Pergamon

Insect Biochemistry and Molecular Biology 28 (1998) 705–715

Insect Biochemistry and Molecular Biology

# Endocrine regulation of de novo aggregation pheromone biosynthesis in the pine engraver, *Ips pini* (Say) (Coleoptera: Scolytidae)

Julie A. Tillman [a,*], Glenn L. Holbrook [b], Paul L. Dallara [c], Coby Schal [b], David L. Wood [c], Gary J. Blomquist [a], Steven J. Seybold [a]

[a] *Department of Biochemistry/330, University of Nevada, Reno, NV 89557-0014, U.S.A.*
[b] *Department of Entomology, North Carolina State University, Box 7613, Raleigh, NC 27695-7613, U.S.A.*
[c] *Division of Insect Biology, Department of Environmental Science, Policy, and Management, University of California, Berkeley, CA 94720-3112, U.S.A.*

Received 18 August 1997; accepted 5 December 1997

**Abstract**

In vivo and in vitro radiotracer studies were conducted with the pine engraver, *Ips pini* (Say) (Coleoptera: Scolytidae), to elucidate the relationships among feeding on host (*Pinus jeffreyi* Grev. & Balf.) phloem, juvenile hormone III (JH III) biosynthesis, and de novo aggregation pheromone (ipsdienol) biosynthesis. The in vivo incorporation of [1-$^{14}$C]acetate into ipsdienol by male *I. pini* increased with increasing dose of topically-applied JH III, demonstrating the stimulatory role by JH III in de novo pheromone production. In vivo incorporation of (*RS*)-[2-$^{14}$C]mevalonolactone into ipsdienol by male *I. pini* was not affected by increasing JH III dose. However, injection of [$^{14}$C]mevalonolactone resulted in significantly higher levels of [$^{14}$C]ipsdienol than those observed in saline-injected controls. This is direct evidence for the mevalonate-based isoprenoid pathway in de novo ipsdienol biosynthesis, and suggests that in this pathway JH III does not influence enzymatically-catalyzed reactions subsequent to the conversion of 3-hydroxy-3-methylglutaryl-coenzyme A to mevalonate. An additional in vivo [$^{14}$C]acetate study demonstrated that de novo ipsdienol biosynthesis is also stimulated by feeding on host phloem. Lastly, an in vitro radiotracer study utilizing L-[*methyl*-$^{3}$H]methionine demonstrated that feeding stimulates JH III biosynthesis by the corpora allata (CA) of male, but not female, *I. pini*. Analysis by radio-high pressure liquid chromatography revealed that JH III is likely the type of juvenile hormone produced by the male CA. These data support a sequence of events leading to feeding-induced de novo pheromone biosynthesis in male *I. pini*: (1) feeding on host phloem; (2) feeding-dependent JH III biosynthesis by the CA; and (3) JH III-stimulated de novo ipsdienol biosynthesis. 

*Keywords:* Endocrine regulation; De novo pheromone biosynthesis; Pine engraver; *Ips pini*; Ipsdienol; 2-Methyl-6-methylene-2,7-octadien-4-ol; Juvenile hormone; *cis*-10,11-Epoxy-3,7,11-trimethyl-*E*2,*E*6-dodecadienoic acid methyl ester; Corpora allata; Isoprenoids

## 1. Introduction

The aggregation pheromone of most western North American populations of the pine engraver, *Ips pini* (Say) (Coleoptera: Scolytidae), consists of an enantiomeric blend [approx. 95–98%-(—)] of the acyclic monoterpene alcohol ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol) (Vité et al., 1972; Stewart, 1975; Birch et al., 1980; Seybold et al., 1992, 1995a) [Fig. 1(A) inset)]. The mechanism of pheromone-based aggregation in *I. pini* and in the related California fivespined ips, *I. paraconfusus* Lanier begins with host selection by males (Anderson, 1948; Wood and Vité, 1961). In western North America, *Pinus jeffreyi* Grev. & Balf., *P. ponderosa* Laws., *P. contorta* spp. *murrayana* (Balf.), and *P. contorta* spp. *latifolia* (Engelm. ex Wats.) are the predominant hosts for *I. pini* (S.L. Wood, 1982; Seybold et al., 1995a). Pioneer males tunnel through the outer bark, begin to feed on phloem tissue, and excavate a nuptial chamber (Clemens, 1916). Shortly thereafter, pheromone

* Corresponding author. Tel.: + 1-702-784-4985; Fax: + 1-702-784-1419; E-mail: jtillman@med.unr.edu

0965-1748/98/$19.00 
PII: S0965-1748(97)00117-3

component(s) accumulate in the Malpighian tubules and posterior region of the gut (Pitman et al., 1965; Wood et al., 1966; Byers, 1983). As the pheromone component(s) volatilize from the fecal pellets in the frass (ingested and uningested phloem and xylem fragments) (Wood, 1962; Wood and Bushing, 1963; Wood et al., 1966), male and female conspecifics detect the pheromone and respond by aggregating on the selected host, resulting in mass attack and colonization (Anderson, 1948; Wood and Vité, 1961).

Studies and reviews of scolytid aggregation pheromone biosynthesis have focused predominantly on biosynthetic pathways involving oxidation of host monoterpene precursors (Hughes, 1973a, b, 1974, 1975; Renwick et al., 1976a, b; Byers et al., 1979; Hendry et al., 1980; Byers, 1981, 1982, 1983, 1989; D.L. Wood, 1982; Borden, 1985; Vanderwel and Oehlschlager, 1987; Gries et al., 1990; Raffa et al., 1993; Vanderwel, 1994). However, some studies have either suggested the possibility of de novo pheromone biosynthesis in *Ips* spp (Hendry et al., 1980) or offered data that directly questioned whether host-derived monoterpenes are the sole precursors for pheromone biosynthesis (Byers, 1981; Byers and Birgersson, 1990). The latter prompted the examination of de novo pheromone biosynthesis in *Ips* spp (Ivarsson et al., 1993; Seybold et al., 1995b). Ivarsson et al. (1993) offered strong indirect evidence for de novo isoprenoid pheromone biosynthesis by the male Eurasian bark beetle, *I. duplicatus* Sahlberg. Production of ipsdienol, and the related monoterpene alcohol, *E*-myrcenol, decreased following treatment of males with compactin, a competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (EC 1.1.1.34). Seybold et al. (1995b) utilized radiotracer techniques to directly demonstrate de novo isoprenoid pheromone biosynthesis by male *I. paraconfusus* and *I. pini*. [1-$^{14}$C]Acetate was incorporated into ipsenol and ipsdienol by male *I. paraconfusus*, and into ipsdienol by male *I. pini*. They also reported preliminary evidence for the incorporation of (*RS*)-[5-$^{3}$H]mevalonolactone [a cyclic form of (3*RS*)-mevalonate] into pheromone components by male *I. paraconfusus* and *I. pini*.

Endocrine regulation of pheromone biosynthesis has been studied in numerous insect systems. Pheromone biosynthesis is regulated by pheromone-biosynthesis-activating-neuropeptide (PBAN) (reviewed in Raina, 1993, 1997) in most lepidopterans; ecdysone in some dipterans (reviewed in Blomquist et al., 1987, 1992); and juvenile hormone (JH) in coleopterans (reviewed in Vanderwel, 1994), some blattodeans (Schal et al., 1990; Chase et al., 1992), and some lepidopterans (Cusson and McNeil, 1989). Studies of scolytids have shown that pheromone biosynthesis in the presence or absence of host monoterpene precursors and without pre-feeding is stimulated by topical treatment with exogenous juvenile hormone III (JH III) or JH analogs (JHAs) (Borden et al., 1969; Hughes and Renwick, 1977a, b; Harring, 1978; Renwick and Dickens, 1979; Bridges, 1982; Conn et al., 1984; Chen et al., 1988). Recently, Ivarsson and Birgersson (1995) utilized compactin and the JHA methoprene to suggest that JH regulates de novo pheromone biosynthesis in male *I. duplicatus*.

In addition to the effect of JH III or its analogs on pheromone production in scolytids, feeding on host phloem also stimulates aggregation pheromone production and/or release in male *I. paraconfusus* (Wood, 1962; Pitman et al., 1965; Wood et al., 1966; reviewed in D.L. Wood, 1982; Vanderwel, 1994; Byers, 1995). Feeding on host phloem (Hughes, 1974), or gut distention induced by air injection (Hughes and Renwick, 1977b), which presumably mimics the physical aspects of feeding, both appear to stimulate the conversion of an exogenous host monoterpene precursor (myrcene) to pheromone in male *I. paraconfusus*. However, these studies did not use radiolabeled substrates and thus were not able to distinguish between synthetic contributions from de novo sources and host monoterpene sources. Lastly, induction of pheromone production during feeding is blocked in male *I. paraconfusus* by treatment with precocene (a chemical allatectomizing agent) (Kiehlmann et al., 1982).

Together, these earlier studies suggest a physiological interplay between feeding on host phloem, JH III titer (likely determined by JH III biosynthesis), and the induction of de novo pheromone biosynthesis in male *Ips* spp. We have employed in vivo and in vitro radiotracer techniques to examine the relationship between these events in male *I. pini*. Radiolabeled acetate and mevalonolactone were used to evaluate the relationship between dose of topically applied JH III and de novo ipsdienol biosynthesis, whereas radiolabeled acetate was used to compare the effects of feeding and topically-applied JH III on de novo ipsdienol biosynthesis. Finally, in vitro JH III biosynthesis by corpora allata (CA) from fed and unfed male and female *I. pini* were compared. Specifically, the aim of our study was to determine the role of JH III as a regulatory link between the behavioral event of phloem feeding and the biochemical event of de novo ipsdienol synthesis.

## 2. Materials and methods

### 2.1. Insects

*Ips pini*-infested *P. jeffreyi* logging debris was collected from the Lassen National Forest, Lassen County, CA (28 October 1994, 1 June 1995, and 27 September 1995, ~ 2000 m elevation, 1.2 km south of Blacks Ridge; T34N, R7E, S10). Insects were reared to the adult stage and collected upon emergence from logs (Browne,

1972). Newly emerged insects were stored on moist filter paper at 4°C and utilized within 14 days of emergence.

### 2.2. Chemicals and materials

Ipsdienol used for a chromatographic standard was obtained from Bedoukian Research Incorporated (Danbury, CT; #P407). Sodium [1-$^{14}$C]acetate (59 mCi/mmol; #12013) and Ecolume biodegradable liquid scintillation solution (#882475) were obtained from ICN Biomedicals (Costa Mesa, CA). (*RS*)-[2-$^{14}$C]Mevalonolactone (58 mCi/mmol; #NEC-679) and L-[*methyl*-$^{3}$H]methionine (200 mCi/mmol; #NET-061) were purchased from NEN Research Products (Wilmington, DE). Acetone (#A949-4) and hexane (#H303-4) were purchased from Fisher Scientific (Pittsburgh, PA), and pentane (#6145-08) was obtained from Mallinckrodt Specialty Chemicals Co. (Paris, KY). *n*-Octanol (#36,056-2) and isooctane (#15,501-2) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Porapak Q (50/80 mesh; #20339) was purchased from Supelco Chromatography Products (Bellefonte, PA). Juvenile hormone III ([ ± ]-*cis*-10,11-epoxy-3,7,11-trimethyl-*E*2,*E*6-dodecadienoic acid methyl ester; #J2000, 75% chemical purity) and saline components for the in vitro assay were obtained from Sigma Chemical Co. (St Louis, MO). The L-15B medium (Munderloh and Kurtti, 1989) used for in vitro assays with *I. pini* corpora allata was purchased from Specialty Media (Lavalette, NJ).

### 2.3. In vivo studies of de novo ipsdienol biosynthesis by male I. pini

#### 2.3.1. Application of Juvenile Hormone III

Specified quantities of JH III dissolved in 0.5 $\mu$l acetone were topically applied to the ventral abdominal cuticle of recently emerged male *I. pini* using a 10 $\mu$l syringe with a 33-gauge blunt-tipped needle (Hamilton Co., Reno, NV). The doses of JH III presented in this paper were not adjusted for the chemical purity of the commercial material (i.e. a 10 $\mu$g dose represents 7.5 $\mu$g of JH III). Control applications consisted of topical treatment with 0.5 $\mu$l acetone.

#### 2.3.2. Administration of sodium [1-$^{14}$C]acetate or (RS)-[2-$^{14}$C]mevalonolactone

Individual insects were injected with ~ 0.2 $\mu$Ci sodium [1-$^{14}$C]acetate or (*RS*)-[2-$^{14}$C]mevalonolactone dissolved in 0.5 $\mu$l saline (0.15 M aqueous sodium chloride) using a 10 $\mu$l syringe with a 33-gauge beveled needle (Hamilton Co., Reno, NV). The insects were injected between the abdominal sternites.

#### 2.3.3. Experimental treatments

The dose–response relationship between JH III and the incorporation of [$^{14}$C]acetate or [$^{14}$C]mevalonolactone into ipsdienol (de novo ipsdienol biosynthesis) was examined. Individual male *I. pini* were topically treated with 0 (control), 1, 10, or 100 $\mu$g JH III (three replicates/treatment and fourteen individuals/replicate). The insects were then incubated at room temperature for 12–16 h (Chen et al., 1988) in glass wool in a screened enclosure in a glass jar with moistened filter paper at the bottom. Thus, the insects were prevented from contacting and ingesting the moist paper. Following incubation, insects from each JH III treatment group were injected with [$^{14}$C]acetate or [$^{14}$C]mevalonolactone. Later, to control for the level of $^{14}$C incorporated into ipsdienol from [$^{14}$C]mevalonolactone, male *I. pini* in twelve replicates (fourteen individuals per replicate) were each injected with 0.5 $\mu$l saline solution. These males had not been previously treated with JH III.

The effect of topical JH III treatment or *P. jeffreyi* phloem feeding on de novo ipsdienol biosynthesis in male *I. pini* was also examined. Individual male *I. pini* were: (1) topically treated with 0.5 $\mu$l of acetone (control) followed by incubation for 12–16 h; (2) fed on *P. jeffreyi* phloem for 48 h; or (3) topically treated with 10 $\mu$g JH III in 0.5 $\mu$l acetone followed by incubation for 12–16 h. Each treatment consisted of four replicates/treatment and fourteen individuals/replicate. Following incubation or phloem feeding, insects were injected with [$^{14}$C]acetate. Phloem for feeding insects prior to [$^{14}$C]acetate injection (treatment 2 above) was prepared by cutting a phloem/bark strip approx. 2.5 cm wide from a shaved *P. jeffreyi* log, removing the remaining bark from the phloem strip, and placing the rolled phloem into a plastic 35 mm film canister. Twenty to twenty-five individuals were placed in each phloem canister.

#### 2.3.4. Collection, extraction, and quantitative analysis of ipsdienol

After [$^{14}$C]acetate, [$^{14}$C]mevalonolactone, or saline injection, each male *I. pini* was immediately inserted into a pre-drilled (approx. 3 mm diameter) hole in a *P. jeffreyi* phloem/bark strip for volatile pheromone collection. These strips were prepared by shaving a *P. jeffreyi* log until 3–5 mm of bark remained, and cutting strips of approx. 30 × 280 mm. After fourteen males were inserted into individual holes in the strip, metal screening was stapled to the phloem and bark surfaces to secure the insects. Phloem/bark strips containing treated beetles were then placed into 50 × 305 × 25 mm plexiglass aeration chambers (designed by D.R. Quilici at University of Nevada, Reno, unpublished). Volatiles released during the ensuing 72 h feeding and pheromone production period by each group of 14 males (one replicate) were trapped on 0.8 g Porapak Q (Byrne et al., 1975) at a flow rate of 100 ml/min.

Porapak Q was extracted in a glass column with 150 ml pentane by gravity flow. The pentane extract was

concentrated to 2 ml by Kuderna–Danish evaporative concentration, and ipsdienol in the samples was quantified by gas chromatography (GC) with *n*-octanol as an internal standard (Seybold et al., 1995a, b). Since each sample was derived from beetles that had ample time to feed and induce pheromone production (72 h), we expected to detect ipsdienol in each sample. If ipsdienol was not detected, this likely reflected an experimental problem with a particular sample, such as insufficient feeding, and that replicate was omitted from the analysis (no more than one replicate per experimental condition was omitted). Following quantitative analysis, three 100 $\mu$l aliquots were removed from each pentane extract. These aliquots were analyzed on a Beckman LS-1701 liquid scintillation analyzer (LSA) using Ecolume scintillation cocktail to determine the total level of $^{14}$C-associated radioactivity in each crude extract. The $^{14}$C counting efficiency was 97%.

*2.3.5. Radio-chromatographic analysis of ipsdienol*

Following Kuderna–Danish evaporative concentration and GC analysis, an aliquot of each pentane extract was fractionated by high pressure liquid chromatography (HPLC) using a Hewlett Packard Series 1050 instrument with a 10 mm × 50 cm Nucleosil 50-5 normal-phase column (Alltech Associates, Inc., Deerfield, IL). Solvent system was hexane:acetone (96:4, vol:vol) at a flow rate of 2 ml/min and mass detection was by UV (235 nm). The aliquot volume from each pentane extract to be fractionated was adjusted to provide a sample load of approx. 5000 dpm of $^{14}$C-associated radioactivity, and the volumes ranged from 0.1 to 1.7 ml. Fractions (every 30 s) were collected using a Pharmacia LKB-FRAC-100 Fraction Collector (Pharmacia Biotech, Uppsala, Sweden). Each fraction was assayed by LSA and the ipsdienol peak was verified in this study by a comparison of HPLC retention time with a reference standard. The amount of $^{14}$C-associated radioactivity in the ipsdienol fractions from each sample aliquot was adjusted by the appropriate volume factor to yield the total $^{14}$C-associated radioactivity from ipsdienol in each original 2 ml sample. In a previous study of *I. pini* performed in this laboratory (Seybold et al., 1995b), the presence of [$^{14}$C]ipsdienol in HPLC fractions was established by the comparison of HPLC and GC retention times with standard ipsdienol as well as by HPLC and gas chromatography–mass spectrometry (GC-MS) analyses of camphanic acid ester diastereomeric derivatives of ipsdienol. In this previous study, a radio-chromatographic peak thought to contain ipsdienol was identified from the crude Porapak extract. Ipsdienol (labeled and unlabeled) was purified by fractionation of several aliquots of the Porapak extract. Fractions containing purified ipsdienol were pooled and reacted with (1*S*)-(—)-camphanic acid to prepare the ipsdienol diastereomeric esters (Seybold, 1992). The ipsdienol–camphanic acid esters were then analyzed by HPLC, LSA, and GC-MS. HPLC co-elution and structural identity of radiolabeled, derivatized ipsdienol with the unlabeled, derivatized standard confirmed the identity of the original radio-chromatographic peak as ipsdienol. Thus, we are confident of our assay for [$^{14}$C]ipsdienol in the current study.

*2.4. In vitro study of juvenile hormone biosynthesis by corpora allata from male and female* I. pini

*2.4.1. Experimental treatments*

Male and female *I. pini* were fed on *P. jeffreyi* phloem for 12 (males only), 24, 48, or 72 h to examine the effect of phloem feeding on JH III biosynthesis by isolated corpora allata (CA). Insects were fed in groups of 20–25 on phloem in film canisters as described earlier. Two different control conditions were included: (1) **Incubated Controls** consisted of groups of 20–25 unfed insects incubated at room temperature in glass wool in a screened enclosure over moistened filter paper in a glass jar (as described earlier) for 12 (males only), 24, 48, or 72 h; and (2) **Unincubated Controls** consisted of unfed insects that were removed directly from refrigerator storage and immediately dissected and assayed.

*2.4.2. Isolation of corpora allata gland pairs and in vitro measurement of juvenile hormone biosynthesis*

After specified feeding or incubation periods (or immediate removal from refrigerator storage [unincubated controls]), male and female *I. pini* were immobilized to a paraffin wax substrate by insertion of an insect pin through the right elytron. Insects were decapitated and CA were dissected from severed heads beneath saline (Kurtti and Brooks, 1976). Corpora allata pairs were isolated and assayed individually in replicate for JH III biosynthesis using a modified partition radiochemical assay (Feyereisen and Tobe, 1981; Holbrook et al., 1997). From the original 20–25 individuals in the fed, incubated control, or unincubated control groups, dissection resulted in 7–12 gland pairs per treatment group, and each gland pair yielded an individual measurement of JH III biosynthesis. Thus, there were 7–12 replicates for each treatment. Isolated gland pairs were pre-incubated for 90 min in L-15B medium containing 100 $\mu$M L-[*methyl*-$^{3}$H]methionine as the only methionine source in the medium. After pre-incubation, when radiolabeled methionine dilutes the endogenous methionine pool within the CA, gland pairs were individually transferred into 20 $\mu$l of fresh medium containing 100 $\mu$M L-[*methyl*-$^{3}$H]methionine in a 6 × 25 mm glass culture tube. After a 6 h incubation at 27°C, the medium in each tube, along with the gland pair, was extracted with 100 $\mu$l isooctane. A 50 $\mu$l aliquot of the isooctane hyperphase was assayed by liquid scintillation analysis (LSA) to quantify JH III biosynthesis by the CA.

*2.4.3. Radio-chromatographic analysis of juvenile hormone produced by male* I. pini

Isooctane extracts from twelve CA gland pairs from 24 h-fed male *I. pini* were pooled and analyzed by radio-HPLC. The HPLC analysis was performed to determine the identity of the radiolabeled in vitro products of the CA and to indicate the type(s) of JH (JH I, II, and/or III) biosynthesized by CA from male *I. pini*. The pooled isooctane extracts were evaporated to near dryness with a stream of nitrogen, and the sample was resuspended in 20 $\mu$l acetonitrile. A 10 $\mu$l aliquot was analyzed on a Beckman HPLC system equipped with a model 125 pump, a model 166 UV detector, and a model 171 radioactive flow detector (all from Beckman Instruments Inc., Fullerton, CA). A Hibar® C18 reverse-phase HPLC column (Econosphere C18, 4.6 mm × 250 mm, 5 $\mu$m particle size; Alltech Associates, Inc., Deerfield, IL) was utilized during sample analysis. The solvent program consisted of a linear gradient of 50–100% acetonitrile in 5 mM HEPES buffer (pH 7.5) at 1 ml/min for a total run time of 50 min. Scintillation fluid for radioactivity detection was run at a flow rate of 3 ml/min. Retention times of assay products were compared with those of synthetic, unlabeled standards of JH III (retention time [Rt] = 24.5 min), JH II (Rt = 27.5 min, JH I (Rt = 30 min), and methyl farnesoate (Rt = 37 min).

*2.5. Statistical analyses for in vivo and in vitro studies*

The relationships between levels of [$^{14}$C]ipsdienol (nCi) and doses of JH III ($\mu$g) ([$^{14}$C]acetate and [$^{14}$C]mevalonolactone as radiotracers) were investigated with linear regression (Sokal and Rohlf, 1995). For these analyses, the doses of JH III were transformed by $\log_{10}(x + 1)$ to remove heteroscedasticity. The levels of [$^{14}$C]ipsdienol measured for each treatment were transformed by $\log_{10}(100y)$ for the same reason (multiplication of $y$ values by 100 to facilitate plotting). The level of [$^{14}$C]ipsdienol from mevalonolactone-injected insects (pooled over all JH III doses) was compared to the level of [$^{14}$C]ipsdienol from saline-injected insects using the non-parametric Mann–Whitney *U*-test (Zar, 1996). The levels of [$^{14}$C]ipsdienol (nCi) in the study comparing topical JH III treatment (10 $\mu$g) with feeding on *P. jeffreyi* phloem were analyzed using two sequential non-parametric methods. Kruskal–Wallis analysis of variance (Kruskal and Wallis, 1952; Sokal and Rohlf, 1995) was used to analyze overall treatment effect ($\alpha = 0.05$) and medians were separated with a multisample median test (comparisonwise $\alpha = 0.05$) (Zar, 1996). Since the non-parametric methods are based on ranks and are independent of the underlying distribution of the data, the levels of [$^{14}$C]ipsdienol were not transformed for the Mann–Whitney *U*-test and Kruskal–Wallis analysis of variance. The Kruskal–Wallis test statistic is symbolized by an *H* (Kruskal and Wallis, 1952).

In the time course study of in vitro JH III biosynthesis, relationships among the levels of feeding time (12 [males only], 24, 48, or 72 h) and feeding substrate (phloem fed or unfed) in male and female *I. pini* were investigated with 2-way ANOVA (Wilkinson, 1990, Systat, Inc., Evanston, IL). Nonorthogonal contrasts (experimentwise $\alpha = 0.05$) were used to separate mean levels of the rate of [$^{3}$H]JH III biosynthesis if *F* tests were significant in the ANOVA. Unfed, incubated controls were included in this analysis; unincubated controls were not included.

## 3. Results

*3.1. In vivo studies of de novo ipsdienol biosynthesis by male* I. pini

*3.1.1. Effect of JH III dose on incorporation of [$^{14}$C]acetate*

Incorporation of [$^{14}$C]acetate into ipsdienol (nCi) by male *I. pini* increased with increasing dose of JH III [Fig. 1(A)], as demonstrated by the significant linear regression ($F_{[1,2]} = 438.2$; $P = 0.0023$; $r^2 = 0.73$) between the two variables. The 10 and 100 $\mu$g JH III treatments yielded the highest levels of stimulation of de novo ipsdienol biosynthesis (means of 0.49 and 2.30 nCi, respectively). In this experiment, one replicate of the 0 $\mu$g JH III (acetone only) treatment did not yield a detectable mass of ipsdienol in the crude extract, hence only two replicates are presented for this treatment.

*3.1.2. Effect of JH III dose on incorporation of [$^{14}$C]mevalonolactone*

Incorporation of [$^{14}$C]mevalonolactone into ipsdienol (nCi) by male *I. pini* was not affected by increasing dose of JH III (Fig. 1(B)), as demonstrated by the non-significant linear regression ($F_{[1,2]} = 1.97$; $P = 0.295$; $r^2 = 0.21$) between the two variables. However, [$^{14}$C]mevalonolactone was incorporated into ipsdienol in all treatments in the experiment, ranging from means of 2.85 nCi in the control (0 $\mu$g JH III, acetone only) to 7.06 nCi in the 100 $\mu$g JH III dose. Since the mevalonolactone regression was not significant, results from mevalonolactone-injected treatment groups were pooled (11 replicates, median = 5.13 nCi) and compared to results from saline-injected control group (12 replicates, median = 0.00125 nCi). This demonstrated that the median radioactivity in ipsdienol in mevalonolactone-injected groups was significantly greater than in the controls [Fig. 1(B), inset; Mann–Whitney *U*-test, $P < 0.0005$; (Zar, 1996)]. In this experiment, one replicate of the 0 $\mu$g JH III treatment did not yield a detectable mass of ipsdienol in the

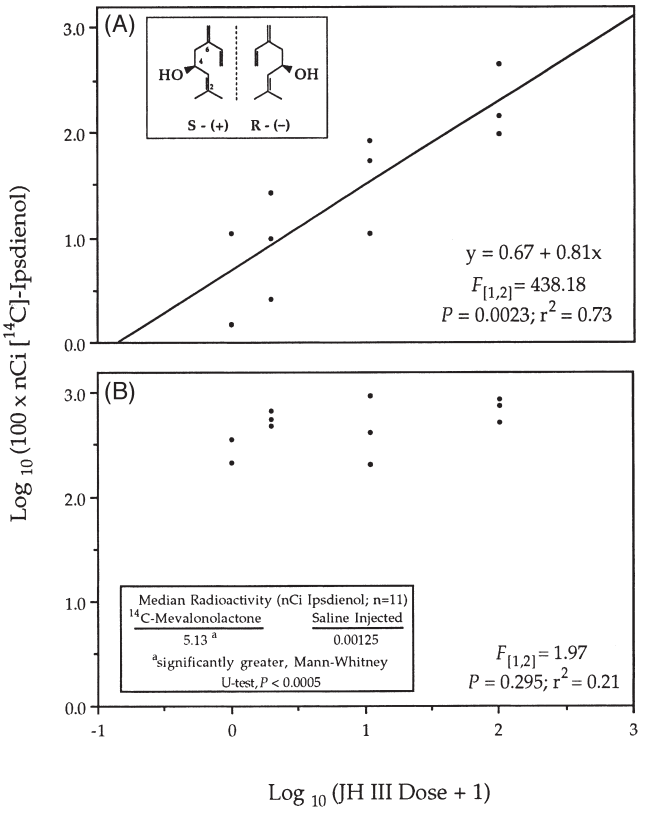


Fig. 1. Effect of juvenile hormone III (JH III) dose on de novo biosynthesis of [$^{14}$C]ipsdienol (nCi) by male *I. pini*. Individual males were topically treated with 0, 1, 10, or 100 $\mu$g JH III; incubated for 12–16 h; injected with ~ 0.2 $\mu$Ci [1-$^{14}$C]acetate (A) or ~ 0.2 $\mu$Ci (*RS*)-[2-$^{14}$C]mevalonolactone (B); and volatiles were collected from groups of fourteen males on Porapak Q, while they fed on *P. jeffreyi* phloem for 72 h. Ipsdienol was purified from pentane extracts of the Porapak by HPLC. Inset (A): the structures of *S*-(+)- and *R*-(—)-ipsdienol. Inset (B): statistical analysis of the level of [$^{14}$C]ipsdienol from [$^{14}$C]mevalonolactone-injected and saline-injected male *I. pini*.

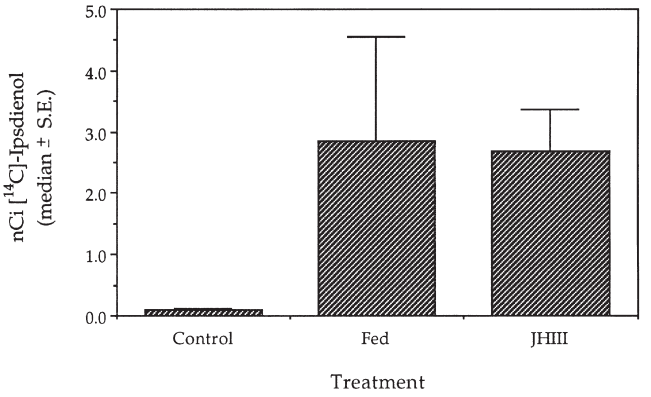


Fig. 2. Effect of feeding on *P. jeffreyi* phloem or juvenile hormone III (JH III) treatment on de novo biosynthesis of [$^{14}$C]ipsdienol (median nCi ± SE) by male *I. pini*. Males were (1) topically treated with 0.5 $\mu$l acetone and incubated for 12–16 h (control); (2) fed on *P. jeffreyi* phloem for 48 h; or (3) topically treated with 10 $\mu$g JH III in 0.5 $\mu$l acetone and incubated for 12–16 h. Individual males were then injected with ~ 0.2 $\mu$Ci [1-$^{14}$C]acetate, and volatiles were collected from groups of fourteen individuals on Porapak Q, while the males fed on *P. jeffreyi* phloem for 72 h. Ipsdienol was purified from pentane extracts of the Porapak by HPLC. The following pairwise comparisons were tested:

| Comparison | Type of test | Significant |
|---|---|---|
| $H_o$: JH III = Feeding<br>$H_A$: JH III≠Feeding | Two-sided | No ($P$ = 0.3715) |
| $H_o$: Acetone = JH III<br>$H_A$: Acetone≠JH III | Two-sided | Yes ($P$ = 0.01428) |
| $H_o$: Acetone = Feeding<br>$H_A$: Acetone≠Feeding | Two-sided | Yes ($P$ = 0.0286) |

The null hypothesis of equality of treatments was rejected ($P < 0.01$). The pairwise comparisons were analyzed using the multisample median test with comparisonwise $\alpha = 0.05$. Error bars indicate standard errors of the medians for each treatment (Sokal and Rohlf, 1995).

crude extract, hence only two replicates are presented for this treatment.

### *3.1.3. Comparative effects of feeding on* P. jeffreyi *phloem or JH III treatment on de novo ipsdienol biosynthesis*

There was a significant treatment effect in this experiment ($H = 7.211$, $P < 0.01$). Pairwise comparisons (Fig. 2) of the stimulatory treatments with each other and each individually with the acetone control indicated that median levels of [$^{14}$C]ipsdienol isolated from the fed and JH III treated insects were not significantly different from each other, whereas median levels of each of these was significantly different from the median level of the control (multisample median test). Male *I. pini* that had fed on *P. jeffreyi* phloem or were topically treated with 10 $\mu$g JH III prior to [$^{14}$C]acetate injection displayed marked increases (approximately 28 and 26 times higher, respectively) in the median levels of [$^{14}$C]ipsdienol (2.84 and 2.68 nCi, respectively) as compared to the median level in the control insects (non-prefed, acetone treated; 0.102 nCi) (Fig. 2). In this experiment, one replicate of the fed male *I. pini* groups did not yield a detectable mass of ipsdienol in the crude extract, hence only three replicates are presented for this treatment.

## *3.2. In vitro study of rate of juvenile hormone biosynthesis by male and female* I. pini

### *3.2.1. Effect of feeding on* P. jeffreyi *phloem on rate of in vitro juvenile hormone biosynthesis*

Feeding on host phloem prior to assay had a significant effect on the rate of JH III biosynthesis (pmol $h^{-1}$/CA pair) by male *I. pini* ($F = 6.519$; $P = 0.013$) [Fig. 3(A)]. Males that had fed for 24 h prior to assay displayed a significantly different mean JH III biosynthesis rate than the corresponding unfed males. The mean rate of JH III biosynthesis in 24 h-fed males was greater than that observed in the 24 h-unfed, incubated control as well as that observed in the 0 h unincubated control [Fig.

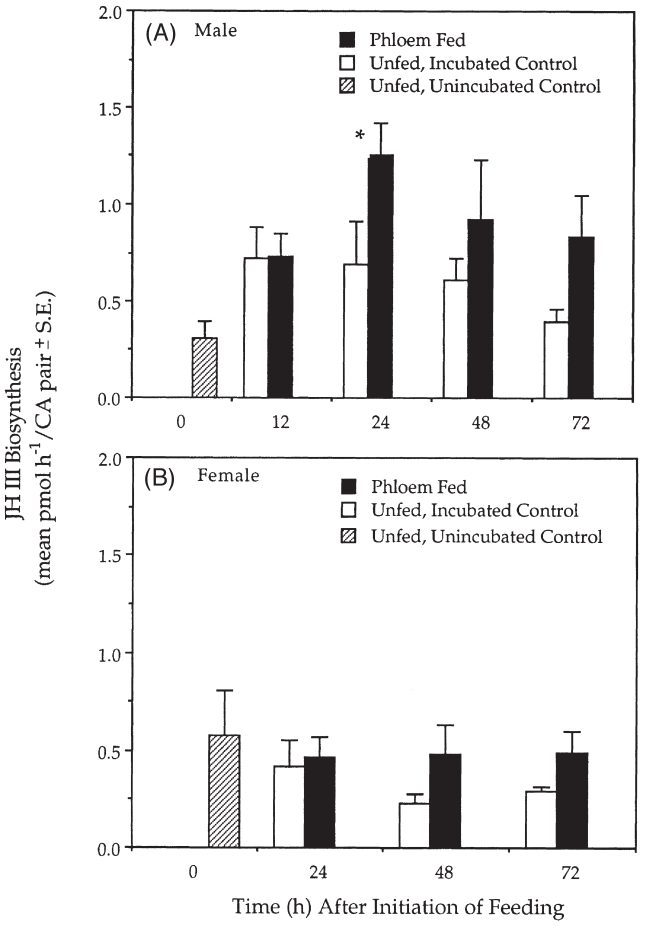


Fig. 3. Effect of feeding on *P. jeffreyi* phloem on the rate of biosynthesis [mean ( ± SE) pmol●h$^{-1}$/CA pair] of JH III by CA dissected from male and female *I. pini*. CA were isolated from both sexes and assayed for in vitro JH III biosynthesis, as measured by the incorporation of L-[$^{3}$H-*methyl*]methionine into JH III. Radioactivity was detected by liquid scintillation analysis. (A) Rates of JH III biosynthesis by CA gland pairs from males fed for 12, 24, 48, or 72 h on *P. jeffreyi* phloem, from unfed males incubated for 12, 24, 48, or 72 h on glass wool, and from unfed, unincubated males. Phloem feeding had a significant effect on the rate of JH III biosynthesis ($F = 6.519$; $P = 0.013$). Time did not have a significant effect ($F = 1.436$; $P = 0.240$) and the interaction between feeding and time was not significant ($F = 0.887$; $P = 0.452$). The * symbol denotes a significant difference between rates of JH III biosynthesis by fed and unfed, incubated males at 24 h. No other comparisons were significant. (B) Rates of JH III biosynthesis by CA gland pairs from females fed for 24, 48, or 72 h on *P. jeffreyi* phloem, from unfed females incubated for 24, 48, or 72 h on glass wool, and from unfed, unincubated females. Neither phloem feeding ($F = 3.357$; $P = 0.073$) nor time ($F = 0.346$; $P = 0.709$) had a significant effect on the rate of JH III biosynthesis. The interaction between feeding and time was not significant ($F = 0.429$; $P = 0.653$). Error bars for both sexes were constructed based on one standard error of the mean for replicate measurements of 7–12 CA pairs.

3(A)]. None of the other pairwise comparisons (12, 48, or 72 h) indicated a significant difference in the rate of JH III biosynthesis between fed and unfed, incubated males. Although JH III biosynthesis rates at 48 and 72 h from the initiation of feeding were not significantly different from the corresponding unfed controls, rates of

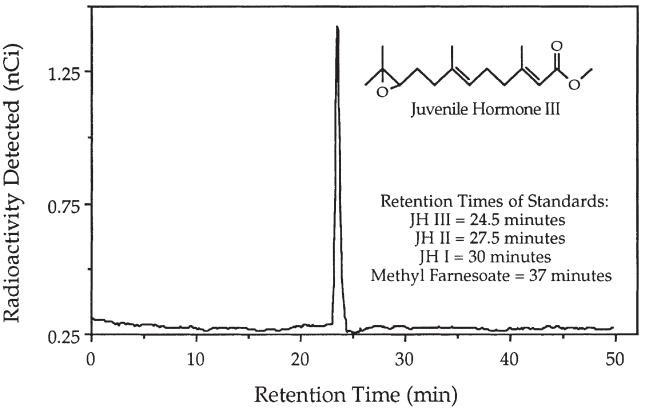


Fig. 4. Radio-HPLC chromatogram of combined isooctane extracts of in vitro JH III biosynthesis assay media from twelve male *Ips pini* CA pairs. CA gland pairs were isolated from males that had fed on *P. jeffreyi* phloem for 24 h, and the gland pairs were incubated in a medium containing 100 $\mu$M L-[*methyl*-$^{3}$H]methionine. The media were then individually extracted with isooctane, and the extracts were combined and analyzed by radio-HPLC. Inset: structure of JH III (*cis*-10,11-epoxy-3,7,11-trimethyl-*E*,*E*-2,6-dodecadienoic acid methyl ester).

JH III biosynthesis by male CA gland pairs from these times remained markedly higher than the corresponding unfed controls [Fig. 3(A)]. In males, time spent feeding did not have a significant effect on JH III biosynthesis ($F = 1.436$; $P = 0.240$) and the interaction between feeding and time was not significant ($F = 0.887$; $P = 0.452$).

Feeding on host phloem prior to assay did not have a significant effect on the rate of JH III biosynthesis (pmol h$^{-1}$/CA pair) by female *I. pini* ($F = 3.357$; $P = 0.073$) [Fig. 3(B)]. Similarly, time spent feeding did not have a significant effect on the rate of JH III biosynthesis ($F = 0.346$; $P = 0.709$) and the interaction between feeding and time was also not significant ($F = 0.429$; $P = 0.653$). For females the rate of JH III biosynthesis from the 0 h unincubated control exceeded all fed treatments and incubated controls [Fig. 3(B)].

### *3.2.2. Radio-chromatographic analysis of juvenile hormone produced by male* I. pini

Radio-HPLC analysis of isooctane extracts from in vitro assays of CA isolated from 24 h-fed male *I. pini* indicated that the type of JH released by the CA is JH III (Fig. 4). A majority (11.3 nCi, approx. 90%) of the total radioactivity detected in the extract eluted at the same retention time (Rt) as the unlabeled JH III standard (Rt = 24.5 min).

## 4. Discussion

Previous studies have shown that both JH III and JHAs stimulated pheromone production in newly emerged scolytids that, subsequent to hormonal treatment, were neither exposed to volatile pheromone pre-

cursors (e.g. myrcene or $\alpha$-pinene) nor fed on phloem (Borden et al., 1969; Hughes and Renwick, 1977a, b; Harring, 1978; Renwick and Dickens, 1979; Bridges, 1982; Conn et al., 1984; Chen et al., 1988; Ivarsson and Birgersson, 1995). Thus, JH III and JHAs induced pheromone production in *Ips* spp. and *Dendroctonus* spp. that were not able to inhale or ingest pheromone precursors. Generally, to explain the results of these studies, JH-induced pheromone production in unfed, newly emerged insects was hypothesized to occur from sequestered monoterpene precursors (Bridges, 1982; Conn et al., 1984; Chen et al., 1988; Vanderwel, 1994). With *Ips* spp., these earlier results can now be partially or fully attributed to induction of de novo pheromone biosynthesis occurring from nutritional reserves accumulated during previous phloem feeding by the larvae or immature (teneral) adults and/or from nutritional reserves derived from the catabolism of flight muscle tissue (Borden and Slater, 1968, 1969; Unnithan and Nair, 1977).

Indirect evidence for JH regulation of de novo pheromone biosynthesis was presented when Ivarsson and Birgersson (1995) combined treatments with compactin and the JHA methoprene to implicate JH in the regulation of de novo ipsdienol production in *I. duplicatus*. Pheromone production was inhibited by injection with compactin (Ivarsson et al., 1993) and was stimulated by topical application of methoprene to control insects (Ivarsson and Birgersson, 1995). Pheromone production in compactin-injected insects was not fully rescued by methoprene treatment. These results indirectly suggested that de novo pheromone production is JH regulated in *I. duplicatus*. Since rescue was not complete, the results also implied that compactin and methoprene may affect the same enzymatically catalyzed step in the synthesis (i.e. the step catalyzed by HMG-CoA reductase).

Our study with male *I. pini* directly demonstrates (using radiotracer techniques) that feeding on host phloem stimulates JH (likely JH III) biosynthesis by the CA, and suggests that JH III (or a secondary hormone or neuropeptide under the control of JH III) stimulates de novo ipsdienol production via the isoprenoid pathway (Seybold et al., 1995b). In vivo radiotracer techniques revealed an increase in the incorporation of [$^{14}$C]acetate into ipsdienol with increasing dose of JH III by male *I. pini* [Fig. 1(A)]. This result demonstrates that JH III stimulates de novo ipsdienol biosynthesis from acetate in a dose-dependent manner. Although the incorporation of $^{14}$C into ipsdienol from [$^{14}$C]mevalonolactone does not increase with increasing JH III dose, radiolabeled mevalonolactone was significantly incorporated into radiolabeled ipsdienol when compared to ipsdienol from saline-injected control insects [Fig. 1(B)]. Since mevalonate is a key isoprenoid intermediate, this directly demonstrates that male *I. pini* utilize the classical isoprenoid pathway in de novo ipsdienol biosynthesis. A comparison of de novo ipsdienol biosynthesis from [$^{14}$C]acetate and [$^{14}$C]mevalonolactone suggests that JH III stimulates enzymatically-catalyzed reaction(s) prior to the reaction that converts mevalonate to 5-phosphomevalonate [catalyzed by mevalonate kinase (EC 2.7.1.36)] in this pathway.

A second in vivo study confirmed the stimulatory effect of JH III on de novo ipsdienol biosynthesis from [$^{14}$C]acetate and demonstrated that feeding on host phloem also stimulates de novo ipsdienol biosynthesis by male *I. pini* (Fig. 2). Both treatments resulted in ~ 25-fold increases over the small quantity of [$^{14}$C]ipsdienol produced by the groups of control (acetone-treated and [$^{14}$C]acetate-injected) males. However, similar quantities of total (radiolabeled and unlabeled) ipsdienol were produced by the groups of males under all three experimental conditions: JH III-treated (mean = 36.8 $\mu$g), phloem-fed (mean = 25.2 $\mu$g), and control insects (mean = 19.7 $\mu$g).

Relative to the mass of ipsdienol, the low level of [$^{14}$C]acetate incorporation into ipsdienol (i.e. de novo pheromone biosynthesis) by control males may have resulted from a combination of rapid metabolism of [$^{14}$C]acetate and a requisite lag time between the initiation of feeding (or JH III treatment) and de novo pheromone accumulation or release. We hypothesize that in male *I. pini* both of these events occur during a 6–12 h time window. The majority of [$^{14}$C]acetate injected into the dampwood termite, *Zootermopsis nevadensis* (Hagen) (Isoptera: Termopsidae) is largely incorporated into anabolic products (e.g. lipids, carbohydrates, proteins) or catabolized to $CO_2$ within the first 6–8 h following injection (G.J. Blomquist and D.R. Quilici, unpublished). If the metabolism of acetate is similar in *I. pini*, then there is only a small time window during which introduced, labeled acetate can be incorporated into anabolic processes. If pheromone biosynthesis is activated after this narrow time window, the labeled acetate would no longer be available. Using a laboratory behavioral assay, Wood and Bushing (1963) found that male *I. paraconfusus* feeding in *P. ponderosa* bark-wood samples produced pheromone in the frass as early as 9–12 h after initiation of feeding, likely co-incident with the appearance of fecal pellets in the frass (Wood et al., 1966). This lag time in *I. paraconfusus* pheromone production between the initiation of feeding and attraction in the behavioral assay was supported by the work of Chen et al. (1988), who chemically detected a trace of pheromone at 12 h after fenoxycarb (JHA) treatment. Maximal production of pheromone by male *I. paraconfusus* occurred at 16 and 20 h after fenoxycarb treatment (Chen et al., 1988). Because the only stimulus for pheromone biosynthesis by control male *I. pini* in our study occurred while they fed on host phloem during the 72 h aeration period immediately **subsequent** to [$^{14}$C]acetate injection, the vast majority of total ipsdienol produced by control males was unlabeled. These males

missed the opportunity to incorporate [$^{14}$C]acetate into ipsdienol because de novo pheromone biosynthesis had not been previously activated by phloem feeding or JH III treatment.

The second in vivo study with male *I. pini* demonstrated that both JH III treatment and phloem feeding stimulate de novo pheromone biosynthesis, thereby revealing a relationship between feeding and JH III titer during the induction of de novo pheromone biosynthesis. This relationship was examined in an in vitro radiotracer study that measured rate of JH III biosynthesis by isolated CA. Juvenile hormone III biosynthesis rates by isolated CA were compared in fed and unfed male and fed and unfed female insects (Fig. 3). This study demonstrated that host phloem feeding [for at least 24 h] stimulates JH III biosynthesis and release from the male CA, and that the type of JH released by CA isolated from male *I. pini* is likely JH III (Fig. 4). Thus, in our experiments with topically-applied racemic JH III, we used the JH that occurs naturally in *I. pini*, but the stereochemistry of the natural JH III remains to be determined.

The rate of JH III biosynthesis by CA from fed male *I. pini* was initially low and then became elevated between 12 and 24 h after feeding [Fig. 3(A)]. This is consistent with the lag time for feeding induced pheromone production noted in previous studies with *I. paraconfusus* (Wood and Bushing, 1963; Wood et al., 1966). Since it supplants the need for feeding induced JH III biosynthesis, it is surprising that JHA topical treatment of *I. paraconfusus* (Chen et al., 1988) and *I. duplicatus* (Ivarsson and Birgersson, 1995) results in a lag time for pheromone production that rivals the lag time for feeding induced pheromone production. However, molecular and biochemical events in addition to JH III biosynthesis, such as JH III transport, biosynthesis and transport of secondary hormones, transcription and translation of genes and gene products for enzymes in the isoprenoid pathway and anatomical transport of isoprenoid intermediates, may also be involved in the time lag. Although not significant, the rate of JH III biosynthesis in fed male *I. pini* remained elevated at the 48 and 72 h time points, while analysis of females revealed uniformly low rates at all time points. The low rate of JH III biosynthesis by CA from fed female *I. pini* is inconsistent with feeding and JH III-induced degeneration of flight muscle tissue in female *I. paraconfusus* (Borden and Slater, 1968, 1969). Ultimately, the hemolymph titer of JH III, not the rate of biosynthesis by the CA, is likely to control ipsdienol biosynthesis and other physiological events in *I. pini*. The JH III titer (regulated independently of synthesis by esterases and hydrolases) should be investigated in future studies of de novo pheromone biosynthesis in *Ips* spp.

The results from our studies with *I. pini* demonstrate a link between the behavioral event of host colonization (phloem feeding) and the biochemical events of JH III biosynthesis and de novo ipsdienol biosynthesis in male *I. pini*. Phloem feeding by the closely-related *I. paraconfusus* initiates a cascade of JH-coordinated physiological events including flight muscle degeneration (Borden and Slater, 1968, 1969; Unnithan and Nair, 1977) and, by inference from studies of other insects (Koeppe et al., 1985), reproductive maturity under the bark of the recently-colonized host tree. The results reported herein with male *I. pini* add another link (de novo pheromone biosynthesis) to the feeding stimulated, JH-coordinated physiological chain of events that ultimately lead to successful host colonization.

## Acknowledgements

We thank the following individuals at University of Nevada, Reno: Dr David R. Quilici for scientific discussion and technical assistance; Fang Lu and Stephanie L. Peterson for technical assistance; and Dr David A. Schooley for valuable discussions about the physiological aspects of juvenile hormone and much advice on its experimental use. We thank Dr Paul Neese working in the laboratory of Dr R.M. Roe at North Carolina State University for assistance with HPLC analysis. We gratefully acknowledge a valuable and thorough statistical review of our paper by Sylvia R. Mori, U.S. Department of Agriculture, Forest Service, Pacific Southwest Research Station, Albany, CA. We also appreciate the assistance of R. Barnhart with the Hat Creek Ranger District of the Lassen National Forest, U.S. Department of Agriculture, Forest Service, and D. Hansen with Roseburg Resources Inc. with collection of the insects used in this study. This is a contribution of the Nevada Agricultural Experiment Station. Portions of this research were supported by the Chemical Ecology of Forest Insects Project at the U.S. Department of Agriculture, Forest Service, Pacific Southwest Research Station, Albany, CA, through Cooperative Research Agreement #PSW-92-0014CA to DLW. Major funding for this research came from United States Department of Agriculture National Research Initiative Competitive Grants #9302089 and #9502551 to SJS.

## References

Anderson, R.F., 1948. Host selection by the pine engraver. J. Econ. Entomol. 41, 596–602.

Birch, M.C., Light, D.M., Wood, D.L., Browne, L.E., Silverstein, R.M., Bergot, B.J., Ohloff, G., West, J.R., Young, J.C., 1980. Pheromonal attraction and allomonal interruption of *Ips pini* in California by the two enantiomers of ipsdienol. J. Chem. Ecol. 6, 703–717.

Blomquist, G.J., Dillwith, J.W., Adams, T.S., 1987. Biosynthesis and endocrine regulation of sex pheromone production in Diptera. In: Prestwich, G.D., Blomquist, G.J. (Eds.), Pheromone Biochemistry. Academic Press, Orlando, FL, pp. 217–250.

Blomquist, G.J., Adams, T.S., Halarnkar, P.P., Gu, P., Mackay, M.E., Brown, L., 1992. Ecdysteroid induction of sex pheromone biosynthesis in the housefly, *Musca domestica*—are other factors involved? J. Insect Physiol. 38, 309–318.

Borden, J.H., 1985. Aggregation pheromones. In Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry, and Pharmacology, vol. 9. Pergamon Press, Oxford, pp. 257–285.

Borden, J.H., Slater, C.E., 1968. Induction of flight muscle degeneration by synthetic juvenile hormone in *Ips confusus* (Coleoptera: Scolytidae). Z. vergl. Physiologie 61, 366–368.

Borden, J.H., Slater, C.E., 1969. Flight muscle volume change in *Ips confusus* (Coleoptera: Scolytidae). Can. J. Zool. 47, 29–32.

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.

Bridges, J.R., 1982. Effects of juvenile hormone on pheromone synthesis in *Dendroctonus frontalis*. Environ. Entomol. 11, 417–420.

Browne, L.E., 1972. An emergence cage and refrigerated collector for wood-boring insects and their associates. J. Econ. Entomol. 65, 1499–1501.

Byers, J.A., 1981. Pheromone biosynthesis in the bark beetle, *Ips paraconfusus*, during feeding or exposure to vapours of host plant precursors. Insect Biochem. 11, 563–569.

Byers, J.A., 1982. Male-specific conversion of the host plant compound, myrcene, to the pheromone, (+)-ipsdienol, in the bark beetle, *Dendroctonus brevicomis*. J. Chem. Ecol. 8, 363–371.

Byers, J.A., 1983. Influence of sex, maturity, and host substances on pheromones in the guts of the bark beetles, *Ips paraconfusus* and *Dendroctonus brevicomis*. J. Insect Physiol. 29, 5–13.

Byers, J.A., 1989. Chemical ecology of bark beetles. Experientia 45, 271–283.

Byers, J.A., 1995. Host-tree chemistry affecting colonization in bark beetles. In: Cardé, R.T., Bell, W.J. (Eds.), Chemical Ecology of Insects 2. Chapman & Hall, New York, pp. 154–213.

Byers, J.A., Birgersson, G., 1990. Pheromone production in a bark beetle independent of myrcene precursor in host pine species. Naturwissenschaften 77, 385–387.

Byers, J.A., Wood, D.L., Browne, L.E., Fish, R.H., Piatek, B., Hendry, L.B., 1979. Relationship between a host plant compound, myrcene and pheromone production in the bark beetle, *Ips paraconfusus*. J. Insect Physiol. 25, 477–482.

Byrne, K.J., Gore, W.E., Pierce, G.T., Silverstein, R.M., 1975. Porapak-Q collection of airborne organic compounds serving as models for insect pheromones. J. Chem. Ecol. 1, 1–7.

Chase, J., Touhara, K., Prestwich, G.D., Schal, C., Blomquist, G.J., 1992. Biosynthesis and endocrine control of the production of the German cockroach sex pheromone 3,11-dimethylnonacosan-2-one. Proc. Natl. Acad. Sci. U.S.A. 89, 6050–6054.

Chen, N.-M., Borden, J.H., Pierce, H.D., 1988. Effect of juvenile hormone analog, fenoxycarb, on pheromone production by *Ips paraconfusus* (Coleoptera: Scolytidae). J. Chem. Ecol. 14, 1087–1098.

Clemens, W.A., 1916. The pine bark beetle (*Ips pini* Say). Cornell University Agricultural Experiment Station, Bulletin 383, 287–298.

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.

Cusson, M., McNeil, J.N., 1989. Involvement of juvenile hormone in the regulation of pheromone release activities in a moth. Science 243, 210–212.

Feyereisen, R., Tobe, S.S., 1981. A rapid partition assay for routine analysis of juvenile hormone release by insect corpora allata. Anal. Biochem. 111, 372–375.

Gries, G., Bowers, W.W., Gries, R., Noble, M., Borden, J.H., 1990. Pheromone production by the pine engraver *Ips pini* following flight and starvation. J. Insect Physiol. 36, 819–824.

Harring, C.M., 1978. Aggregation pheromones of the European fir engraver beetles *Pityokteines curvidens*, *P. spinidens*, and *P. vorontzovi* and the role of juvenile hormone in pheromone biosynthesis. Z. ang. Ent. 85, 281–317.

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 (London) 284, 485.

Holbrook, G.L., Chiang, A.-S., Schal, C., 1997. Improved conditions for culture of biosynthetically active cockroach corpora allata. In Vitro Cellular and Developmental Biology—Animal 33, 452–458.

Hughes, P.R., 1973a. *Dendroctonus*: production of pheromones and related compounds in response to host monoterpenes. Z. ang. Ent. 73, 294–312.

Hughes, P.R., 1973b. 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.

Hughes, P.R., 1975. Pheromones of *Dendroctonus*: origin of $\alpha$-pinene oxidation products present in emergent adults. J. Insect Physiol. 21, 687–691.

Hughes, P.R., Renwick, J.A.A., 1977a. Hormonal and host factors stimulating pheromone synthesis in female western pine beetles, *Dendroctonus brevicomis*. Physiol. Entomol. 2, 289–292.

Hughes, P.R., Renwick, J.A.A., 1977b. Neural and hormonal control of pheromone biosynthesis in the bark beetle, *Ips paraconfusus*. Physiol. Entomol. 2, 117–123.

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., 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. Molec. Biol. 23, 655–662.

Kiehlmann, E., Conn, J.E., Borden, J.H., 1982. 7-Ethoxy-6-methoxy-2,2-dimethyl-2*H*-1-benzopyran. Org. Prep. Proc. Int. 14, 337–342.

Koeppe, J.K., Fuchs, M., Chen, T.T., Hunt, L.-M., Kovalick, G.E., Briers, T., 1985. The role of juvenile hormone in reproduction. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry, and Pharmacology, vol. 8. Pergamon Press, Oxford, pp. 165–203.

Kruskal, W.H., Wallis, W.A., 1952. Use of ranks in one-criterion variance analysis. J. Am. Statist. Assn. 47, 583–621.

Kurtti, T.J., Brooks, M.A., 1976. The dissociation of insect embryos for cell culture. In vitro 12, 141–146.

Munderloh, U.G., Kurtti, T.J., 1989. Formulation of medium for tick cell culture. Exp. Appl. Acarol. 7, 219–229.

Pitman, G.B., Kliefoth, R.A., Vité, J.P., 1965. Studies on the pheromone of *Ips confusus* (LeConte). II. Further observations on the site of production. Contrib. Boyce Thompson Inst. 23, 13–17.

Raffa, K.F., Phillips, T.W., Salom, S.M., 1993. Strategies and mechanisms of host colonization by bark beetles. In: Schowalter, T.D., Filip, G.M. (Eds.), Beetle–Pathogen Interactions in Conifer Forests. Academic Press, London, pp. 103–128.

Raina, A.K., 1993. Neuroendocrine control of sex pheromone biosynthesis in Lepidoptera. A. Rev. Entomol. 38, 329–349.

Raina, A.K., 1997. Control of pheromone production in moths. In: Cardé, R.T., Minks, A.K. (Eds.), Insect Pheromone Research: New Directions. Chapman & Hall, New York, pp. 21–41.

Renwick, J.A.A., Dickens, J.C., 1979. Control of pheromone production in the bark beetle, *Ips cembrae*. Physiol. Entomol. 4, 377–381.

Renwick, J.A.A., Hughes, P.R., Krull, I.S., 1976a. 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., Pitman, G.B., Vité, J.P., 1976b. Oxi-

dation products of terpenes identified from *Dendroctonus* and *Ips* bark beetles. J. Insect Physiol. 22, 725–727.

Schal, C., Burns, E.L., Blomquist, G.J., 1990. Endocrine regulation of female contact sex pheromone production in the German cockroach, *Blattella germanica*. Physiol. Entomol. 15, 81–91.

Seybold, S.J., 1992. The role of chirality in the olfactory-directed aggregation behavior of pine engraver beetles in the genus *Ips* (Coleoptera: Scolytidae). Ph.D. thesis, University of California at Berkeley.

Seybold, S.J., Ohtsuka, T., Wood, D.L., Kubo, I., 1995a. The 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., Quilici, D.R., Tillman, J.A., Vanderwel, D., Wood, D.L., Blomquist, G.J., 1995b. De novo biosynthesis of the aggregation pheromone components ipsenol and ipsdienol by the pine engraver beetles *Ips paraconfusus* Lanier and *Ips pini* (Say) (Coleoptera: Scolytidae). Proc. Natl. Acad. Sci. U.S.A. 92, 8393–8397.

Seybold, S.J., Teale, S.A., Wood, D.L., Zhang, A., Webster, F.X., Lindahl, K.Q., Kubo, I., 1992. The role of lanierone in the chemical ecology of *Ips pini* (Coleoptera: Scolytidae) in California. J. Chem. Ecol. 18, 2305–2329.

Sokal, R.R., Rohlf, F.J., 1995. Biometry—The Principles and Practice of Statistics in Biological Research, 3rd ed. Freeman, New York.

Stewart, T.E. 1975. Volatiles isolated from *Ips pini*: isolation, identification, enantiomeric composition, biological activity, and the enantiomeric composition of other insect pheromone alcohols and bicyclic ketals. M.Sc. thesis, College of Environmental Science and Forestry, SUNY, Syracuse, New York.

Unnithan, G.C., Nair, K.K., 1977. Ultrastructure of juvenile hormone-induced degenerating flight muscles in a bark beetle, *Ips paraconfusus*. Cell Tiss. Res. 185, 481–490.

Vanderwel, D., 1994. Factors affecting pheromone production in beetles. Arch. Insect Biochem. Physiol. 25, 347–362.

Vanderwel, D., Oehlschlager, C.A., 1987. Biosynthesis of pheromones and endocrine regulation of pheromone production in Coleoptera. In: Prestwich, G.D., Blomquist, G.J. (Eds.), Pheromone Biochemistry. Academic Press, Orlando, FL, pp. 175–215.

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

Wilkinson, L., 1990. SYSTAT: The System for Statistics. SYSTAT, Inc., Evanston, IL.

Wood, D.L., 1962. The attraction created by males of a bark beetle, *Ips confusus* (LeConte) attacking ponderosa pine. Pan-Pac. Entomol. 38, 141–145.

Wood, D.L., 1982. The role of pheromones, kairomones, and allomones in the host selection and colonization behavior of bark beetles. A. Rev. Entomol. 27, 411–446.

Wood, D.L., Bushing, R.W., 1963. The olfactory response of *Ips confusus* (LeConte) (Coleoptera: Scolytidae) to the secondary attraction in the laboratory. Can. Ent. 95, 1066–1078.

Wood, D.L., Vité, J.P., 1961. Studies on host selection behavior of *Ips confusus* (LeConte) (Coleoptera: Scoytidae) attacking *Pinus ponderosa*. Contrib. Boyce Thompson Inst. 21, 79–95.

Wood, D.L., Browne, L.E., Silverstein, R.M., Rodin, J.O., 1966. Sex pheromones of bark beetles—I. Mass production, bio-assay, source, and isolation of the sex pheromone of *Ips confusus* (LeC.). J. Insect Physiol. 12, 523–536.

Wood, S.L., 1982. The bark and ambrosia beetles of North and Central America (Coleoptera: Scolytidae), a Taxonomic Monograph. Great Basin Naturalist Memoirs, No. 6, Brigham Young University.

Zar, J.H. 1996. Biostatistical Analysis, 3rd Edition. Prentice Hall, Upper Saddle River, New Jersey.