Naturwissenschaften (2004) 91:324–328
DOI 10.1007/s00114-004-0523-y

SHORT COMMUNICATION

Christopher I. Keeling · Gary J. Blomquist · Claus Tittiger

Coordinated gene expression for pheromone biosynthesis in the pine engraver beetle, *Ips pini* (Coleoptera: Scolytidae)

Received: 9 December 2003 / Accepted: 18 March 2004 / Published online: 29 April 2004

Abstract In several pine bark beetle species, phloem feeding induces aggregation pheromone production to coordinate a mass attack on the host tree. Male pine engraver beetles, *Ips pini* (Say) (Coleoptera: Scolytidae), produce the monoterpenoid pheromone component ipsdienol de novo via the mevalonate pathway in the anterior midgut upon feeding. To understand how pheromone production is regulated in this tissue, we used quantitative real-time PCR to examine feeding-induced changes in gene expression of seven mevalonate pathway genes: acetoacetyl-coenzyme A thiolase, 3-hydroxy-3-methylglutaryl coenzyme A synthase, 3-hydroxy-3-methylglutaryl coenzyme A reductase, mevalonate 5-diphosphate decarboxylase, isopentenyl-diphosphate isomerase, geranyl-diphosphate synthase (GPPS), and farnesyl-diphosphate synthase (FPPS). In males, expression of all these genes significantly increased upon feeding. In females, the expression of the early mevalonate pathway genes (up to and including the isomerase) increased significantly, but the expression of the later genes (GPPS and FPPS) was unaffected or decreased upon feeding. Thus, feeding coordinately regulates expression of the mevalonate pathway genes necessary for pheromone biosynthesis in male, but not female, midguts. Furthermore, basal mRNA levels were 5- to 41-fold more abundant in male midguts compared to female midguts. This is the first report of coordinated regulation of mevalonate pathway genes in an invertebrate model consistent with their sex-specific role in de novo pheromone biosynthesis.

Electronic Supplementary Material Supplementary material is available in the online version of this article at http://dx.doi.org/10.1007/s00114-004-0523-y.

C. I. Keeling (✉) · G. J. Blomquist · C. Tittiger
Department of Biochemistry,
University of Nevada Reno,
Mail Stop 330, Reno, NV 89557-0014, USA
e-mail: ckeeling@mac.com
Tel.: +1-775-7841734
Fax: +1-775-7841419

Introduction

Bark beetles (Coleoptera: Scolytidae) are significant pests of conifer forests. Most use an aggregation pheromone to coordinate a mass attack and colonize a host tree. Upon phloem feeding, male pine engraver beetles, *Ips pini* (Say), produce a pheromone that is released from the frass and which attracts conspecifics of both sexes. The major pheromone component produced is the monoterpenoid ipsdienol (2-methyl-6-methyleneocta-2,7-dien-4-ol, Fig. 1) (Vité et al. 1972; Birch et al. 1980).

Although pheromone biosynthesis in bark beetles was originally thought to involve oxidation of host monoterpenes, there is now solid evidence that de novo biosynthesis is the major source of pheromone, particularly in *Ips* spp. (reviewed in Seybold and Tittiger 2003). Male *I. pini* synthesize ipsdienol de novo (Seybold et al. 1995) via the mevalonate pathway (Fig. 1) in the anterior midgut (Hall et al. 2002). Phloem feeding by males induces both biosynthesis of juvenile hormone III (JH III) in the corpora allata (Tillman et al. 1998) and de novo ips- dienol biosynthesis. Topical application of JH III increases both 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMG-S) and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-R) mRNA levels in male *I. pini* (Hall et al. 2002; J.A. Tillman et al., unpublished; J.C. Bearfield et al., unpublished). These studies support the paradigm that feeding stimulates JH III biosynthesis in the corpora allata, with increased JH III titers stimulating pheromone production in the anterior midgut. However, apart from in situ hybridizations of HMG-R (Hall et al. 2002), no studies have shown that feeding directly increases mevalonate pathway gene mRNA levels in this pheromone-producing tissue.

Recently, an EST project in *I. pini* recovered representations of seven mevalonate pathway genes: acetoacetyl-coenzyme A thiolase (AACT), HMG-S, HMG-R, mevalonate 5-diphosphate decarboxylase (MPDC), isopentenyl-diphosphate isomerase (IPPI), geranyl-diphosphate synthase (GPPS), and farnesyl-diphosphate synthase (FPPS) (Eigenheer et al. 2003). This new sequence in-

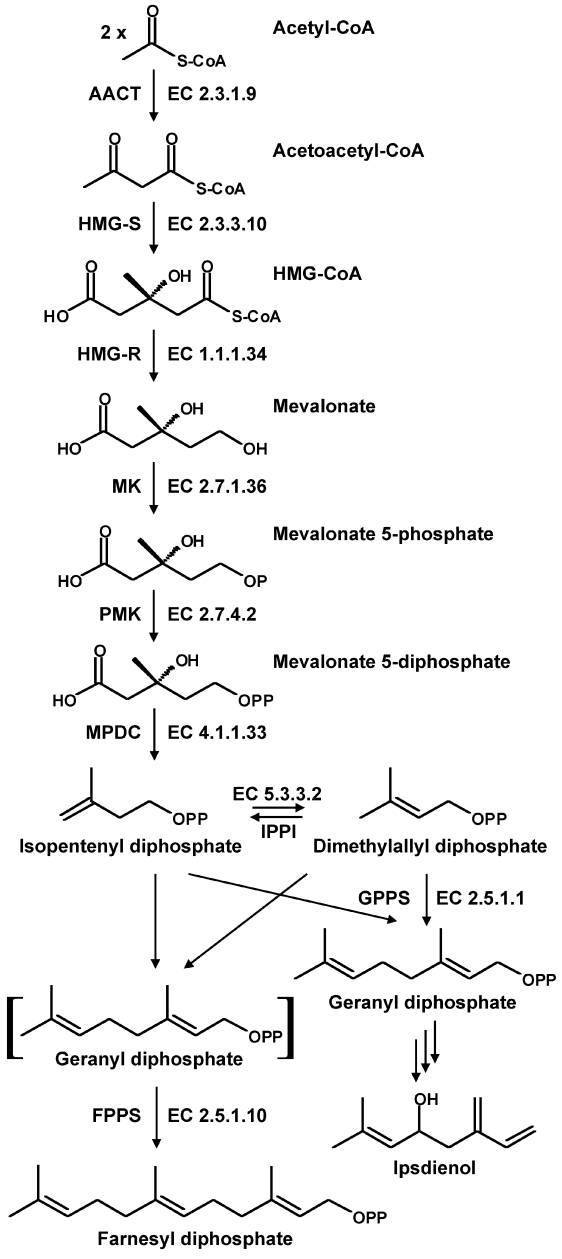

Fig. 1 Mevalonate pathway and ipsdienol biosynthesis. *MK* mevalonate kinase, *PMK* phosphomevalonate kinase

formation allowed us to examine how feeding affects the expression of these mevalonate pathway genes in the midgut, thus providing a better understanding of how de novo pheromone biosynthesis is regulated.

Method and materials

Tissue samples

Logging slash of Jeffrey pine (*Pinus jeffreyi* Grev. & Balf.) infested with *Ips pini* were obtained from the Sierra Nevada in California and Nevada, USA, and placed in a greenhouse to allow the emergence of adult beetles (Browne 1972). Upon emergence, beetles were typed and sexed following the procedures of Wood (1982) and kept at 4°C on moist paper towels. Holes (3 mm diameter) were drilled into the phloem of un-infested Jeffrey pine bolts (15–20 cm diameter × 100 cm). One beetle was placed head-first into each hole and held in with metal screening. Male and female beetles were placed in separate bolts. Bolts were then placed vertically at room temperature for 4, 8, 16, or 32 h. Simultaneously, unfed beetles were kept at room temperature in the dark in 60-ml plastic containers, five beetles per container. After incubation, fed beetles were removed from the bark and their anterior midguts excised in water. Midguts were gently purged of their contents and then immediately frozen in liquid nitrogen and stored at −80°C. Each fed/unfed time-point pair was replicated three times and each replicate contained tissue from five midguts.

Analysis of gene expression by real-time PCR

Total RNA was extracted using the RNeasy Mini plant kit (Qiagen) and a 20% portion was reverse-transcribed using Superscript III RNase H^- reverse transcriptase and random primers (Invitrogen). Relative gene expression at each time-point was determined using the ΔΔCT method (Livak and Schmittgen 2001). Cytoplasmic actin, which is unaffected by feeding or JH III treatment in *I. pini* midguts (C.I. Keeling et al., unpublished), was used as the endogenous control. EST sequences (see Electronic Supplementary Material; Eigenheer et al. 2003) were used to design real-time PCR primers. Where multiple ESTs existed for a gene, the contig sequence was used. Additional sequence information was obtained as follows: HMG-R (Hall et al. 2002), HMG-S (J.C. Bearfield et al., unpublished), and GPPS and FPPS (A. Gilg-Young et al., unpublished). Gene-specific primers with minimal potential for primer–dimer formation were identified using Vector NTI software (version 7.1, InforMax) from primers suggested by Primer Express software (Applied Biosystems) (see Electronic Supplementary Material). Real-time PCR data were acquired on an ABI Prism 7000 sequence detection system using SYBR Green I PCR Master Mix with universal thermocycler conditions according to the manufacturer's protocol (Applied Biosystems). Template and primer concentrations were optimized and amplification efficiencies determined for each gene. Dissociation curves for each product were examined for non-specific amplification. Correlations between genes were examined independent of treatment and time point for each sex. JMP software (SAS Institute, Cary, N.C., USA) was used for statistical analysis.

Results

Both males and females fed significantly on phloem. After 32 h, males had excavated nuptial chambers and females had tunneled 10–15 mm. Early mevalonate pathway genes were significantly upregulated in the midguts of both males and females within 4 h of being placed in the phloem (Fig. 2a–e). The later genes, GPPS and FPPS, were upregulated in males and unregulated or downregulated in females (Fig. 2f–g). Although the early genes were upregulated by similar amounts in both sexes, males had 5- to 10-fold higher basal levels for these genes (Fig. 2h). For GPPS, basal levels were 41-fold more abundant in males and were induced upon feeding only in males. In addition, basal expression levels of FPPS were 6-fold higher in males and feeding reduced FPPS expression in females.

Genes in the mevalonate pathway were coordinately regulated by feeding. Strong correlations in males were found between all genes (Table 1). In females, expression

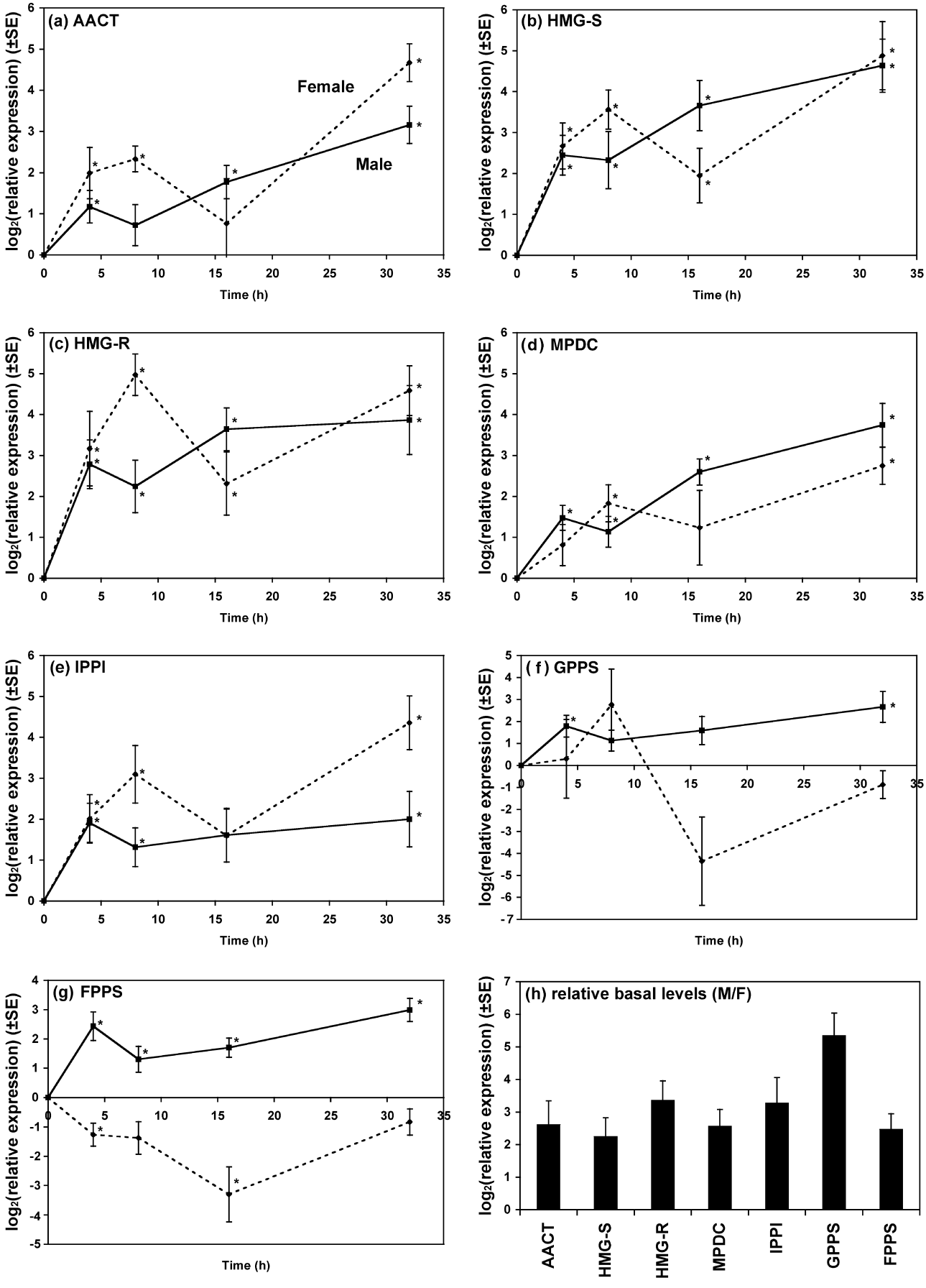
(a) AACT
Female
Male
log2(relative expression) (±SE)
Time (h)
(b) HMG-S
(c) HMG-R
(d) MPDC
(e) IPPI
(f) GPPS
(g) FPPS
(h) relative basal levels (M/F)
AACT
HMG-S
HMG-R
MPDC
IPPI
GPPS
FPPS

Table 1 Correlations between gene expression profiles. r^2 values in bold indicate a statistically significant linear regression, $P<0.05$

Males	Females						
	AACT	HMG-S	HMG-R	MPDC	IPPI	GPPS	FPPS
AACT		**0.776**	**0.818**	**0.828**	**0.787**	0.036	-0.060
HMG-S	**0.885**		**0.891**	**0.740**	**0.921**	0.005	**-0.225**
HMG-R	**0.818**	**0.954**		**0.802**	**0.900**	0.020	**-0.232**
MPDC	**0.936**	**0.952**	**0.885**		**0.779**	0.031	-0.081
IPPI	**0.794**	**0.861**	**0.855**	**0.793**		0.003	**-0.217**
GPPS	**0.782**	**0.881**	**0.881**	**0.829**	**0.836**		**0.428**
FPPS	**0.788**	**0.890**	**0.877**	**0.836**	**0.844**	**0.867**	

patterns of the early mevalonate pathway genes were strongly correlated with each other, GPPS was only weakly correlated with FPPS, and FPPS was weakly negatively correlated with HMG-S, HMG-R, and IPPI.

Discussion

All the mevalonate pathway genes examined were significantly upregulated by feeding in male anterior midguts. This is consistent with previous studies that show de novo pheromone production from radiolabeled acetate and mevalonolactone (Tillman et al. 1998; Hall et al. 2002) and HMG-R mRNA levels (Hall et al. 2002; Seybold and Tittiger 2003) increase upon feeding in *I. pini* males, and that males produce negligible amounts of ipsdienol prior to feeding (Pureswaran et al. 2000). Interestingly, several of these genes were also significantly upregulated by feeding in female midguts. However, basal transcript levels were significantly lower, and GPPS and FPPS were not significantly upregulated by feeding in females, consistent with the low, but detectable, quantity of ipsdienol produced by females upon feeding (Pureswaran et al. 2000).

The significant increase in male FPPS mRNA levels with feeding was somewhat surprising because greater quantities of this enzyme would draw mevalonate pathway products away from the C_{10} products from which ipsdienol is biosynthesized. However, C_{15} and higher products are necessary for protein glycosylation and isoprenylation, modifications that may be necessary to biosynthesize and excrete the large quantities of pheromone produced. Why FPPS mRNA levels decreased in fed females is unclear.

The modest induction of GPPS in males, and the large difference in basal levels of all observed mevalonate pathway genes between sexes, particularly GPPS, might suggest that the later steps of pheromone biosynthesis are not as highly regulated by feeding as the early steps, but rather are regulated developmentally. No genes have been characterized for the biochemical steps between geranyl diphosphate and ipsdienol (Fig. 1). However, a male-specific terpene synthase activity that is likely involved in this pathway has recently been reported (Martin et al. 2003). In comparison to the more than 15-fold induction of HMG-R mRNA levels we observed in fed males, this terpene synthase activity is induced approximately 2-fold by feeding or JH III treatment, but the activity of untreated males was nearly 5-fold higher than that of untreated females (Martin et al. 2003). This suggests that some pheromone biosynthesis genes are more strongly regulated developmentally rather than environmentally.

The strong correlations in mRNA levels between genes (Table 1) suggest that the mevalonate pathway is coordinately regulated by feeding at the transcript level through a common transcription factor. Despite this coordinate regulation, gene expression was more complex than anticipated. The lower basal transcript levels and the lack of induction for GPPS and FPPS transcripts in females suggest that the mevalonate pathway is also modulated by sex-specific, developmentally regulated, factors. In *Drosophila*, HMG-R expression is tightly controlled during development (Van Doren et al. 1998). mRNA levels and enzymes of the mevalonate pathway are coordinately regulated in other organisms, including humans (Rosser et al. 1989), rats (Olivier et al. 1999) and cockroaches (Casals et al. 1996). The promoter regions of some human mevalonate pathway genes share common regulatory elements, suggesting their potential to be coordinately regulated (Bishop et al. 1998). An *I. pini* genomic library is currently being screened for conserved promoter sequences of mevalonate pathway and JH III-responsive genes.

Tillman et al. (1998) found JH III biosynthesis by the corpora allata increased significantly only in males upon feeding, and only at 24 h. However, we have shown that the mevalonate pathway genes were upregulated within 4 h of feeding in both sexes. This suggests either that measuring JH III biosynthesis does not accurately predict JH III titers or that something else is involved in regulating the mevalonate pathway upon feeding. However, topical application of JH III clearly increases pheromone production and HMG-R and HMG-S mRNA in at least three bark beetle species (Seybold and Tittiger 2003). Accurate measurement of JH III titer would clarify the feeding-induced changes in JH III titers and confirm the role of JH III in feeding-induced pheromone biosynthesis.

Fig. 2 a–g Expression profiles of mevalonate genes upon phloem feeding relative to unfed beetles. *Dotted lines* females, *solid lines* males. An *asterisk* beside a data point indicates a significant difference between fed and unfed beetles (*t*-test, $P<0.05$); **h** relative basal (unfed) expression levels between males and females. For all genes, mRNA levels were significantly higher in males (*t*-test, $P<0.001$)

In this study, we have only examined changes in mRNA levels with feeding. However, it is well established in vertebrates that mevalonate pathway enzymes, particularly HMG-R, are also posttranscriptionally and posttranslationally regulated (Goldstein and Brown 1990). A proteomics-based examination of pheromone biosynthesis in *I. pini* midguts is now planned in order to clarify the regulation of this pathway at the protein level.

Acknowledgements We thank J.C. Bearfield and A. Gilg-Young for sharing unpublished sequence data and designing real-time primers for GPPS, J.C. Bearfield and G.M. Hall for assistance with collecting the beetles, J. Rowe and C. Osborne at the Nevada Genomics Center for assistance with the real-time PCR instrument, and E. Plettner for comments on an earlier version of this manuscript. This work was supported by a Canadian NSERC postdoctoral fellowship to C.I.K., the USDA (2001-35302-11035), the NSF (IBN 0316370), the Nevada Agricultural Experiment Station (publication no. 03031391), the Nevada Biomedical Research Infrastructure Network (P20 RR 16464), and represents research coordinated in part through USDA-CSREES Multistate Research Project W-189. This research was conducted in accordance with the current laws of the USA.

References

Birch MC, Light DM, Wood DL, Browne LE, Silverstein RM, Bergot BJ, Ohloff G, West JR, Young JC (1980) Pheromonal attraction and allomonal interruption of *Ips pini* in California by the two enantiomers of ipsdienol. J Chem Ecol 6:703–717

Bishop RW, Chambliss KL, Hoffman GF, Tanaka RD, Gibson KM (1998) Characterization of the mevalonate kinase 5′-untranslated region provides evidence for coordinate regulation of cholesterol biosynthesis. Biochem Biophys Res Comm 242: 518–524

Browne LE (1972) An emergence cage and refrigerated collector for wood-boring insects and their associates. J Econ Entomol 65:1499–1501

Casals N, Buesa C, Piulachs M-D, Cabañó J, Marrero PF, Bellés X, Hegardt FG (1996) Coordinated expression and activity of 3-hydroxy-3-methylglutaryl coenzyme A synthase and reductase in the fat body of *Blattela germanica* (L.) during vitellogenesis. Insect Biochem Mol Biol 26:837–843

Eigenheer AL, Keeling CI, Young S, Tittiger C (2003) Comparison of gene representation in midguts from two phytophagous insects, *Bombyx mori* and *Ips pini*, using expressed sequence tags. Gene 316:127–136

Goldstein JL, Brown MS (1990) Regulation of the mevalonate pathway. Nature 343:425–430

Hall GM, Tittiger C, Andrews GL, Mastick GS, Kuenzli M, Luo X, Seybold SJ, Blomquist GJ (2002) Midgut tissue of male pine engraver, *Ips pini*, synthesizes monoterpenoid pheromone component ipsdienol de novo. Naturwissenschaften 89:79–83

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}{}_T$ method. Methods 25:402–408

Martin D, Bohlmann J, Gershenzon J, Francke W, Seybold SJ (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

Olivier LM, Chambliss KL, Gibson KM, Krisans SK (1999) Characterization of phosphomevalonate kinase: chromosomal localization, regulation, and subcellular targeting. J Lipid Res 40:672–679

Pureswaran DS, Gries R, Borden JH, Pierce HD Jr (2000) Dynamics of pheromone production and communication in the mountain pine beetle, *Dendroctonus ponderosae* Hopkins, and the pine engraver, *Ips pini* (Say) (Coleoptera: Scolytidae). Chemoecology 10:153–168

Rosser DSE, Ashby MN, Ellis JL, Edwards PA (1989) Coordinate regulation of 3-hydroxy-3-methylglutaryl-coenzyme A synthase, 3-hydroxy-3-methylglutaryl-coenzyme A reductase, and prenyltransferase synthesis but not degradation in HepG2 cells. J Biol Chem 264:12653–12656

Seybold SJ, Tittiger C (2003) Biochemistry and molecular biology of de novo isoprenoid pheromone production in the Scolytidae. Annu Rev Entomol 48:425–453

Seybold SJ, Quilici DR, Tillman JA, Vanderwel D, Wood DL, Blomquist GJ (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

Tillman JA, Holbrook GL, Dallara PL, Schal C, Wood DL, Blomquist GJ, Seybold SJ (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

Van Doren M, Broihier HT, Moore LA, Lehmann R (1998) HMG-CoA reductase guides migrating primordial germ cells. Nature 396:466–469

Vité JP, Bakke A, Renwick JAA (1972) Pheromones in *Ips* (Coleoptera: Scolytidae): occurrence and production. Can Entomol 104:1967–1975

Wood SL (1982) The bark and ambrosia beetles of north and central America (Coleoptera: Scolytidae), a taxonomic monograph. Brigham Young University, Salt Lake City, Utah, USA