host phloem, while extract from hormone-treated males elicited only a weak response (Borden et al. 1969).

The induction of mevalonate pathway genes in male bark beetles often precedes an increase in protein production, enzyme activity, and pheromone production (Tillman et al. 2004; Bearfield et al. 2006). In male *I. confusus*, either JH III treatment or feeding is sufficient to increase *HMGS*, *HMGR*, and *GPPS* transcript levels in the anterior midgut (Fig. 2). At the 4 h and 8 h time points, the transcript levels of the fed-group were slightly lower and likely due to the time necessary for JH III to be synthesized and released following feeding. At the 16 hr and 32 hr time points, however, expression levels of JH III-treated males began to decline likely due to the hormone dose being metabolized while the fed beetles showed large increases in all three genes studied (Fig. 2). Although each gene shows a modest induction of transcript at 32 hr following JH III treatment, the relative expression patterns at 32 hr post-feeding are 100 to 200-fold higher than unfed controls (Fig. 2). In addition to the transcriptional effects of JH III, these results suggest that a supplemental factor released following feeding may further induce the expression of mevalonate pathway genes in male *I. confusus*.

Feeding induces the coordinated gene expression of several earlier steps in monoterpene pheromone biosynthesis in both male and female *I. pini*, including *HMGS* and *HMGR*, but *GPPS*, a later step in the pathway, is induced only in males (Keeling et al. 2004). In male *I. confusus*, the differences in transcriptional regulation between JH III treatment and feeding were generally more pronounced further along the biosynthetic pathway and the disparity was most dramatic at 32 hr for all three genes (Fig. 2). The increases in relative expression with feeding were ~65-, 55-, and 120-fold above JH III-treated males for *IcHMGS*, *IcHMGR*, and *IcGPPS*, respectively (Fig. 2). Therefore, it appears that in *Ips* species, the early biosynthetic steps are more coordinately regulated by both feeding and JH III treatment than the terminal steps in the mevalonate pathway. A developmental factor may also contribute to the alternate regulation of pheromone biosynthesis due to the higher basal levels of mevalonate pathway genes. Specifically, basal *GPPS* mRNA levels in male *I. pini* are 400–500-fold above female levels (Keeling et al. 2006), suggesting that the last steps of the pathway are more



**Fig. 5** Effect of feeding and JH III treatment on GPPS enzyme activity in male *I. confusus* (top) and *I. pini* (bottom). An asterisk denotes a significant difference in enzyme activity relative to acetone-treated controls (Dunnnett's method,  $P < 0.05$ )

developmentally regulated than *HMGS* and *HMGR*. In *I. confusus*, however, all genes studied here demonstrated only a two-fold difference in basal transcript levels (data not shown). Although not studied in as much detail, the same coordinate regulation between sexes does not exist in *D. jeffreyi* as only males demonstrate an induction of *HMGS* and *HMGR* with JH III treatment (Hall et al. 2002b; Tittiger et al. 2000, 2003). Male *D. jeffreyi* produce a mevalonate-derived aggregation pheromone component, frontalin, in response to exogenous treatment with JH III (Barkawi et al. 2003)

In *I. confusus*, feeding also caused an increase in *HMGS*, *HMGR*, and *GPPS* enzymatic activity in male beetles (Figs. 3, 4, and 5). Although mRNA levels were induced by JH III treatment in male *I. confusus* (Fig. 2), there was no concomitant increase in enzyme activity (Figs. 3, 4, and 5) or production of ipsdienol and ipsenol in treated insects. Similarly, the mRNA induction of *HMGR* with JH III treatment in male *I. paraconfusus*, measured by northern blot analysis, is not followed by a subsequent increase in enzymatic activity of *HMGR* or pheromone production (Tillman et al. 2004). In this study, we also determined that the enzymatic activity of *HMGS* and *GPPS* in *I. pini* and *I. confusus* are not uniformly regulated by JH III. Unlike *I. pini*, the activity of *HMGS* and *GPPS* in male *I. confusus* was not influenced by hormone treatment.

Tillman et al. (2004) demonstrated a difference in the regulation of pheromone production between species in the *pini* and *grandicollis* subgeneric groups. The work presented here further supports the existence of a dichotomy in the regulation of pheromone production among *Ips* species. It has also been suggested that feeding by male *I. paraconfusus* may cause the release of a brain hormone by *corpora cardiaca* which, in turn, may stimulate pheromone production (Hughes and Renwick 1977; Lu 1999). Interestingly, in preliminary experiments brain extracts from fed male *I. confusus* were sufficient to cause a modest increase in ipsenol production in JH III-treated males (unpub. data). Further study will likely reveal regulatory factors influencing pheromone biosynthesis in members of the *grandicollis* group of bark beetles.

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