



## Review

## Pheromone production in bark beetles

Gary J. Blomquist\*, Rubi Figueroa-Teran, Mory Aw, Minmin Song, Andrew Gorzalski, Nicole L. Abbott, Eric Chang, Claus Tittiger

Biochemistry and Molecular Biology, University of Nevada, Reno, NV 89557, USA

## ARTICLE INFO

## Article history:

Received 17 June 2010

Received in revised form

28 July 2010

Accepted 30 July 2010

## Keywords:

Bark beetles

Pheromone production

Ipsdienol

Ips pini

Dendroctonus ponderosae

Microarrays

## ABSTRACT

The first aggregation pheromone components from bark beetles were identified in 1966 as a mixture of ipsdienol, ipsenol and verbenol. Since then, a number of additional components have been identified as both aggregation and anti-aggregation pheromones, with many of them being monoterpenoids or derived from monoterpenoids. The structural similarity between the major pheromone components of bark beetles and the monoterpenes found in the host trees, along with the association of monoterpenoid production with plant tissue, led to the paradigm that most if not all bark beetle pheromone components were derived from host tree precursors, often with a simple hydroxylation producing the pheromone. In the 1990s there was a paradigm shift as evidence for de novo biosynthesis of pheromone components began to accumulate, and it is now recognized that most bark beetle monoterpenoid aggregation pheromone components are biosynthesized de novo. The bark beetle aggregation pheromones are released from the frass, which is consistent with the isoprenoid aggregation pheromones, including ipsdienol, ipsenol and frontalin, being produced in midgut tissue. It appears that *exo-brevocomin* is produced de novo in fat body tissue, and that verbenol, verbenone and verbenene are produced from dietary  $\alpha$ -pinene in fat body tissue. Combined biochemical, molecular and functional genomics studies in *Ips pini* yielded the discovery and characterization of the enzymes that convert mevalonate pathway intermediates to pheromone components, including a novel bifunctional geranyl diphosphate synthase/myrcene synthase, a cytochrome P450 that hydroxylates myrcene to ipsdienol, and an oxidoreductase that interconverts ipsdienol and ipsdienone to achieve the appropriate stereochemistry of ipsdienol for pheromonal activity. Furthermore, the regulation of these genes and their corresponding enzymes proved complex and diverse in different species. Mevalonate pathway genes in pheromone producing male *I. pini* have much higher basal levels than in females, and feeding induces their expression. In *I. duplicatus* and *I. pini*, juvenile hormone III (JH III) induces pheromone production in the absence of feeding, whereas in *I. paraconfusus* and *I. confusus*, topically applied JH III does not induce pheromone production. In all four species, feeding induces pheromone production. While many of the details of pheromone production, including the site of synthesis, pathways and knowledge of the enzymes involved are known for *Ips*, less is known about pheromone production in *Dendroctonus*. Functional genomics studies are under way in *D. ponderosae*, which should rapidly increase our understanding of pheromone production in this genus. This chapter presents a historical development of what is known about pheromone production in bark beetles, emphasizes the genomic and post-genomic work in *I. pini* and points out areas where research is needed to obtain a more complete understanding of pheromone production.

© 2010 Elsevier Ltd. All rights reserved.

## 1. Introduction

Insects in the family Scolytidae (Coleoptera) are among the most significant and economically important pest insects in the United States. Bark beetles are the most destructive pests of sawtimber and pulpwood in the northern hemisphere. They kill millions of cubic

meters of conifer trees in production forests and also act as keystone species in natural ecosystems by initiating the process of nutrient cycling of mature trees. The current outbreak of mountain pine beetles in western Canada is due in part to increased mature pine stands and favorable climate, and is an order of magnitude greater in area than previous outbreaks (Kurz et al., 2008).

Bark beetles use aggregation pheromones to coordinate mass attacks on host pine. Feeding induces production of an aggregation pheromone in the pioneer sex that attracts both sexes to “mass attack” the tree. For *Ips* spp., the male is the pioneer sex and the first

\* Corresponding author.

E-mail address: [garyb@cabnr.unr.edu](mailto:garyb@cabnr.unr.edu) (G.J. Blomquist).

to bore into the bark and feed on phloem. *Dendroctonus* spp. attacks on host trees begin with the arrival of one or a few pioneer females and both sexes can contribute to the aggregation pheromone complex. The tree responds systemically by producing a defensive resin containing toxic mono-, sesqui-, and diterpenoid chemicals to pitch the beetles out (Steele et al., 1995; Phillips and Croteau, 1999). The combination of toxins and increased resin flow volume is usually sufficient for a healthy tree to kill the beetles. However, under stressful conditions (e.g. drought) or outbreak situations as is now occurring in Western North America, resin production may not be sufficient to stop the infestation (Rudinsky, 1962; Kurz et al., 2008). Mass attacks result in extensive gallery construction in the phloem and the introduction of beetle-associated fungi. Both factors reduce water and nutrient flow and contribute to tree mortality (Seybold et al., 2000). Egg laying, hatching, development through larval instars, pupation, and eclosion to teneral adults all occur within the phloem of the brood tree. Some bark beetle species are very aggressive and can attack and overcome live trees whereas other species concentrate on recently dead branches or trees.

## 2. Origin of pheromones: plant precursors versus de novo

The first bark beetle aggregation pheromone was identified in *I. paraconfusus* as a mixture of ipsdienol, ipsenol and *cis*-verbenol (Silverstein et al., 1966). This was also the first multicomponent insect pheromone described. In the genus *Ips*, the pheromone components ipsdienol and ipsenol are extensively used, with varying ratios of both the *S* and *R* enantiomers of both ipsdienol and ipsenol (Borden, 1985). The IUPAC name for ipsdienol, 2-methyl-6-methylene-2,7-octadien-4-ol, led to the name ipsdienol rather than ipstrienol, even though there are three double bonds in the molecule (Silverstein, personal communication). The structural similarity between the plant monoterpenoids myrcene and  $\alpha$ -pinene to the pheromone components ipsdienol and verbenol led early investigators to propose that bark beetles produced their pheromone components by the hydroxylation of plant derived precursors (Hughes, 1973, 1974) (Fig. 1). In addition, at that time monoterpene synthesis was associated with plant tissues and not described in Animalia. Studies in the 1970s and 1980s led to the conclusion that monoterpene pheromone components in bark beetles were produced from the carbon skeletons of host oleoresin precursors

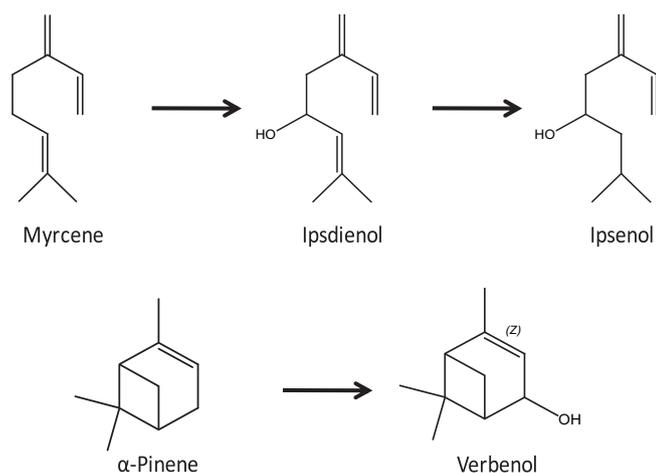
(Hughes, 1973, 1974; Byers et al., 1979; Hendry et al., 1980; Borden, 1985; Vanderwel, 1994). Hendry and colleagues (1980) appeared to have presented unequivocal evidence for the host origin of the monoterpene carbon skeleton when they showed that, when exposed to  $^2\text{H}$ -myrcene, adult male *I. paraconfusus* converted this labeled host hydrocarbon to  $^2\text{H}$ -ipsenol and  $^2\text{H}$ -ipsdienol.

Studies in the late 1980s and the 1990s began challenging the view that bark beetle terpenoid pheromone components were formed exclusively from host precursors. Labeled mevalonate injected into *I. typographus* was incorporated into volatile extracts, and radioactivity was associated with preparative GC fractions that co-eluted with the hemiterpenoid pheromone component, 2-methyl-3-buten-2-ol (Lanne et al., 1989). Byers and Birgersson (1990) presented evidence that there was not enough myrcene in the diet of some *Ips* species to account for the amount of pheromone produced. Theories were advanced that perhaps beetles sequestered myrcene or its hydroxylated products during larval stages, to provide sufficient monoterpenoids for pheromone production (Vanderwel, 1994). Ivarsson et al. (1993) showed that compactin, a statin that inhibits 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) (Nakamura and Abeles, 1985), inhibited the production of ipsdienol and *E*-myrcenol in *I. duplicatus*, providing indirect evidence for de novo biosynthesis. Compactin did not inhibit the synthesis of *cis*-verbenol, consistent with *cis*-verbenol being derived from host tree  $\alpha$ -pinene. Direct biochemical evidence that bark beetles synthesize the acyclic monoterpene pheromones ipsdienol and ipsenol *de novo* via the mevalonate pathway was obtained by demonstrating the incorporation of  $^{14}\text{C}$ -acetate and  $^{14}\text{C}$ -mevalonolactone into both components in *I. pini* and *I. paraconfusus* (Seybold et al., 1995a) (Fig. 2). Furthermore, the enantiomeric ratios of the radiolabeled components match the enantiomeric compositions found in nature (Fig. 2C) (Seybold et al., 1995b). When exposed to myrcene vapors, male *I. pini* produced ipsdienol, but analysis on a chiral column showed that it was a racemic mixture (Lu, 1999) rather than the approximate 95:5 ( $-/+$ ) enantiomeric blend that is produced by feeding or JH III-treated western *I. pini* and functions as the major pheromone component. Work using radio-labeled precursors was extended to *D. jeffreyi* and other *Dendroctonus* species (Barkawi et al., 2003) showing that radioactivity from labeled acetate, mevalonate and isopentenol were incorporated into frontalin in *Dendroctonus* spp. It now appears that most bark beetle pheromone components are produced *de novo* (Fig. 3), although a few, including the  $\alpha$ -pinene derived verbenol, verbenone and verbenene, and the *n*-heptane derived 1- and 2-heptanol are still thought to be derived from host tree precursors (Fig. 4).

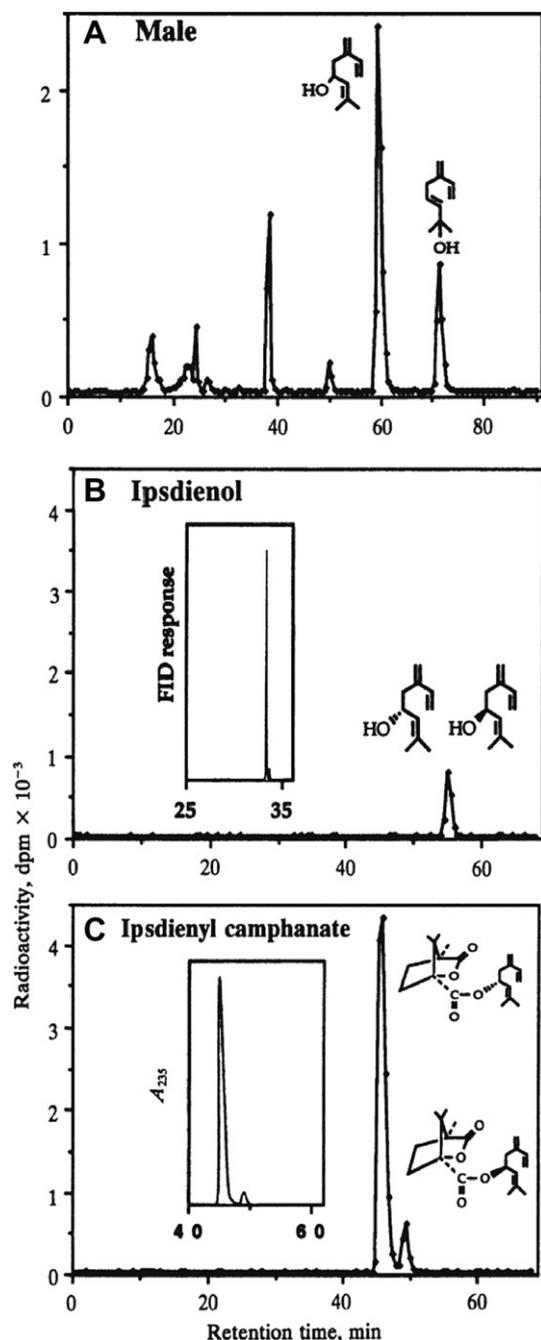
## 3. Metabolic pathways: pheromone production in *Ips* spp.

### 3.1. Ipsdienol and ipsenol

*In vivo* radiochemical studies demonstrated that labeled acetate and mevalonolactone were incorporated into ipsdienol and ipsenol in several *Ips* species (Seybold et al., 1995a; Tillman et al., 1998). The most likely pathway to account for this was the mevalonate pathway (Fig. 5) to form isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) which then condense to form geranyl diphosphate prior to being converted to myrcene and then to ipsdienol and ipsenol. Biochemical studies with isolated midgut tissue demonstrated the conversion of geranyl diphosphate to myrcene (Martin et al., 2003). Numerous earlier studies had demonstrated that myrcene was converted to both ipsdienol and ipsenol (reviewed in Seybold and Tittiger, 2003). Fish et al. (1984) and Vanderwel (unpublished data) showed that ipsdienol could



**Fig. 1.** The first bark beetle pheromone components identified were ipsdienol, ipsenol and verbenol (Silverstein et al., 1966). Because of their similarity to myrcene and  $\alpha$ -pinene, it was suggested that the pheromone components arose from monoterpenes (Hughes, 1973, 1974).



**Fig. 2.** Direct evidence for the de novo biosynthesis of bark beetle pheromones came from the demonstration that radiolabeled acetate, injected into male *I. pini*, labeled ipsdienol, as demonstrated by radio-HPLC (2A) (Seybold et al., 1995a). The isolated ipsdienol (2B) was separated into the (+) and (–) enantiomers as camphanate derivatives, and was comprised of approximately 95:5 (–/+) isomers (2C), the approximate composition of the pheromone from western *I. pini* (Seybold et al., 1995a).

be converted to ipsdienone, reduced to ipsenone, and then converted to ipsenol.

In vertebrate systems, the two enzymes 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) and 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS) are pivotal to the regulation of carbon flow to isoprenoids, and these were the first enzymes examined in bark beetle pheromone biosynthesis (Tittiger et al., 1999a,b, 2003). Using PCR and primers designed from conserved regions for these genes from other metazoans, HMGR was isolated. Both feeding and JH III markedly up-regulated HMGR transcript level and enzyme

activity in *I. pini*, and based on the important regulatory role of HMGR in other organisms, it was assumed that HMGR was the key regulatory step in bark beetle pheromone production (Tillman et al., 2004). Later work using genomic techniques, described below, demonstrated that many of the steps in pheromone production in *I. pini* are coordinately regulated and led to the isolation, expression and characterization of key enzymes.

#### 4. Pheromone production in *Dendroctonus* spp.

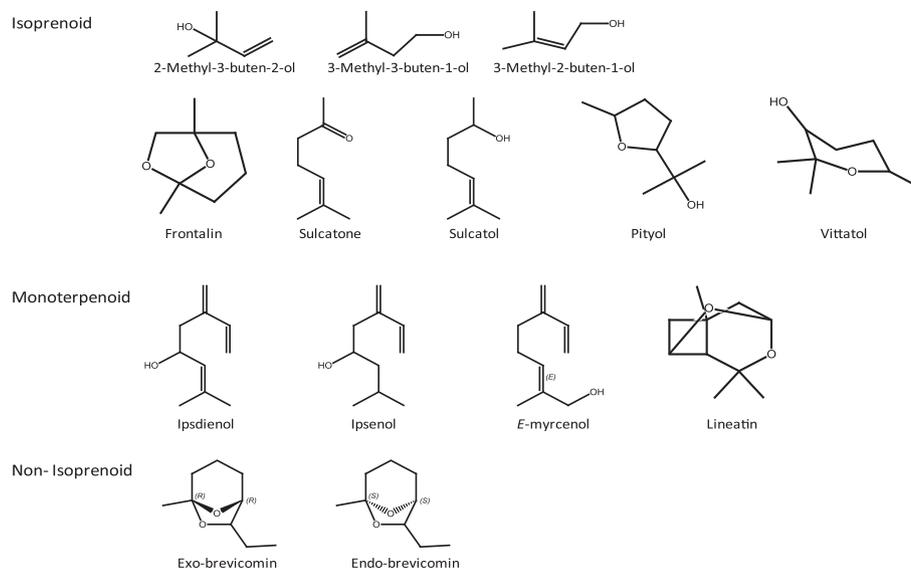
The bicyclic acetals frontalin and *exo*- and *endo*-brevicomin along with hydroxylation products of  $\alpha$ -pinene are often part of *Dendroctonus* spp. pheromones. Frontalin is an anti-aggregation pheromone in some species and an aggregation pheromone in others (Borden, 1985). In *D. ponderosae* the pheromone is still not fully characterized, although three major components are known: frontalin is an anti-aggregation pheromone and *exo*-brevicomin and *trans*-verbenol are aggregation pheromone components (Conn et al., 1984; Hunt et al., 1986; Greis et al., 1990; Pureswaran et al., 2000). The roles of other components, e.g. myrcenol and *endo*-brevicomin, are not as clearly understood (Hunt et al., 1986). *exo*-Brevicomin, frontalin, and verbenol are apparently synthesized via different metabolic pathways as summarized below.

##### 4.1. Frontalin

Frontalin [(1*S*,5*R*)-1,5-dimethyl-6,8-dioxabicyclo[3.2.1]octane], a bicyclic acetal, is produced by *D. ponderosae* males after they arrive in the host tree and may function as a spacing factor signaling “the tree is full” to other beetles (Pureswaran et al., 2000). It is made using the mevalonate pathway in the anterior midgut of male beetles (Hall et al., 2002b). Barkawi et al. (2003) showed that both  $^{14}\text{C}$  labeled mevalonolactone and isopentenol injected into the abdomen of pheromone-producing male *D. jeffreyi* results in production of  $^{14}\text{C}$ -labeled frontalin. Furthermore, in situ hybridization showed that HMGR, which encodes a key regulatory enzyme in the mevalonate pathway, has high transcript levels mostly in midgut tissue of *D. jeffreyi*, showing that the midgut is the site of pheromone biosynthesis. Prior to these studies, the precursor to frontalin was shown to be 6-methylhept-6-en-2-one (6MHO) (Vanderwel et al., 1992; Perez et al., 1996). However its origin and the enzymes responsible for its synthesis and its conversion to frontalin remain unknown. Several metabolic routes leading to this precursor have been hypothesized and include the following (Fig. 6).

- 1- Because mevalonate is readily incorporated into frontalin (Barkawi et al., 2003), 6MHO production may involve a shunt of carbons at the GPP stage in the pathway. Geranyl diphosphate (GPP) could be cleaved by a dioxygenase to yield sulcatol, which in turn could be converted by an isomerase to 6MHO.
- 2- 6MHO could originate from farnesyl diphosphate (FPP), farnesene or geranylgeranyl diphosphate (Fig. 6), following the action of a cleavage enzyme and an isomerase reactions as above. Both *FPPS* and *GGPPS* are up-regulated in *D. ponderosae* male midguts after males join females in nuptial chambers (Aw et al., 2010).
- 3- Another route has been proposed by Francke and Schulz (personal communication) in which 6MHO originates from isopentenyl diphosphate (IPP) by the addition of three carbons from acetoacetyl-CoA.

As for the final steps converting 6MHO to frontalin, a two step reaction mediated first by an epoxidase/P450 followed by a cyclase is possible (Perez et al., 1996), but remains unconfirmed.



**Fig. 3.** Bark beetle pheromones now thought to be produced primarily *de novo* and mainly through the mevalonate pathway. Because host tree monoterpenes can be converted to pheromone components, it is likely that a small amount of some of these components could also arise from host tree precursors.

#### 4.2. *exo*-Brevicommin

*exo*-Brevicommin (exo-7-Ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]octane) is a bicyclic acetal that synergizes the aggregation effect of female-produced *trans*-verbenol. It is produced by newly emerged males (but not females), and production drops when the males arrive at a new tree and mate (Pureswaran et al., 2000). *exo*-Brevicommin is likely synthesized *de novo* by epoxidation and cyclization of its precursor, 6-(*Z*)-nonen-2-one (Vanderwel et al., 1992; Francke et al., 1996). 6-(*Z*)-Nonen-2-one is likely fatty-acid derived, either via desaturation and limited  $\beta$ -oxidation of long chain fatty acyl precursors, as preceded in pheromone biosynthesis in Lepidoptera (Rafaeli and Jurenka, 2003), or else directly via a thioesterase II-mediated termination of fatty acid biosynthesis at a 10-carbon intermediate, desaturated to (*Z*)-7-decenoyl-CoA, which would then be oxidized to a 3-keto-(*Z*)-7-decenoyl intermediate and then decarboxylated (Vanderwel et al., 1992). Conversion of 6-(*Z*)-nonen-2-one to *exo*-brevicommin likely involves a cytochrome P450-mediated formation of a keto-epoxide intermediate that is subsequently cyclized either enzymatically or possibly without an enzyme catalyst (Fig. 7) (Belles et al., 1969).

#### 4.3. *trans*-Verbenol

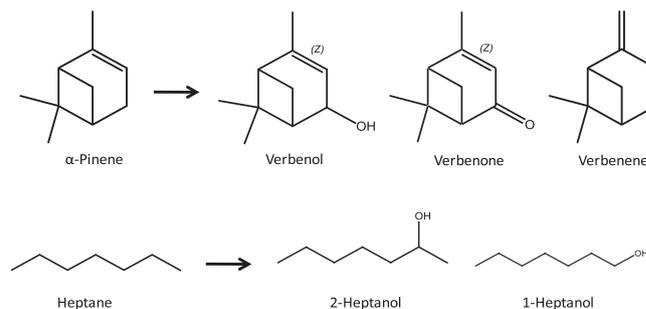
*trans*-Verbenol (4,7,7-trimethylbicyclo[3.1.1]hept-3-en-2-ol) is a bicyclic monoterpene alcohol. It is the major female-produced aggregation pheromone component. Production likely begins upon feeding on a new host tree and ceases upon mating (Pureswaran et al., 2000). It is most likely produced via cytochrome P450-mediated hydroxylation of the host tree resin component,  $\alpha$ -pinene. Female *D. ponderosae* produce a range of hydroxylated  $\alpha$ -pinene products, including *trans*-verbenol and myrtenol (Pierce et al., 1987; Greis et al., 1990), indicating multiple P450 enzymes, one of which produces the pheromone component, (-)-*trans*-verbenol (Greis et al., 1990). There is evidence supporting *de novo* biosynthesis in *D. frontalis* (Renwick et al., 1973), so *de novo* biosynthesis, likely via the mevalonate pathway, is a formal possibility in *D. ponderosae* as well (C. Keeling, personal communication).

#### 4.4. Other components

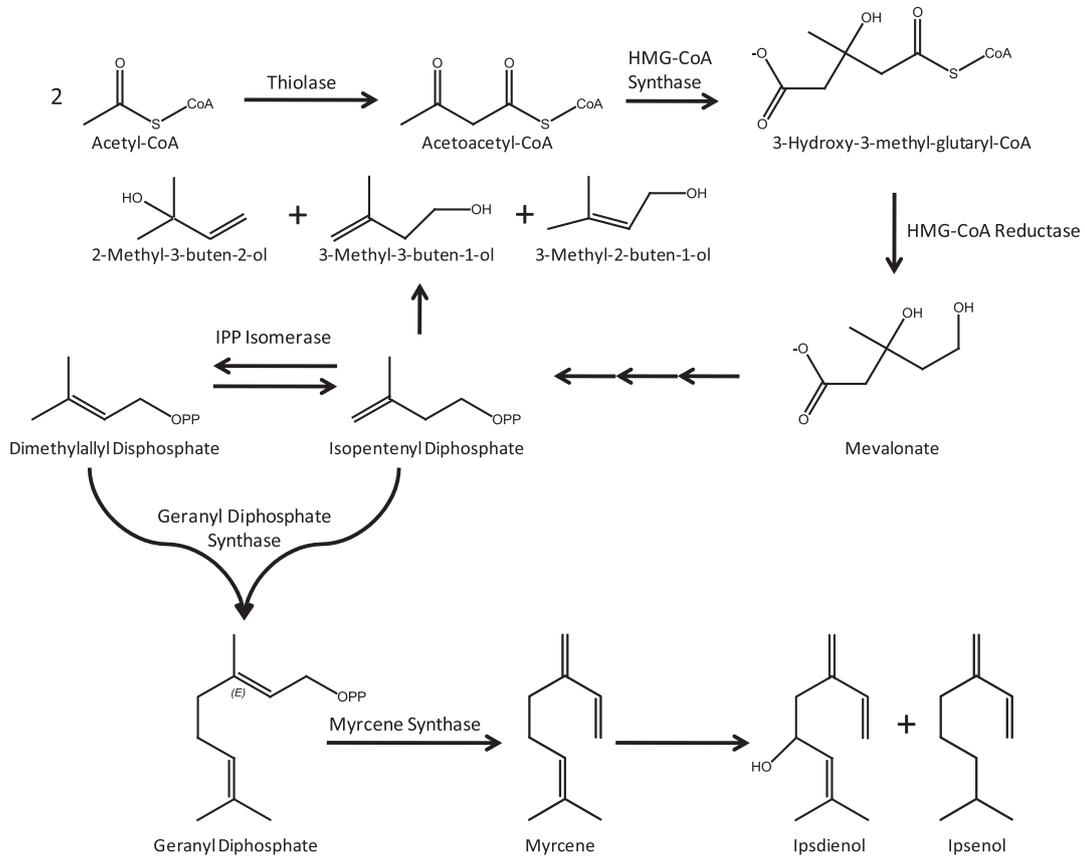
In addition to the three major chemicals described above, other volatiles are present in *D. ponderosae* with more or less well-defined roles. *endo*-Brevicommin is produced by males in a pattern parallel to that of *exo*-brevicommin, but at much lower levels (Pureswaran et al., 2000). Given that its precursor, 6-(*E*)-nonen-2-one is a stereoisomer of the *exo*-brevicommin precursor (Vanderwel et al., 1992), both brevicommins may be produced by the same enzyme. Its role as a semiochemical is unclear, though it is a significant aggregation pheromone component in *D. frontalis* (Sullivan et al., 2007). Verbenone is thought to be an auto-oxidation product of verbenol and may work as an anti-aggregation signal along with frontalin (Lindgren and Miller, 2002). Other components, including *cis*-verbenol, ipsdienol and myrcenol (Hunt et al., 1986; Pierce et al., 1987) have less well-defined roles. Host tree volatiles also synergize beetle-produced pheromone components (Pureswaran et al., 2000).

#### 5. Site of pheromone production in bark beetles

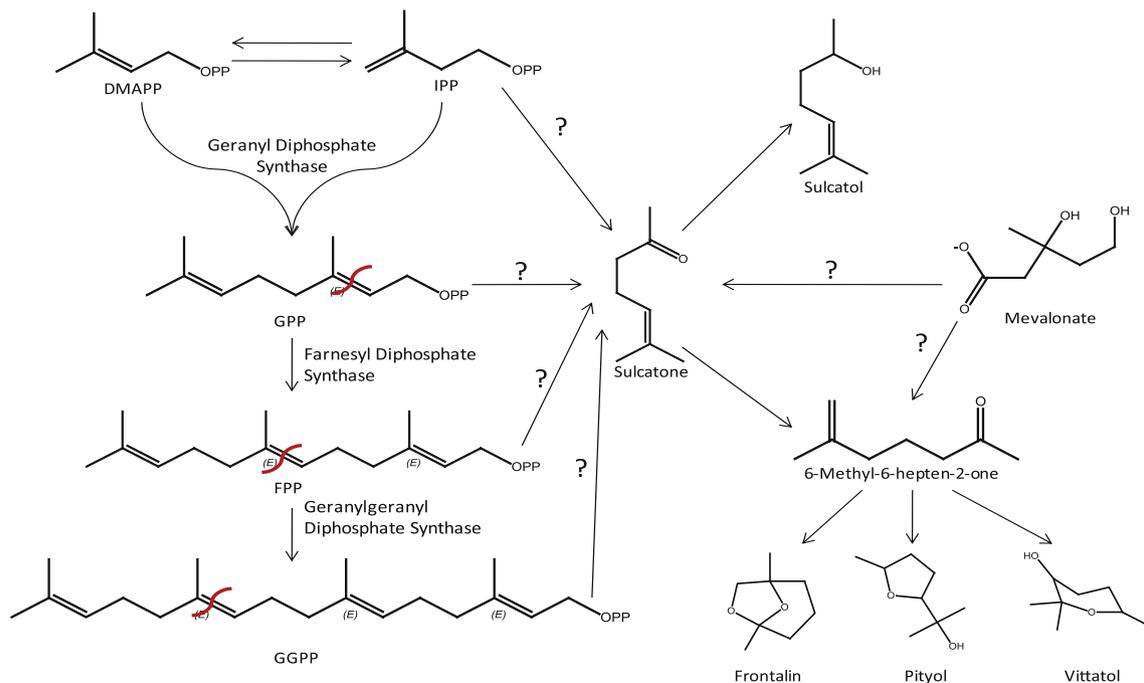
Many insects have specialized cells in the abdomen or use epidermal cells to produce pheromone. Bark beetle pheromones are often associated with frass, suggesting a different site of



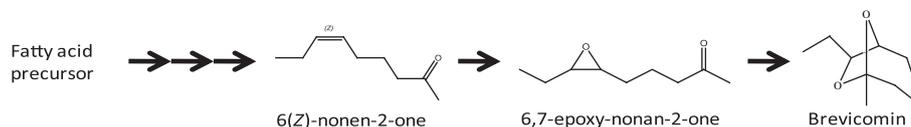
**Fig. 4.** It appears that verbenol, verbenone and verbenene arise from the hydroxylation of host tree derived  $\alpha$ -pinene and that 1- and 2-heptanol from host tree derived *n*-heptane.



**Fig. 5.** Mevalonate pathway leading to the production of hemiterpenes and monoterpenes.



**Fig. 6.** Potential pathways leading to sulcatone and 6-methyl-6-hepten-2-one and frontalin. Radiolabeling studies (Barkawi et al., 2003) demonstrated the incorporation of acetate, mevalonolactone and isopentenol into frontalin. 6-Methyl-6-hepten-2-one has been shown to be converted to frontalin (Perez et al., 1996).



**Fig. 7.** Proposed biosynthetic pathway to brevicomin. 6(Z)-nonen-2-one is presumably epoxidized to the corresponding epoxide and then cyclized to brevicomin (Vanderwel et al., 1992).

synthesis. Early studies showed that pheromones were isolated from the hindguts of various bark beetles (Pitman, 1969; Byers and Wood, 1980; Hughes, 1973). Based on the location of pheromone in the hindgut and the histology and anatomy of gut tissue, particularly the cellular diversity and features which show a secretory richness (Diaz et al., 2000), suggest the midgut as a possible location of pheromone production. Studies with *I. paraconfusus* examined incorporation of radiolabeled acetate into pheromone precursors throughout various body regions in an attempt to localize the site of pheromone synthesis (Ivarsson et al., 1998). They implicated the metathorax, which would contain the midgut, as the site of pheromone biosynthesis of *I. paraconfusus*.

The site of pheromone synthesis in *I. pini* was determined through a combination of molecular and biochemical experiments. Inhibition of HMGR in *I. duplicatus*, showed reduction in ipsdienol synthesis (Ivarsson et al., 1993), and topical treatment of unfed male *I. pini* and *I. paraconfusus* with JH III resulted in a marked increase in HMGR transcripts (Tillman et al., 2004). Therefore HMGR was used as a marker for mevalonate derived pheromone biosynthesis in bark beetles.

Hall et al. (2002a) used *in situ* hybridization to show that HMGR mRNA was very abundant in midguts of JH III-treated male *I. pini* (Fig. 8a–d). HMGR expression increases in the anterior midgut when beetles were treated with JH III, as compared with the acetone control (Fig. 8a and b) (Hall et al., 2002a). To rule out other portions of the alimentary canal as pheromone producing tissue, the authors isolated alimentary tracts from control (Fig. 8d) and JH III treated beetles (Fig. 8c), which showed, similar to the sagittal view of the beetle, that JH III treatment raised HMGR transcript levels in the anterior midgut.

Biochemical assays then confirmed that the *I. pini* midgut makes the pheromone ipsdienol. Alimentary canals from fed and JHIII-treated males were dissected into proventriculus, midgut, and hindgut. The tissues were incubated with radiolabeled acetate,

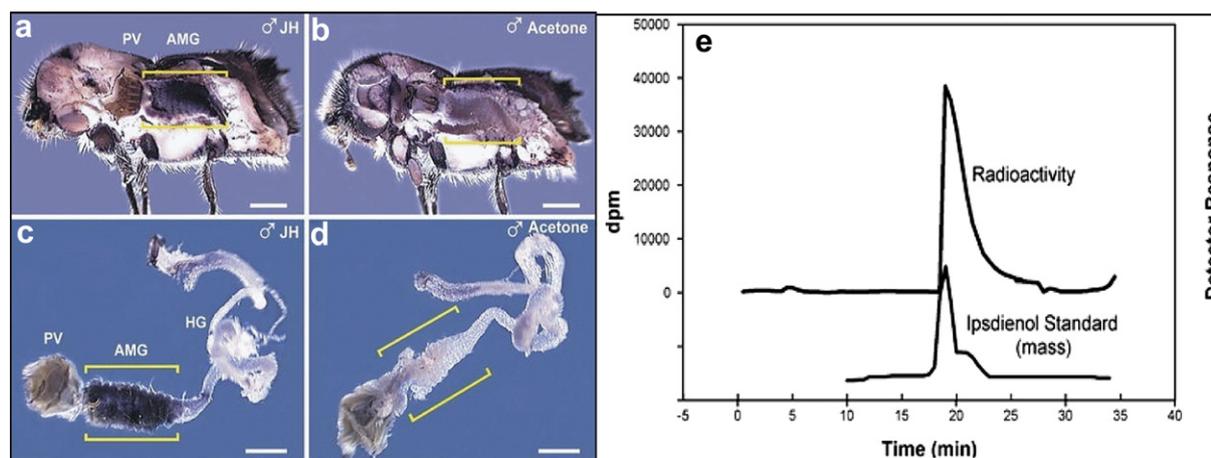
extracted, and analyzed by radio-HPLC. Most of the radioactivity in the pentane:ether extract from midgut tissue was associated with the peak corresponding to an ipsdienol standard (Fig. 8e) thus showing that pheromone biosynthesis occurs in the anterior midgut (Hall et al., 2002a). A similar approach showed that the monoterpene pheromone, frontalin, is also produced in midgut tissue in *D. jeffreyi*, Hall et al. (2002b).

Nardi et al. (2002) performed an ultrastructural examination of the beetle midgut and the endodermal cells from both *D. jeffreyi* and *I. pini*. Using electron microscopy, the smooth endoplasmic reticulum (SER) of the JH III treated male midgut cells were found to be large and arranged in microcrystalline arrays, structures not seen in the negative controls for both *I. pini* and *D. jeffreyi*. The formation of microcrystalline arrays of SER in the pheromone producing cells is the main distinguishing subcellular characteristic inferring involvement of the SER in pheromone biosynthesis, and this abundant SER is associated with isoprenoid lipid biosynthesis. It is known that cytochrome P450s involved in pheromone production are associated with the endoplasmic reticulum (Sandstrom et al., 2006, 2008), but at this point the subcellular localization of the other pheromone biosynthetic enzymes in bark beetles is unknown.

Earlier studies suggested that bark beetle pheromones could be produced by gut tract bacteria (Brand et al., 1975) or fungus (Brand et al., 1976). It is difficult to reconcile JH regulated pheromone production with a bacterial or fungal source of pheromone. The genomic work done (described below) in *I. pini* in which mRNA used to construct cDNA libraries of mRNAs with poly-A tails provides further evidence against a major role of gut bacteria in pheromone production.

## 6. Endocrine regulation pheromone production

Endocrine regulation of pheromone biosynthesis in insects is mediated by pheromone biosynthesis activating neuropeptide



**Fig. 8.** *In situ* hybridization of JH III induced HMGR expression in *I. pini* with the sagittal view exposed showing the proventriculus (PV) and anterior midgut (AMG) of a JH III-treated (a) and acetone treated (b) male. Isolated alimentary canal of the male treated with JH III (c) and the male treated with acetone (d). Radio HPLC (e) of pentane-diethyl ether extract of midgut tissue from five fed male *I. pini* incubated with [ $^{14}$ C] acetate as described Hall et al. (2002a).

(PBAN) in Lepidoptera, by ecdysteroids in the house fly and presumably other Diptera, and by JH III in some Coleoptera, including some bark beetles (Blomquist et al., 2005). The endocrine regulation of pheromone production in bark beetles is complex and not fully understood. In male *I. pini*, feeding on phloem stimulates the corpora allata (CA) to synthesize and release JH III which in turn induces ipsdienol production (Tillman et al., 1998). Application of JH III to male *I. duplicatus* (Ivarsson and Birgersson, 1995) and *I. pini* (Tillman et al., 1998) results in increased pheromone production. However, in two other *Ips* species, *I. paraconfusus* and *I. confusus*, JH III does not induce pheromone production (Tillman et al., 2004; Bearfield et al., 2009).

Studies prior to the 1990s had the difficult task of reconciling the JH III-mediated increase in pheromone production with the expectation that the carbon skeleton of pheromone components was derived from the diet. It was known that bark beetles possess the mechanism to detoxify monoterpenes and it was thought the products of these processes were used directly as pheromones. Since the recognition that ipsdienol and ipsenol are made de novo in bark beetles, work has concentrated on what steps in the process are controlled. Ivarsson and Birgersson (1995) showed that a JH analog increases ipsdienol and *E*-myrcenol production in *I. duplicatus*, whereas exposure to myrcene did not increase pheromone production. The same report also showed Hez-PBAN has no effect on pheromone production in *I. duplicatus*. In *I. pini*, Tillman et al. (1998, 2004) showed that topically applied JH III increased pheromone production and markedly upregulated *HMGR*. Interestingly, in two other *Ips* species, *I. paraconfusus* and *I. confusus*, topically applied JH III did not increase pheromone production (Tillman et al., 1998; Bearfield et al., 2009).

*HMGR* transcript levels increased in both *I. pini* and *I. paraconfusus* when treated with JH III, but *HMGR* enzyme activity remained low even though the transcript level increased in *I. paraconfusus*. This result suggested different post-transcriptional *HMGR* regulation by JH III in *I. paraconfusus* and *I. pini*. Besides *HMGR*, other enzymes from the mevalonate pathway were also studied. The effect of JH III on *HMGS* transcript levels in *I. pini* was studied using real time PCR and Northern blotting (Bearfield et al., 2006). In both males and females, JH III stimulated mRNA levels in the first 8 h. While male mRNA levels continued to increase, female mRNA levels started to decrease after the first 8 h. Furthermore, male transcript levels were higher than the female at every time point examined. These data suggested the coordinate regulation of mevalonate pathway genes by JH III in *I. pini*. Bearfield et al. (2009) further showed different regulatory mechanisms in comparisons between *I. pini* and *I. confusus*, another member of grandicollis group. By using radio-labeling studies, activities of key mevalonate pathway enzymes including *HMGS*, *HMGR*, and *GPPS* were measured. Feeding up-regulated these enzyme activities in both species, but JH III only increased their activities in *I. pini* and not in *I. confusus*. However, RT-PCR showed JH III and feeding up-regulated the mRNA levels in both species. These data suggest at least an additional factor functioning post-transcriptionally to regulate pheromone production.

Antennae also play a role in regulation in *I. pini* (Ginzel et al., 2007). Male beetles without antenna had higher levels of pheromone, a phenomena first demonstrated in the Colorado potato beetle (Dickens et al., 2002). Following JH III treatment, beetles with antennae removed showed higher enzyme activities for *HMGS*, *HMGR*, and *GPPS* than beetles with intact antennae. mRNA levels are also higher in male beetles without antennae. This suggests negative feedback at the transcription level. It was proposed that this mechanism prevents the beetles from overcrowding.

## 7. Functional genomics: *I. pini*

Both feeding and topical JH III application up-regulate pheromone production in *I. pini* (Tillman et al., 1998) and up-regulate transcript and enzyme activity for both *HMGS* and *HMGR* (Tillman et al., 2004). Agreement between biochemical and molecular data for mevalonate pathway genes confirmed that molecular techniques could identify late-step genes. A functional genomics survey in this situation is most useful because late step enzymes may be cryptic (i.e. dioxygenase) or else members of large gene families (e.g. P450s). The strategy to combine functional genomics, molecular biology, and biochemistry was applied to both *I. pini* and *D. ponderosae*. These were the first microarray-based studies of the developmental and hormonal regulation of insect pheromone biosynthesis, and in addition to answering a number of questions about the JH regulation of pheromone biosynthesis, has led to a more complete understanding of pheromone production.

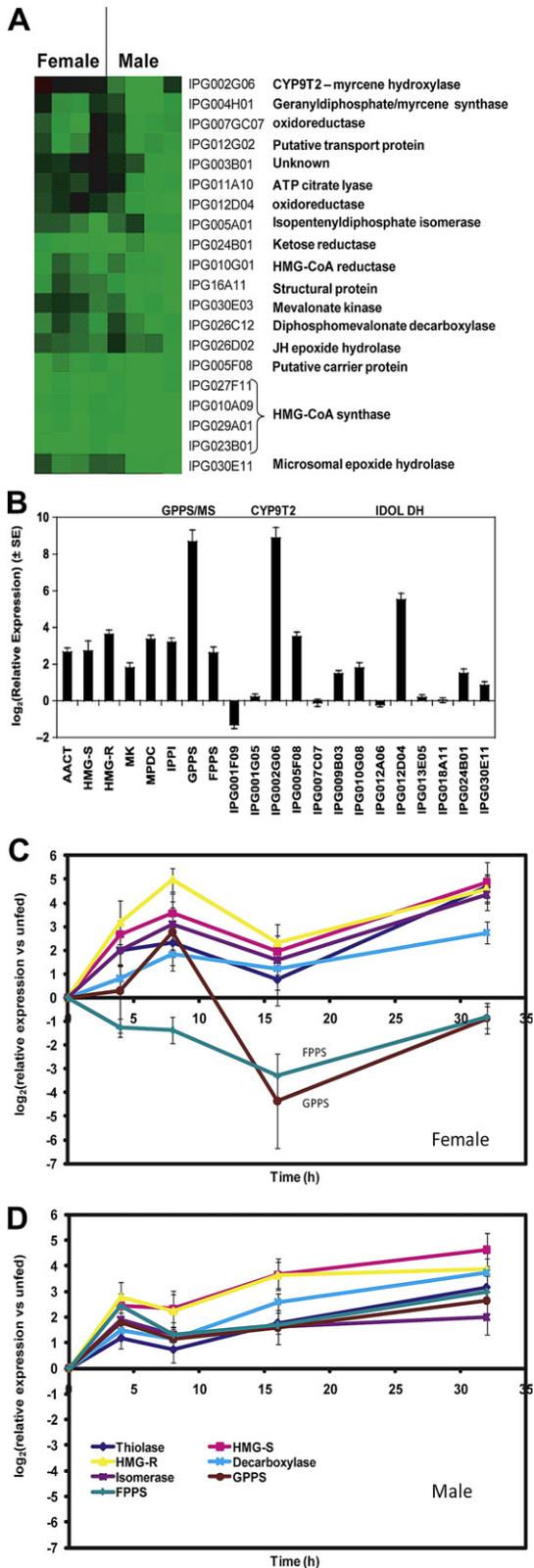
A small-scale EST project (curtailed because of high redundancy) recovered 574 tentatively unique genes expressed in anterior midguts of JH III-treated *I. pini* males (Eigenheer et al., 2003). Microarrays were prepared and hybridized to cDNA from midguts of fed or JH III-treated beetles (Fig. 9A) (Keeling et al., 2004, 2006). Clustering analysis of the microarray data confirmed the coordinate regulation of mevalonate pathway genes by JH III (Keeling et al., 2004, 2006). Other genes with similar expression profiles sorted into a 'pheromone biosynthetic' cluster with the mevalonate pathway genes and were obvious candidates for late-step reactions. They included a novel bifunctional *GPPS*/myrcene synthase, a cytochrome P450 (*CYP9T2*), an oxidoreductase that uses ipsdienol as a substrate and a number of other potentially important genes in pheromone production (Fig. 9A). They are described below.

While the microarray data could suggest candidate genes, other molecular data helped narrow the search. In addition to induction by JH III or feeding, two other criteria, elevated expression levels in males compared to females and mRNA localization to the midgut, became apparent as important markers of putative pheromone-biosynthetic genes. For example, qRT-PCR analyses of expression profiles (Fig. 9C) confirmed the effect of feeding on the up-regulation of mevalonate pathway genes. Surprisingly, many of the mevalonate genes were up-regulated in both males and females, but expression levels in males were always higher than in females. These criteria helped identify ipsdienol dehydrogenase (*IDOL-DH*) from among three other oxidoreductases in the pheromone-biosynthetic gene cluster.

The detailed expression analyses provided clues about the sex-specificity of ipsdienol production. A comparison of basal levels of the mevalonate genes (Fig. 9D) partially explains why males alone produce pheromone as males have a 6 to 40 fold higher basal levels of mevalonate pathway genes. In addition, a key gene in monoterpene production, *GPPS*, has low basal levels in females and is down-regulated by JH III. Thus, females have a lower flux through the metabolic pathway and do not divert carbon into the ipsdienol-biosynthetic pathway.

### 7.1. Geranyl diphosphate synthase/myrcene synthase (*GPPS*/*MS*)

Geranyl diphosphate synthase (*GPPS*) catalyzes the condensation of dimethylallyl diphosphate (*DMAPP*) and isopentenyl diphosphate (*IPP*) to form geranyl diphosphate (*GPP*). Geranyl diphosphate is the precursor of monoterpenes, a large family of naturally occurring  $C_{10}$  compounds predominately found in plants. Martin et al. (2003) showed myrcene synthase activity in *I. paraconfusus*. Conclusive evidence for de novo monoterpene biosynthesis in an animal was obtained by describing *I. pini* *GPPS* (Gill et al., 2005). *GPPS* expression levels are regulated by JH III in



**Fig. 9.** The 'pheromone biosynthetic' gene cluster based on the analysis of microarray data showing the effect of JH III on mevalonate pheromone biosynthesis genes in *I. pini* (Eigenheer et al., 2003). qRT-PCR analyses show the effect of feeding on selected (A) mevalonate genes from female (B) and male (C) midguts (Keeling et al., 2004), and the basal levels of selected genes in males compared to females (D) (Keeling et al., 2006).

a dose- and time-dependent manner, almost exclusively in the anterior midgut of male *I. pini* similar to other mevalonate pathway genes involved in pheromone biosynthesis. The functionally expressed recombinant enzyme produced geranyl diphosphate as its major product. A three-dimensional structural model of GPPS showed that the insect enzyme has the sequence and structural motifs common to *E*-isoprenyl diphosphate synthases, and moreover, interactions between key residues at or near the floor of the binding pocket limit product size to C<sub>10</sub> molecules (Gilg et al., 2005; unpublished).

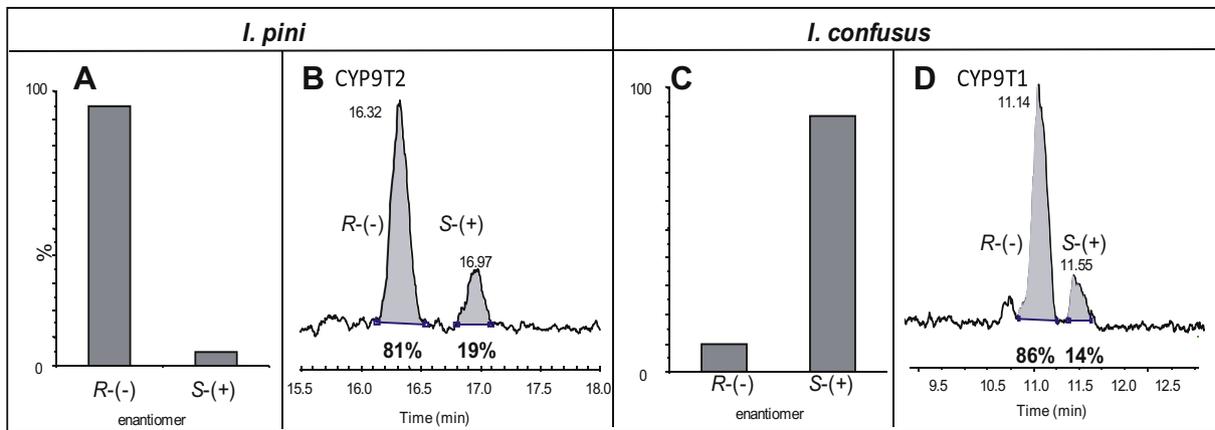
In pheromone-producing male *I. pini*, the acyclic monoterpene myrcene is required for the production of the major aggregation pheromone component, ipsdienol. Surprisingly, monoterpene synthase activity, first demonstrated in *I. confusus* (Martin et al., 2003), was shown to be associated with expressed GPPS (Gilg et al., 2009). Enzyme assays were performed on recombinant GPPS to determine the presence of monoterpene synthase activity, and the reaction products were analyzed by coupled GC-MS. The functionally expressed recombinant enzyme produced both GPP from IPP and DMAPP and myrcene from GDP, making it a bifunctional enzyme. This unique isoprenyl diphosphate synthase possesses the functional plasticity that is characteristic of terpene biosynthetic enzymes of plants, contributing toward the current understanding of product specificity of the isoprenoid pathway.

## 7.2. Cytochrome P450 (CYP9T2) produces an 81:19 (–/+) ipsdienol

Cytochrome P450 monooxygenases (P450s) constitute a diverse superfamily of enzymes that play a crucial role in the metabolism of a wide range of both endogenous and foreign compounds. Insects generally have approximately one hundred cytochrome P450 genes, so preliminary identification of *I. pini* myrcene hydroxylase relied on expression profiling. CYP9T2 was the only P450 among at least four assayed that had an expression pattern consistent with pheromone biosynthesis (Fig. 9). A full-length cDNA clone was isolated and expressed in Sf9 cells. A functional assay using microsomes of Sf9 cells infected with baculoviral constructs encoding CYP9T2 and housefly (*Musca domestica*) P450 reductase demonstrate that CYP9T2 is a myrcene hydroxylase that converts myrcene to ipsdienol (Sandstrom et al., 2006).

The major monoterpene aggregation pheromone component released by *I. pini* is 95% (4*R*)-(–)-ipsdienol (Fig. 10A), whereas recombinant CYP9T2 produced 81% *R*-(–)-ipsdienol (Fig. 10B). More recent data (Sandstrom et al., 2008) demonstrated that the expressed ortholog (CYP9T1) from *I. confusus* produces a similar ratio of *R*-(–) and *S*-(+) ipsdienol (85% *R*-(–)) (Fig. 10D) as does CYP9T2, even though the pheromone blend from *I. confusus* is approximately a 10/90 *R*-(–)/*S*-(+) ratio (Fig. 10C). These data strongly suggest that enzymatic steps downstream from the hydroxylation step are required to produce the final enantiomeric blend.

Three lines of evidence argue for a role of ipsdienone (Fig. 11) in achieving the final 95:5 (–/+) enantiomeric composition in the western *I. pini* pheromone. First, in examining pheromone producing tissue, Ivarsson et al. (1997) isolated much of the labeled C<sub>10</sub> product as ipsdienone. Secondly, Vanderwel et al. (unpublished) demonstrated that deuterium labeled *S*-(+)-ipsdienol was converted to ipsdienone, and that ipsdienone was selectively converted to the *R*-(–) ipsdienol. Finally, our recent demonstration that the product of CYP9T2 produced an 81:19 (–/+) ipsdienol composition argues for other genes to control the final enantiomeric composition. Domingue et al. (2006) selected lines of *I. pini* with either primarily the *R*-(–) enantiomer (western *I. pini*) or the *S*-(+) enantiomer (eastern *I. pini*), and created F1 and F2 progeny. In the analysis of the results from this work, they conclude that



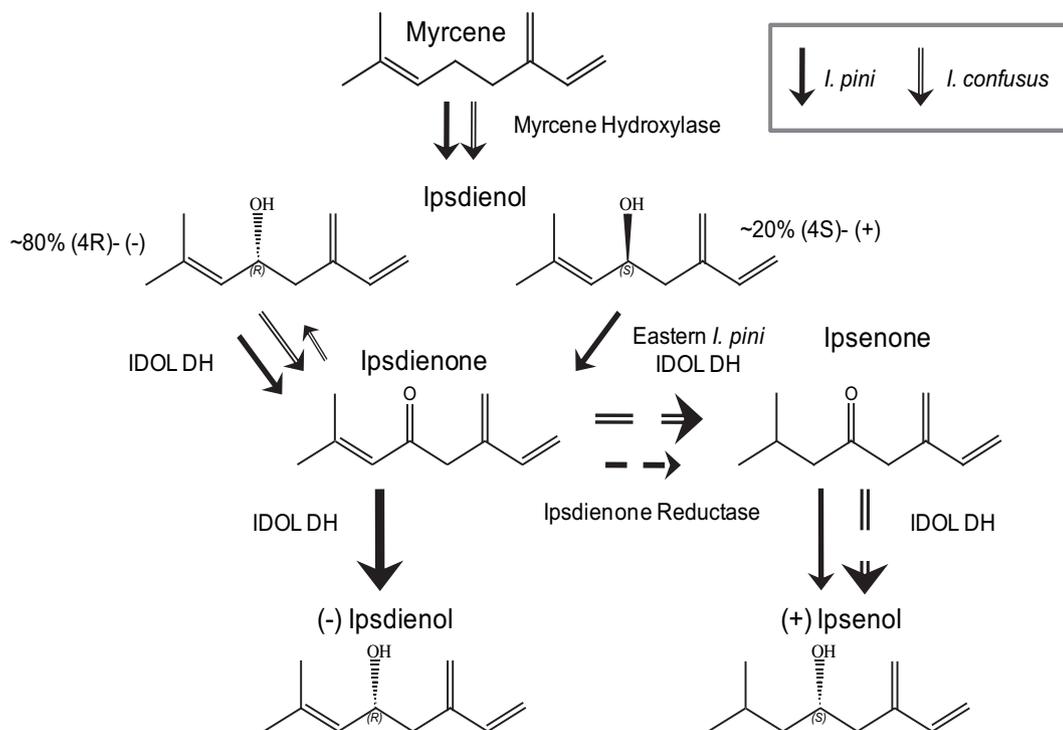
**Fig. 10.** Graphs A and C represent the  $R(-)/S(+)$  ratios of the ipsdienol pheromone components used by *I. pini* and *I. confusus*. GC-MS traces (B and D) show expressed CYP9T2/1 produce predominantly the  $R(-)$  enantiomer of ipsdienol.

dominance at one autosomal locus explains much of the variation in ipsdienol blend between the divergently selected lines, although later work (Domingue and Teale, 2008) suggested the situation might be more complex.

### 7.3. Identification of the Ipsdienol Dehydrogenase (IDOL DH)

A male *I. pini* midgut tissue microarray hybridized to cDNA from JH III-treated beetles identified three oxidoreductases whose expression patterns were similar to known pheromone biosynthetic genes. The gene represented by EST IPG012D04 had the third highest basal transcript level in males compared to females (Fig. 9D) and was specific to the fed male anterior midgut. Full-length cDNAs of this oxidoreductase, now called ipsdienol

dehydrogenase (IDOL DH) and homologues from eastern *I. pini* and *I. confusus* were cloned and sequenced. Homology searches of IDOL DH identified it as a short chain dehydrogenase/reductase (SDR). SDRs constitute a large family of NAD(P)(H)-dependent oxidoreductases and have important roles in the metabolism of lipids, amino acids, carbohydrates, cofactors, hormones and xenobiotics as well as in redox sensor mechanisms (Kavanagh et al., 2008). An NADP(H) assay and coupled GC-MS analysis demonstrated that recombinant IDOL DH could readily oxidize  $R(-)$  ipsdienol to ipsdienone and to stereo-specifically catalyze the reverse reaction. The recombinant enzyme also accepts other substrates including the pheromone precursor ipsenone to  $R(-)$  ipsenol (Figueroa Teran, Welch, Blomquist and Tittiger, unpublished data). Further studies are being conducted to measure the kinetic properties of



**Fig. 11.** Current thoughts on how the stereochemistry of ipsdienol and ipsenol are achieved in *I. pini* and *I. confusus*. There is good evidence that an ipsdienol/ipsdienone dehydrogenase (IDOL DH) oxidizes both  $(-)$  and  $(+)$  ipsdienol to ipsdienone and then stereospecifically reduces ipsdienone to  $(-)$  ipsdienol (Figueroa-Teran et al., unpublished results). In *I. confusus*, we hypothesize that only the  $(-)$  ipsdienol is oxidized to ipsdienone, which is then reduced to ipsenone and then converted to ipsenol.

this protein. The preliminary results suggest that this enzyme plays the key role in determining the final stereochemistry of ipsdienol and ipsenol.

#### 7.4. Functional genomics: *D. ponderosae*

To better understand *Dendroctonus* pheromone production at the molecular level, we conducted a functional genomics study of the mountain pine beetle, *D. ponderosae* (Aw et al., 2010). The scope of this study was larger than that for *I. pini*, partly because of the expected increase in complexity of gene expression in the mountain pine beetle pheromone system. MPB pheromone production levels are also lower than those in *I. pini*, complicating microarray analyses. Thus the *D. ponderosa* EST project involved two cDNA libraries, one including cDNAs from midguts and fat bodies of juvenile hormone (JH) III-treated and –untreated insects because of the known role for JH III in stimulating pheromone biosynthesis in the Coleoptera (Tillman et al., 1998), while the other included cDNAs from insects that were fed or unfed, but not stimulated with applied hormone and thus could be expected to have more biologically normal expression patterns. Following single-pass Sanger sequencing of 12,461 templates, cleansing and clustering, these libraries yielded 1201 contigs and 2833 singlets representing 4034 tentative unique genes distributed across a broad spectrum of the midgut/fat body transcriptome.

In order to begin to identify pheromone-biosynthetic genes, custom oligonucleotide microarrays were used to compare expression patterns of the 4034 TUGs among 11 biological states that spanned the beetle's life history and pheromone component profile (Aw et al., 2010). Four biological replicate pools were generated for each of the 11 states. The microarray data were

confirmed by quantitative (Real-Time) RT-PCR (qRT-PCR) amplification of nine genes, including mostly P450s and mevalonate pathway genes, using first strand cDNA templates prepared from the same RNA used for microarray hybridizations.

The precedent established in *I. pini* (Keeling et al., 2004, 2006) led to the expectation that pheromone biosynthetic genes would be coordinately regulated. These included mevalonate pathway genes involved in frontalin production, and lipid-metabolizing genes (e.g. acetyl-CoA carboxylase, acyl-carrier protein (ACP), ACP transferase,  $\alpha$ -ketoacyl ACP reductase, desaturases, acetyl-CoA synthetase, enoyl hydratase, etc.) for *exo*-brevicomin production. Surprisingly no coordinate regulation was observed among the mevalonate pathway or lipid-metabolizing genes. The lack of coordination may be due to several factors, including the fact that two tissues (midguts and fat bodies) were surveyed simultaneously, while the components are known to be synthesized in one (frontalin in midguts) or the other (*exo*-brevicomin in fat bodies) (Song et al. unpublished results).

The lack of coordinate regulation supports the assertion by Keeling et al. (2006) that microarray data alone are not reliable indicators of a gene's potential role. Nevertheless the data were still useful indicators for preliminary identification of pheromone-biosynthetic genes. For example, *exo*-brevicomin biosynthesis requires a reaction that is likely catalyzed by a cytochrome P450, as epoxides are often formed by P450s. One microarray cluster of three genes with a "male-enriched" expression profile contains a cytochrome P450 (*CYP6CR1*). qRT-PCR profiling indicates that *CYP6CR1* has expression characteristics consistent with *exo*-brevicomin biosynthesis (G. Song and Tittiger, unpublished data), suggesting that it may carry out the epoxidation step. Similarly, frontalin biosynthesis requires carbon to be shunted from the

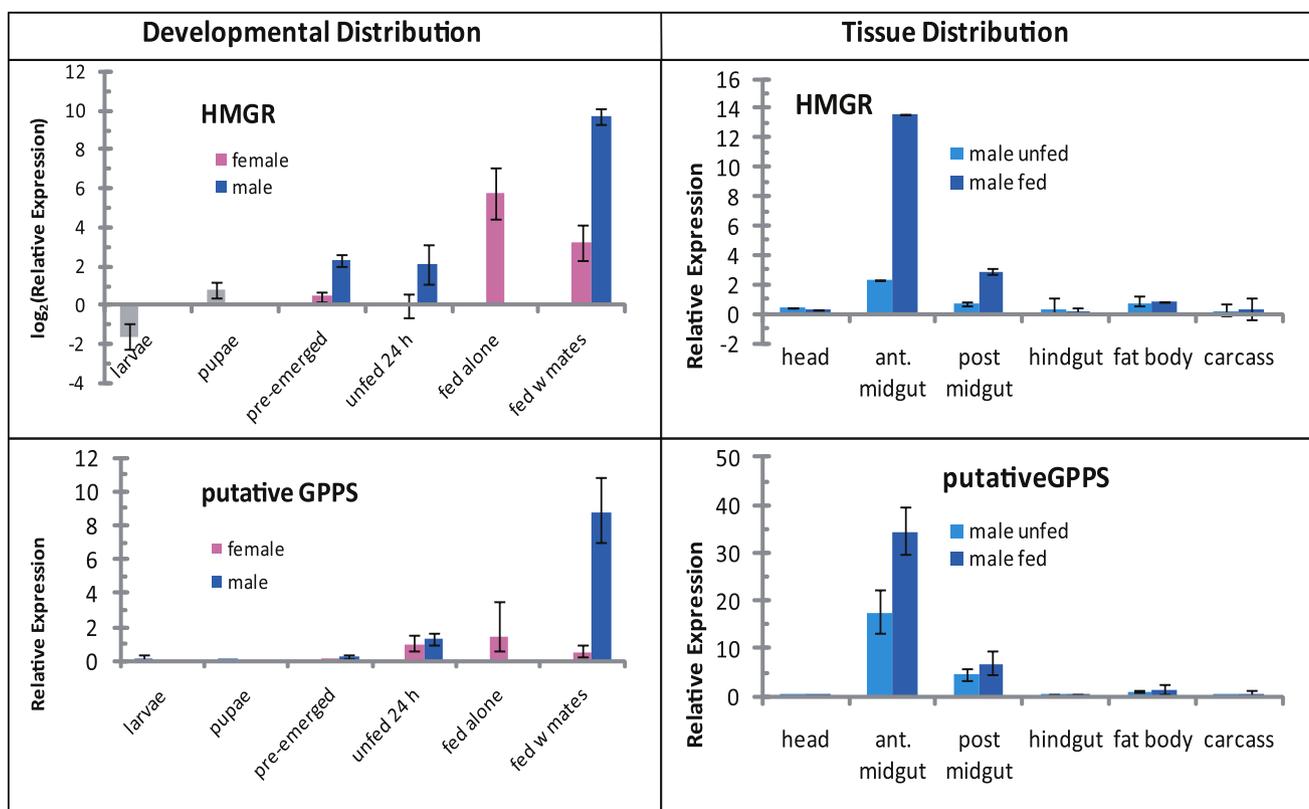


Fig. 12. qRT-PCR analysis of *HMGR* and putative *GGPPS*. Relative mRNA levels are shown with respect to development (A) and tissue distribution in males (B). Note that the basal expression levels of the putative *GGPPS* are much higher than those for *HMGR*.

mevalonate pathway at some point. The microarray data showed reasonably coordinate regulation between *HMGR*, *HMGS* (both encoding enzymes acting early in the pathway) and a putative *GGPPS* (Fig. 12). The expression profiles of all three genes are consistent with frontalin production. The activity of the putative *GGPPS* must be determined as it may provide clues as to where carbon is diverted from the mevalonate into the frontalin biosynthetic pathway. Both *CYP6CR1* (putative epoxidase) and *contig126* (putative *GGPPS*) are current subjects of post-genomic experiments to confirm their functions.

Candidate genes involved in  $\alpha$ -pinene hydroxylation were also tentatively identified and work is underway to express and characterize them.

## 8. Evolution of pheromone production

The evolution of pheromone biosynthesis is complicated by several factors including the various involved organisms (beetles, trees, nematodes, associated microbes, predators, etc.), their corresponding interests and metabolic abilities. Despite this, it may be possible to detect general trends. Biochemical studies appear to support Symonds and Elgar's (2008) suggestion that minor changes to existing metabolic pathways are sufficient to generate a hugely diverse suite of pheromone components. Indeed, differences in *I. pini* pheromone composition are attributed to a single genetic locus (Domingue et al., 2006) or a few loci (Domingue and Teale, 2008). The research summarized in this chapter also indicates both how changes to single enzyme evidently changes pheromone composition, or at least the potential for synthesis. However, it is important to remember that the involved pathways are part of a metabolic web, and that perturbation at one node may exert pressure on others.

The link between many pheromones and host tree resin components was suggested almost as soon as the first bark beetle pheromones were identified as monoterpenoid alcohols (Hughes, 1973). Indeed, the fact that monoterpenes are toxic solvents used by trees to protect against herbivory (Lewinsohn et al., 1991; Steele et al., 1995; Phillips and Croteau, 1999), coupled with the well-known phenomenon of metabolic modification to increase the solubility of toxic chemicals (Berenbaum, 2002), suggests a clear evolutionary path by which hydroxylated monoterpenes became aggregation pheromone components. An ancestral bark beetle likely evolved resin detoxification mechanisms before aggregation pheromone biosynthesis became an important aspect of its progeny's life history. The excreted monoterpene alcohol(s) would have exerted selective evolutionary pressure for conspecifics that were able to sense the presence of the metabolite and respond to its meaning that a host had potentially been compromised. While this would have raised intra-specific competition between the pioneer and its followers, that cost would have obviously outweighed (at least initially) by the contribution of additional beetles in mitigating the danger of a healthy tree.

Thus, the product of the detoxification reaction effectively became the aggregation pheromone component in these ancestral beetles and we see evidence for this in modern bark beetles. For example, there is no evidence supporting de novo verbenol production, suggesting that pheromonal verbenol is derived either from host tree  $\alpha$ -pinene or else from endosymbionts (Renwick et al., 1976; Rao et al., 2003). Even those species which synthesize monoterpenoid alcohols de novo retain the need to detoxify resin. The situation with ipsdienol is not so clear because of stereochemical considerations (see below), but inhaled myrcene is readily converted to ipsdienol in *Ips* spp. Here, host tree precursors may join the same metabolic pool as de novo precursors and are thus indistinguishable to downstream enzymes (Hendry et al., 1980;

Ivarsson and Birgersson, 1995; Lu, 1999). Exogenously applied myrcene was converted to a racemic mixture of ipsdienol in *I. pini* (Lu, 1999), suggesting that all mechanisms of myrcene accumulation are not treated the same.

One implication of the above scenario is that some "detoxification" enzymes have taken a secondary biological role as "pheromone biosynthetic" enzymes. It predicts that pheromone-biosynthetic enzymes may be distinguishable from detoxification enzymes based on alterations in enzyme activity and/or regulation. For example, a detoxification enzyme may be expected to have a broad substrate range and be induced by those substrates, whereas a pheromone-biosynthetic enzyme may show a strong preference for the pheromone precursor and perhaps coordinate hormonal regulation with other pheromone-biosynthetic (non-detoxifying) enzymes. The difference between the two may conceivably occur following simple point mutations in coding or regulatory regions, respectively. The fact that *I. pini* *CYP9T2* (myrcene hydroxylase) is not induced by myrcene (A. Griffith and Tittiger, unpublished data) and is clearly pheromone-biosynthetic (Sandstrom et al., 2008), yet also hydroxylates  $\alpha$ -pinene (Sandstrom, 2007), may be evidence for this evolutionary scheme.

A second implication is that enantiomeric constraints on "pheromonal" blends are a comparatively recent development in *Ips* spp, and that more primitive species may not share those constraints. The enantiomeric ratios of ipsdienol produced by *CYP9T2* (*I. pini*) and *CYP9T1* (*I. confusus*) are nearly identical despite the fact that the enantiomeric ratios of pheromonal ipsdienol are almost diametrically opposed in the two species, so the P450s themselves are not important to establish their pheromone blend ratios (Sandstrom et al., 2008). For *I. pini*, at least one oxidoreductase, *IDOL-DH*, catalyzes the interconversion of (+)- and (–)-ipsdienol (and ipsenol) via a ketone intermediate. Genetic drift of this enzyme may be important for the divergence of enantiomeric ratios of ipsdienol among eastern and western populations. This hypothesis is currently being tested by comparing *IDOL-DH* sequences, expression patterns, and activities in different *I. pini* populations. Similarly lanierone is an aggregation pheromone component of eastern *I. pini* populations, but is not a pheromone component of western populations (Miller et al., 1997); indeed, it is produced in western populations typically towards the end of an attack. It is probably derived from ipsdienol (or ipsenol) via a series of cyclization/decarboxylation/oxidation reactions, although its biosynthetic pathway is not yet characterized. Thus, lanierone production appears to be a relatively recent addition to pheromone components.

While the observation that cytochromes P450 are not necessarily important for the enantiomeric ratios of pheromonal ipsdienol (Sandstrom et al., 2008) contradicts earlier predictions (Renwick et al., 1976; Seybold et al., 2000), there is strong indirect evidence supporting stereo-selective P450-mediated reactions in the case of  $\alpha$ -pinene hydroxylation in *Dendroctonus* spp. (Greis et al., 1990). Female (*trans*-verbenol producing) *D. ponderosae* appear to have two distinct  $\alpha$ -pinene hydroxylating pathways. The first pathway does not discriminate between  $\alpha$ -pinene enantiomers and likely plays a detoxification role, while second pathway appears specific for (–)- $\alpha$ -pinene and therefore produces the bulk of the pheromone component, (–)-*trans*-verbenol (Pierce et al., 1987). The P450s in the two pathways may be closely related.

De novo production requires supporting precursor biosynthesis and therefore at least one enzyme upstream of the P450 to have changed activity. For ipsdienol, this would be acquisition of monoterpenoid biosynthetic ability. Monoterpene biosynthesis is rare among the Metazoa, but appears to have evolved at least twice in the Coleoptera: in the Scolytidae and Chrysomelidae (or once in

their common ancestor and lost multiple times) (Burse et al., 2007). Given that ipsdienol/ipsenol pheromone components are common in *Ips* spp., but are virtually absent in *Dendroctonus*, it is possible that this ability arose after these genera diverged. *I. pini* GPPS appears to have derived from an ancestral GGPPS (Gilg et al., 2005). Primary structure differences between these enzymes result in differences in the substrate binding pocket such that the pocket for GPPS is smaller and less selective than those for FPPS or GGPPS. Furthermore, the GPPS can also accept GPP as a substrate to produce myrcene (Gilg et al., 2009).

Frontalin production also requires a novel activity to lead to the 6-methyl-6-hepten-2-one precursor. Beyond solid experimental evidence that frontalin is of isoprenoid origin with 6-methylhept-6-en-2-one as a late precursor (Perez et al., 1996; Barkawi et al., 2003), the metabolic pathway to frontalin is not clear. A dioxygenase is proposed to act on an isoprenoid precursor to produce 6-methylhept-6-en-2-one, but it is perhaps significant that a monoterpene precursor may not be required if the proposed dioxygenase can accept longer chain isoprenoids (Fig. 6). *Dendroctonus* spp. may thus lack the GPPS and/or monoterpene synthase required for ipsdienol production, which perhaps explains why a GPPS has not yet been identified in *Dendroctonus*, despite considerable effort (Aw et al., 2010).

Possible intermediates following dioxygenase activity are sulcatone or sulcatol, which could potentially isomerize to 6-methylhept-6-en-2-one. However, limited experimental evidence suggests neither are direct precursors (Perez et al., 1996), leaving the possibility that the isomerization reaction occurs prior to the dioxygenase, or that a dioxygenase is not required at all. Interestingly, sulcatone is related to (S)-3,7-dimethyl-2-oxo-oct-6-ene-1,3-diol, the male pheromone of the Colorado potato beetle (*Lepidotarsa decemlineata*) (Dickens et al., 2002), and is apparently broadly distributed in the Insecta (Pherobase), although its de novo biosynthesis in insects has not been established. Frontalin biosynthesis in *Dendroctonus* spp may thus represent an adaptation of an ancestral sulcatone biosynthetic activity prior to the divergence of the Scolytidae and Chrysomelidae, perhaps driven by pressure exerted by predators or intraspecific competition. The absence of frontalin biosynthesis in *Ips* spp. is interesting. Some species can convert 6-methylhept-6-en-2-one to frontalin if it is provided to them (Perez et al., 1996), so the P450(s) that epoxidize 6-methylhept-6-en-2-one may be ancestral, whereas the reactions to produce the chemical may have been lost.

*trans*-Verbenol and ipsdienol are examples of how environmental factors may contribute to pheromone evolution. In contrast, frontalin and *exo*-brevicomin are both semiochemicals with no clear link to a detoxification processes. In this respect they are analogous to lepidopteran or dipteran pheromones, which are derived from endogenous pathways. While frontalin and *exo*-brevicomin are often grouped together because of their structural similarity (Symonds and Elgar, 2004; Symonds and Wertheim, 2005), their precursors arise from very different pathways: frontalin is clearly isoprenoid and synthesized in the midgut (Barkawi et al., 2003) whereas *exo*-brevicomin is likely fatty acid-derived (Vanderwel et al., 1992; Perez et al., 1996) and synthesized in the fat body (Song et al., unpublished data). Yet the two chemicals may be metabolically linked because their precursors (6-methylhept-6-en-2-one and 6-(Z)-nonene-2-one) are structurally very similar and may be epoxidized by the same P450.

Brevicomin biosynthesis may represent an unusual example of the well-documented use of lipid metabolites as insect pheromones. Interestingly, similar to frontalin, the brevicomins are produced among various *Dendroctonus* spp., but are absent from *Ips* spp. (Symonds and Elgar, 2004), though some *Ips* beetles may produce *exo*-brevicomin if they are provided the appropriate

precursor (Perez et al., 1996). The *endo*- and *exo*-isomers may be semiochemicals, depending on the species (Silverstein et al., 1968; Francke et al., 1996; Poland and Borden, 1998; Paine et al., 1999; Sullivan et al., 2007), and their appearance depends on the presence of (*E*)- or (*Z*)-non-6-ene-2-one precursors, respectively (Vanderwel et al., 1992). Their production may therefore depend on related desaturases that produce *trans*- or *cis*- desaturated fatty acid precursors, especially if downstream enzymes do not discriminate between the precursors. This may be an example of an alteration in desaturase activity driving changes in pheromone chemistry analogous to the situation observed in various Lepidoptera (Roelofs and Rooney, 2003; Lienard et al., 2008).

## 9. Evolution of the regulation of pheromone production

Early experiments on pheromone production noted the role of juvenile hormone (JH) III in stimulating ipsdienol biosynthesis in *I. confusus* (Borden et al., 1969), and this role was further confirmed in *I. pini* and *D. jeffreyi* (Tillman et al., 1998). The general paradigm that feeding stimulates JH III biosynthesis in pioneer beetles, which in turn stimulates the anterior midgut to synthesize aggregation pheromone components, still holds true in several bark beetle species (Borden et al., 1969; Bridges, 1982; Tillman et al., 1998, 2004; Tittiger et al., 2003). However, recent studies of pheromone-biosynthetic genes in *I. pini* show that regulation is often more complex than originally anticipated.

Production of pheromones from host tree precursors (i.e. *trans*-verbenol) may not be regulated by JH III at all, but may instead be induced by the presence of their precursors in a classic detoxification response. This regulatory scheme may reflect a presumed ancestral state. Better control of the signal could be achieved with pheromone-biosynthetic genes under hormonal regulation. Juvenile hormone III is important in adult Coleoptera, particularly in the context of maturation and homeostasis (Graham et al., 1996; Wyatt and Davey, 1996). The required machinery present in an ancestral beetle was probably adapted for use in regulating communication.

Initial hormonal regulation may have been limited to entry or branch points in the pathway, analogously to the regulation of vertebrate cholesterol biosynthesis by HMGR (Goldstein and Brown, 1990). However, coordinate regulation of pheromone-biosynthetic genes provides even closer control, both opening the gates and widening the corridors for carbon to flow into pheromone production without dealing with potential bottle necks caused by un-regulated steps. Indeed, the entire mevalonate pathway in *I. pini* up to the GPPS/myrcene synthase branch point is induced by JH III (Keeling et al., 2004), as are downstream enzymes (Sandstrom, 2007). Coordinate regulation may also have arisen due to biochemical necessity: with the appearance of GPPS/MS arising from an ancestral GGPPS, beetle tissues would have begun producing their own toxin (myrcene), which would have been dealt with by a P450 (CYP9T2) already involved in hydroxylating ingested myrcene. There would have been significant pressure for CYP9T2 to be coordinately regulated with GPPS/MS.

Other regulatory schemes are clearly present. Basal expression levels of *I. pini* pheromone-biosynthetic genes are significantly higher in males (the pheromone-producing sex) than females, and GPPS/MS is not induced by JH III in females as it is in males. Both observations indicate developmental and sex-specific influences (Keeling et al., 2006). Similarly, teneral male *D. jeffreyi* HMGR mRNA levels are not induced by JH III to the same extent as those in fully mature insects (Barkawi, 2002), suggesting developmental regulation. An antennally-mediated negative-feedback regulation in *I. pini* suggests possible neural influences (Ginzel et al., 2007) similar to that observed in *L. decemlineata* (Dickens et al., 2002). Finally, mevalonate pathway genes show a pulse of mRNA

induction in fed male *I. pini*, whereas unfed, JH III-treated males show a steady increase in mRNA levels over time (Keeling et al., 2004, 2006). Perhaps most striking is the separation of regulation into transcriptional and post-transcriptional steps as observed in *I. confusus* and *I. paraconfusus*. In those insects, JH III-treatment of unfed males elevates mRNA of some mevalonate pathway genes but does not stimulate enzymatic activity of their corresponding products, or pheromone biosynthesis (Bearfield, 2004; Tillman et al., 2004). Instead, a secondary factor, hypothesized to be a peptide hormone, produced upon feeding, appears necessary for enzyme activity and concomitant pheromone production (Bearfield et al., 2009). Finally, as noted above, some pheromone components are not regulated by JH III, and not all are produced in the midgut (e.g. *exo-brevicommin*). These examples illustrate that regulation of pheromone production is complex and diverse, even within a given genus.

Our understanding of pheromone production and evolution in *Ips* spp. has made giant strides forward in the past decade with the use of a genomics approach. It is likely that similar gains in our understanding of *Dendroctonus* pheromone production will be made as the current genomics approach (Aw et al., 2010; Keeling personal communication).

## Acknowledgements

Work described from the author's laboratories was funded in part by USDA grants 2005-04891 and 2009-05200, NSF grants 9985828, 0642182 and 07192279, and a Nevada Agriculture Experiment Station McIntyre-Stennis grant. The authors thank the Whittell Forest managers for permission to obtain beetles from infested trees.

## References

- Aw, T., Schlauch, K., Keeling, C.I., Young, S., Bearfield, J.C., Blomquist, G.J., Tittiger, C., 2010. Functional genomics of the mountain pine beetle (*Dendroctonus ponderosae*) midguts and fat bodies. *BMC Genomics* 11 (215).
- Barkawi, L.S., 2002. Biochemical and molecular studies of aggregation pheromones of bark beetles in the genus *Dendroctonus* (Coleoptera: Scolytidae), with special reference to the Jeffrey pine beetle, *Dendroctonus jeffreyi* Hopkins. Ph.D. Dissertation. Biochemistry, University of Nevada, Reno. 220 pp.
- Barkawi, L.S., Francke, W., Blomquist, G.J., Seybold, S.J., 2003. Frontalin: de novo biosynthesis of an aggregation pheromone component by *Dendroctonus* spp. bark beetles (Coleoptera: Scolytidae). *Insect Biochem. Mol. Biol.* 33, 773–788.
- Bearfield, J.C., 2004. Understanding juvenile hormone's mode of action in the pine engraver beetle, *Ips pini* (Coleoptera: Scolytidae). Ph.D. Dissertation. Biochemistry and Molecular Biology, University of Nevada, Reno. 230 pp.
- Bearfield, J.C., Keeling, C.I., Young, S., Blomquist, G.J., Tittiger, C., 2006. Isolation, endocrine regulation and mRNA distribution of the 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMG-S) gene from the pine engraver, *Ips pini* (Coleoptera: Scolytidae). *Insect Mol. Biol.* 15, 187–195.
- Bearfield, J.C., Henry, A.G., Tittiger, C., Blomquist, G.J., Ginzel, M.D., 2009. Two regulatory mechanisms of monoterpenoid pheromone production in *Ips* spp. of bark beetles. *J. Chem. Ecol.* 35, 689–697.
- Belles, T.E., Brownlee, R.G., Silverstein, R.M., 1969. Synthesis of brevicomin, principal sex attractant in the frass of the female western pine beetle. *Tetrahedron* 25, 5149–5153.
- Berenbaum, M.R., 2002. Postgenomic chemical ecology: from genetic code to ecological interactions. *J. Chem. Ecol.* 28, 873–896.
- Blomquist, G.J., Jurenka, R.A., Schal, C., Tittiger, C., 2005. Biochemistry and molecular biology of pheromone production. In: Gilbert, L.I., Iatrou, K., Gill, S.S. (Eds.), *Comprehensive Molecular Insect Sciences*, vol. 3. Elsevier, pp. 705–752.
- Borden, J.H., Nair, K.K., Slater, C.E., 1969. Synthetic juvenile hormone: induction of sex pheromone production in *Ips confusus*. *Science* 166, 1626–1627.
- Borden, J.H., 1985. Aggregation pheromones. In: Kerkut, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 9, pp. 257–285.
- Brand, J.M., Bracke, J.W., Markovetz, A.J., Wood, D.L., Browne, L.E., 1975. Production of verbenol pheromone by a bacterium isolated from bark beetles. *Nature (Lond.)* 254, 136–137.
- Brand, J.M., Bracke, J.W., Britton, L.N., Markovetz, A.J., Barras, S.J., 1976. Bark beetle pheromones: production of verbenone by a mycangial fungus of *Dendroctonus frontalis*. *J. Chem. Ecol.* 2, 195–199.
- Bridges, J.R., 1982. Effects of juvenile hormone on pheromone synthesis in *Dendroctonus frontalis*. *Environ. Entomol.* 11, 417–420.
- Burse, A., Schmidt, A., Frick, S., Kuhn, J., Gershenzon, J., Boland, W., 2007. Iridoid biosynthesis in *Chrysomelina* larvae: Fat body produces early terpenoid precursors. *Insect Biochem. Mol. Biol.* 37, 255–265.
- Byers, J.A., Wood, D.L., 1980. Antibiotic-induced inhibition of pheromone synthesis in a bark beetle. *Science* 213, 763–764.
- Byers, J.A., Wood, D.L., Browne, L.E., Fish, R.H., Piatek, B., 1979. Relationship between a host plant compound, myrcene, and pheromone production in the bark beetle, *Ips paraconfusus*. *J. Insect Physiol.* 25, 477–482.
- Byers, J.A., Birgersson, G., 1990. Pheromone production in a bark beetle independent of myrcene precursor in host pine species. *Naturwissenschaften* 77, 385–387.
- Conn, J.E., Borden, J.H., Hunt, D.W.A., Holman, J., Whitney, H.S., Spanier, O.J., Pierce, H.D., Oehlschlager, A.C., 1984. Pheromone production by axenically reared *Dendroctonus ponderosae* and *Ips paraconfusus* (Coleoptera: Scolytidae). *J. Chem. Ecol.* 10, 281–290.
- Diaz, E., Cisneros, R., Zuniga, G., 2000. Comparative anatomical and histological study of the alimentary canal of the *Dendroctonus frontalis* (Coleoptera: Scolytidae). *Complex. Ann. Entomol. Soc. Am.* 93, 303–311.
- Dickens, J.C., Oliver, J.E., Hollister, B., Davis, J.C., Klun, J.A., 2002. Breaking a paradigm: male-produced aggregation pheromone for the Colorado potato beetle. *J. Exp. Biol.* 205, 1925–1933.
- Domingue, M., Starmer, W., Teale, S., 2006. Genetic control of the enantiomeric composition of ipsdienol in the pine engraver, *Ips pini*. *J. Chem. Ecol.* 32, 1005–1026.
- Domingue, M., Teale, S., 2008. The genetic architecture of pheromone production between populations distant from the hybrid zone of the pine engraver, *Ips pini*. *Chemoecology* 17, 255–262.
- Eigenheer, A.L., Keeling, C.I., 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.
- Fish, R.H., Browne, L.E., Bergot, J., 1984. Pheromone biosynthetic pathways: conversion of ipsdienol to (-)-ipsdienol, a mechanism for enantioselective reduction in the male bark beetle, *Ips paraconfusus*. *J. Chem. Ecol.* 10, 1057–1064.
- Francke, W., Schroder, F., Philipp, P., Meyer, H., Sinnwell, V., Gries, G., 1996. Identification and synthesis of new bicyclic acetals from the mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Col.: Scol. Biorg. Med. Chem. 4, 363–374).
- Gilg, A.B., Bearfield, J.C., Tittiger, C., Welch, W.H., Blomquist, G.J., 2005. Isolation and functional expression of the first animal geranyl diphosphate synthase and its role in bark beetle pheromone biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* 102, 9760–9765.
- Gilg, A.B., Tittiger, C., Blomquist, G.J., 2009. Unique animal prenyltransferase with monoterpene synthase activity. *Naturwissenschaften* 96, 731–735.
- Ginzel, M.D., Bearfield, J.C., Keeling, C.I., McCormack, C.C., Blomquist, G.J., Tittiger, C., 2007. Antennally-mediated negative-feedback regulation of pheromone production in the pine engraver beetle, *Ips pini*. *Naturwissenschaften* 94, 61–64.
- Goldstein, J.L., Brown, M.S., 1990. Regulation of the mevalonate pathway. *Nature* 343, 425–430.
- Graham, L.A., Bendena, W.G., Walker, V.K., 1996. Juvenile hormone regulation and developmental expression of a *Tenebrio* desiccation stress protein gene. *Develop. Genet.* 18, 296–305.
- Greis, G., Leufven, A., LaFontaine, J.P., Pierce, H.D., Borden, J.H., Vanderwel, D., Oehlschlager, A.C., 1990. New metabolites of  $\alpha$ -pinene produced by the mountain pine beetle, *Dendroctonus ponderosae* (Coleoptera: Scolytidae). *Insect Biochem.* 20, 365–371.
- Hall, G.M., Tittiger, C., Andrews, G.L., Mastick, G.S., Kuenzli, M., Luo, X., Seybold, S.J., Blomquist, G.J., 2002a. Midgut tissue of male pine engraver, *Ips pini*, synthesizes monoterpenoid pheromone component ipsdienol de novo. *Naturwissenschaften* 89, 79–83.
- Hall, G.M., Tittiger, C., Blomquist, G.J., Andrews, G., Mastick, G., Barkawi, L.S., Bengoa, C., Seybold, S.J., 2002b. Male Jeffrey pine beetle, *Dendroctonus jeffreyi*, synthesizes the pheromone component frontalin in anterior midgut tissue. *Insect Biochem. Mol. Biol.* 32, 1525–1532.
- Hendry, L.B., Piatek, B., Browne, L.E., Wood, D.L., Byers, J.A., Fish, R.H., Hicks, R.A., 1980. *In vivo* conversion of a labelled host plant chemical to pheromones of the bark beetle, *Ips paraconfusus*. *Nature* 284, 485.
- Hughes, P.R., 1973. Effect of  $\alpha$ -pinene exposure on *trans*-verbenol synthesis in *Dendroctonus ponderosae* Hopk. *Naturwissenschaften* 60, 261–262.
- Hughes, P.R., 1974. Myrcene: a precursor of pheromones in *Ips* beetles. *J. Insect Physiol.* 20, 1271–1275.
- Hunt, D.W.A., Borden, J.H., Pierce Jr., H.D., Slessor, K.N., King, G.G.S., Czyzewska, E.K., 1986. Sex-specific production of ipsdienol and myrcenol by *Dendroctonus ponderosae* (Coleoptera: Scolytidae) exposed to myrcene vapors. *J. Chem. Ecol.* 12, 1579–1586.
- Ivarsson, P., Schlyter, F., 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.
- Ivarsson, P., 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.
- Ivarsson, P., Blomquist, G.J., Seybold, S.J., 1997. *In vitro* production of the pheromone intermediates ipsdienone and ipsenone by the bark beetles *Ips pini* (Say) and *I. paraconfusus* Lanier (Coleoptera: Scolytidae). *Naturwissenschaften* 84, 454–457.

- Ivarsson, P., Tittiger, C., Blomquist, C., Borgeson, C.E., Seybold, S.J., Blomquist, G.J., Hogberg, H.E., 1998. Pheromone precursor synthesis is localized in the metathorax of *Ips paraconfusus* Lanier (Coleoptera: Scolytidae). *Naturwissenschaften* 85, 507–511.
- Kavanagh, K., Jornvall, H., Persson, B., 2008. The SDR superfamily: functional and structural diversity within a family of metabolic and regulatory enzymes. *Cell. Mol. Life Sci.* 65, 3895–3906.
- Keeling, C.I., Bearfield, J.C., Young, S., Blomquist, G.J., 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.
- Keeling, C.I., Blomquist, G.J., Tittiger, C., 2004. Coordinated gene expression for pheromone biosynthesis in the pine engraver beetle, *Ips pini* (Coleoptera: Scolytidae). *Naturwissenschaften* 91, 324–328.
- Kurz, W.A., Dymond, C.C., Stinson, G., Rampley, G.J., Neilson, E.T., Carroll, A.L., Ebata, T., Safranyik, L., 2008. Mountain pine beetle and forest carbon feedback to climate change. *Nature* 452, 987–990.
- Lanne, B.S., Ivarsson, P., Johnson, P., Bergstrom, G., Wassgren, A.B., 1989. Biosynthesis of 2-methyl-3-buten-2-ol, a pheromone component of *Ips typographus* (Coleoptera: Scolytidae). *Insect Biochem.* 19, 163–168.
- Lewinsohn, E., Gijzen, M., Croteau, R., 1991. Defense mechanisms of conifers. *Plant Physiol.* 96, 44–49.
- Lienard, M., Strandh, M., Hedenstrom, E., Johansson, T., Lofstedt, C., 2008. Key biosynthetic gene subfamily recruited for pheromone production prior to the extensive radiation of Lepidoptera. *BMC Evolution. Biol.* 8, 270.
- Lindgren, B.S., Miller, D.R., 2002. Effect of verbenone on five species of bark beetles (Coleoptera: Scolytidae) in lodgepole pine forests. *Environ. Entomol.* 31, 759–761.
- Lu, F., 1999. Origin and endocrine regulation of pheromone biosynthesis in the pine bark beetles, *Ips pini* (Say) and *Ips paraconfusus* Lanier (Coleoptera: Scolytidae). Ph.D. Dissertation. Biochemistry, University of Nevada, Reno. 152 pp.
- Martin, D., Bohlmann, J., Gershenzon, J., Franke, W., 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.
- Miller, D.R., Gibson, K.E., Raffa, K.F., Seybold, S.J., Teale, S.A., Wood, D.L., 1997. Geographic variation in response of pine engraver, *Ips pini*, and associated species to pheromone, lanierone. *J. Chem. Ecol.* 23, 2013–2031.
- Nardi, J.B., Gilg-Young, A., Ujhelyi, E., Tittiger, C., Lehane, M.J., Blomquist, G.J., 2002. Specialization of midgut cells for synthesis of male isoprenoid pheromone components in two scolytid beetles, *Dendroctonus jeffreyi* and *Ips pini*. *Tissue Cell* 226, 1–11.
- Nakamura, C.E., Abeles, R.H., 1985. Mode of interaction of  $\beta$ -hydroxy- $\beta$ -methylglutaryl coenzyme A reductase with strong binding inhibitors: compactin and related compounds. *Biochemistry* 24, 1364–1376.
- Paine, T.D., Millar, J.G., Hanlon, C.C., Hwang, J.S., 1999. Identification of semi-chemicals associated with Jeffrey pine beetle, *Dendroctonus jeffreyi*. *J. Chem. Ecol.* 25, 433–453.
- Perez, A.L., Gries, R., Gries, G., Oehlschlager, A.C., 1996. Transformation of per-sumptive precursors to frontalin and *exo*-Brevicomin by bark beetles and the West Indian Sugarcane Weevil (Coleoptera). *Bioorg. Med. Chem.* 4, 445–450.
- Phillips, M.A., Croteau, R.B., 1999. Resin-based defenses in conifers. *Trends Plant Sci.* 4, 184–190.
- Pierce, H.D., Conn, J.E., Oehlschlager, A.C., Borden, J.H., 1987. Monoterpene metabolism in female mountain pine beetles, *Dendroctonus ponderosae* Hopkins, attacking ponderosa pine. *J. Chem. Ecol.* 13, 1455–1480.
- Pitman, G.B., 1969. Pheromone response in pine bark beetles: influence of host volatiles. *J. Chem. Ecol.* 166, 905–906.
- Poland, T.M., Borden, J.H., 1998. Disruption of secondary attraction of the spruce beetle, *Dendroctonus rufipennis*, by pheromones of two sympatric species. *J. Chem. Ecol.* 24, 151–166.
- Pureswaran, D.S., Gries, R., Borden, J.H., Pierce Jr., H.D., 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.
- Rafaeli, A., Jurenka, R.A., 2003. PBAN regulation of pheromone biosynthesis in female moths. In: Blomquist, G.J., Vogt, R.G. (Eds.), *Insect Pheromone Biochemistry and Molecular Biology*. Elsevier Academic Press, Amsterdam, pp. 107–136.
- Rao, S.C., Rao, R., Agrawal, R., 2003. Enhanced production of verbenol, a highly valued food flavourant, by an intergeneric fusant strain of *Aspergillus niger* and *Penicillium digitatum*. *Biotech. Appl. Biochem.* 37, 145–147.
- Renwick, J.A.A., Hughes, P.R., Krull, I.S., 1976. Selective production of *cis*- and *trans*-verbenol from (-)- and (+)-alpha pinene by a bark beetle. *Science* 191, 199–201.
- Renwick, J.A.A., Hughes, P.R., Ty, T.D., 1973. Oxidation products of pinene in the bark beetle, *Dendroctonus frontalis*. *J. Insect Physiol.* 19, 1735–1740.
- Roelofs, W.L., Rooney, A.P., 2003. Molecular genetics and evolution of pheromone biosynthesis in Lepidoptera. *Proc. Natl. Acad. Sci. U.S.A.* 100, 9179–9184.
- Rudinsky, J.A., 1962. Ecology of Scolytidae. *Annu. Rev. Entomol.* 7, 327–348.
- Sandstrom, P., 2007. Monoterpene metabolism by bark beetle cytochromes P450. Ph.D. Dissertation. Biochemistry and Molecular Biology, University of Nevada, Reno. 216 pp.
- Sandstrom, P., Ginzl, M.D., Bearfield, J.C., Welch, W.H., Blomquist, G.J., Tittiger, C., 2008. Myrcene hydroxylases do not determine enantiomeric composition of pheromonal ipsdienol in *Ips* spp. *J. Chem. Ecol.* 34, 1584–1592.
- Sandstrom, P., Welch, W.H., Blomquist, G.J., Tittiger, C., 2006. Functional expression of a bark beetle cytochrome P450 that hydroxylates myrcene to ipsdienol. *Insect Biochem. Mol. Biol.* 36, 835–845.
- Seybold, S.J., Quilici, D.R., Tillman, J.A., Vanderwel, D., Wood, D.L., Blomquist, G.J., 1995a. De novo biosynthesis of the aggregation pheromone components ipse-nol and ipsdienol by the pine bark beetles *Ips paraconfusus* Lanier and *Ips pini* (Say) (Coleoptera: Scolytidae). *Proc. Natl. Acad. Sci. U.S.A.* 92, 8393–8397.
- Seybold, S.J., Ohtsuka, T., Wood, D.L., Kubo, I., 1995b. Enantiomeric composition of ipsdienol: a chemotaxonomic character for North American populations of *Ips* spp. In the *pini* subgeneric group (Coleoptera: Scolytidae). *J. Chem. Ecol.* 21, 995–1016.
- Seybold, S.J., Tittiger, C., 2003. Biochemistry and molecular biology of de novo isoprenoid pheromone production in the Scolytidae. *Ann. Rev. Entomol.* 48, 425–453.
- Seybold, S.J., Bohlmann, J., Raffa, K.F., 2000. Biosynthesis of coniferophagous bark beetle pheromones and conifer isoprenoids: evolutionary perspective and synthesis. *Can. Entomol.* 132, 697–753.
- Silverstein, R.M., Rodin, J.O., Wood, D.L., 1966. Sex attractants in frass produced by male *Ips confusus* in ponderosa pine. *Science* 154, 509–510.
- Silverstein, R.M., Brownlee, R.G., Bellas, T.E., Wood, D.L., Browne, L.E., 1968. Brevicomin: principal sex attractant in the frass of the female western pine beetle. *Science (New York, N.Y.)* 159, 889–891.
- Steele, C.L., Lewinsohn, E., Croteau, R., 1995. Induced oleoresin biosynthesis in grand fir as a defense against bark beetles. *Proc. Natl. Acad. Sci. U.S.A.* 92, 4164–4168.
- Sullivan, B.T., Shepherd, W.P., Pureswaran, D.S., Tashiro, T., Mori, K., 2007. Evidence that (+)-endo-brevicomin is a male-produced component of the Southern pine beetle aggregation pheromone. *J. Chem. Ecol.* 33, 1510–1527.
- Symonds, M., Elgar, M., 2004. The mode of pheromone evolution: evidence from bark beetles. *Proc. Royal Soc. London B* 271, 839–846.
- Symonds, M.R., Elgar, M.A., 2008. The evolution of pheromone diversity. *Trends Ecol. Evol.* 23, 220–228.
- Symonds, M.R.E., Wertheim, B., 2005. The mode of evolution of aggregation pheromones in *Drosophila* species. *J. Evol. Biol.* 18, 1253–1263.
- Tillman, J.A., Holbrook, G.L., Dallara, P.L., Schal, C., Wood, D.L., Blomquist, G.J., 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.
- Tillman, J.A., Lu, F., Goodard, L.M., Donaldson, Z., Dwinell, S.C., Tittiger, C., Hall, G.M., Storer, A.J., Blomquist, G.J., Seybold, S.J., 2004. Juvenile hormone regulates de novo isoprenoid aggregation pheromone biosynthesis in pine bark beetles, *Ips* spp. (Coleoptera: Scolytidae), through transcriptional control of HMG-CoA reductase. *J. Chem. Ecol.* 30, 2459–2494.
- Tittiger, C., Blomquist, G.J., Ivarsson, P., Borgeson, C.E., Seybold, S.J., 1999a. Juvenile hormone regulation of HMGR gene expression in the bark beetle *Ips paraconfusus* (Coleoptera: Scolytidae): implications for male aggregation pheromone biosynthesis. *Cell. Mol. Life Sci.* 55, 121–127.
- Tittiger, C., O'Keefe, C., Bengoa, C.S., Barkawi, L., Seybold, S.J., Blomquist, G.J., 1999b. Isolation and endocrine regulation of HMG-CoA synthase cDNA from the male Jeffrey pine beetle, *Dendroctonus jeffreyi*. *Insect Biochem. Mol. Biol.* 30, 1203–1211.
- Tittiger, C., Barkawi, L.S., Bengoa, C.S., Blomquist, G.J., Seybold, S.J., 2003. Structure and juvenile hormone-mediated regulation of the HMG-CoA reductase gene from the Jeffrey pine beetle, *Dendroctonus jeffreyi*. *Mol. Cell. Endocrinol.* 199, 11–21.
- Vanderwel, D., 1994. Factors affecting pheromone production in beetles. *Arch. Insect Biochem. Physiol.* 25, 347–362.
- Vanderwel, D., Gries, G., Singh, S.M., Borden, J.H., Oehlschlager, A.C., 1992. (*E*)- and (*Z*)-6-nonen-2-one: biosynthetic precursors of *endo*- and *exo*-brevicomin in two bark beetles (Coleoptera: Scolytidae). *J. Chem. Ecol.* 18, 1389–1404.
- Wyatt, G.R., Davey, K.G., 1996. Cellular and molecular actions of juvenile hormone. II. Roles of juvenile hormone in adult insects. *Adv. Insect Physiol.* 26, 2–155.