PRODUCTION AND REGULATION OF OXYGENATED TERPENE PHEROMONES IN THE BARK BEETLES *DENDROCTONUS PONDEROSAE* HOPKINS AND *IPS PARACONFUSUS* LANIER

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Production and regulation of oxygenated terpene pheromones in the bark beetles Dendroctonus ponderosae Hopkins and Ips paraconfusus Lanier

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ABSTRACT

Dendroctonus ponderosae Hopkins and *Ips paraconfusus* Lanier of both sexes produced their full complement of terpene alcohols at normal to elevated levels in the absence of readily-culturable microorganisms. However, there was some evidence that microbial involvement may be required by male *I. paraconfusus* to produce ipsenol and ipsdienol at normal levels. Increased levels of certain terpene alcohols found in axenically-reared or streptomycin-fed beetles suggest that symbiotic microorganisms may be responsible for breaking down pheromones and other terpene alcohols. There was considerable evidence for microbial involvement in the production of the antiaggregation pheromone verbenone in *D. ponderosae*. This compound was not produced in quantifiable levels by axenically-reared or streptomycin-fed beetles exposed to *alpha*-pinene as vapours or through feeding, but was found in wild *D. ponderosae* exposed to *alpha*-pinene through feeding on bolts of lodgepole pine. The demonstrated ability of 2 species of yeasts, *Hansenula capsulata* Wickerham and *Pichia pinus* (Holst) Phaff, which are closely associated with *D. ponderosae*, to convert verbenols to verbenone *in vitro* is supportive of the hypothesis that microorganisms produce the verbenone in *D. ponderosae*. Polysubstrate monooxygenase enzymes were implicated in the production of pheromones and other terpene alcohols from host monoterpenes by *D. ponderosae*. This finding is not inconsistent with the hypothesis that microorganisms are involved in producing their hosts' pheromones, since these reactions could be catalyzed by monooxygenase enzymes present in the beetles, in their symbiotic microorganisms, or both. The host tree monoterpene, *alpha*-pinene, was found to autoxidize to *trans*- and *cis*-verbenol and verbenone. This process may occur at behaviourally significant levels under certain situations. The terpene alcohol, ipsdienol, identified in the course of this study as being produced in large quantities by male *D. ponderosae*, was found to reduce the response of beetles of both sexes to attractive semiochemicals. It is concluded that oxygenated terpene pheromones of *D. ponderosae* and *I. paraconfusus* are produced in several ways, including: 1) synthesis by the beetles, probably as a result of detoxication of host tree monoterpenes by

polysubstrate monooxygenase enzymes; 2) synthesis by microorganisms associated with the beetles, which probably occurs in the guts and the galleries; and 3) autoxidation of host tree monoterpenes.

DEDICATION

To Edna Hunt and Frank Hunt in appreciation of their encouragement and support over the course of my education.

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I. GENERAL INTRODUCTION

Anderson (1948) was the first to demonstrate that a bark beetle, *Ips pini* (Say), was capable of attracting other members of the same species. Then, 21 years ago, Wood *et al.* (1966) isolated and Silverstein *et al.* (1966) identified and synthesized the first bark beetle pheromones, those of *Ips paraconfusus* (Lanier). Chemical communication among scolytids is now known to involve a wide variety of compounds, and many bark beetle pheromones have been isolated, identified, synthesized, and their activity confirmed in laboratory and field bioassays (Borden 1982, 1985).

Although the pheromone chemistry of many bark beetle species is now fairly well understood, there is still relatively little known about the mechanisms of pheromone production. Several studies have found that microorganisms associated with bark beetles are capable of *in vitro* production of some of the same compounds which are utilized as pheromones by their hosts (Brand *et al.* 1975, 1976; Chararas *et al.* 1980; Leufvén *et al.* 1984). However, there is little evidence that microbe-produced compounds are used in nature as pheromones by the host beetles, or that these compounds are being produced by the symbionts in quantities which are biologically significant to the host beetles.

The question of microbial involvement in pheromone production by bark beetles has seldom been examined by studying the pheromone-producing capabilities of beetles with reduced levels of microorganisms. Chararas *et al.* (1980) and Byers and Wood (1981) have studied pheromone production in bark beetles which had been fed antibiotics. However, this approach is questionable since the treatment of insects with dietary antibiotics can produce adverse physiological effects (Greenberg 1970). The development of a technique for rearing bark beetles axenically (Bedard 1966; Whitney and Spanier 1982) has provided an opportunity to study the production of pheromones by bark beetles which are free of readily culturable microorganisms, without the use of antibiotics.

A group of enzymes called polysubstrate monooxygenases (PSMO's), formerly called mixed function oxidases (mfo's), are involved in catalyzing reactions which cause lipophilic compounds, such as monoterpenes, to become more hydrophilic and more easily excretable (Brattsten, 1979). Although it has been hypothesized that polysubstrate monooxygenase enzymes are involved in terpene alcohol

pheromone production in bark beetles as a by-product of monoterpene detoxication (White *et al.* 1980), experimental evidence is inconclusive. White *et al.* (1979) reported that microsomal fractions of *Dendroctonus terebrans* (Olivier) could produce *alpha*-pinene oxide, and other unidentified compounds, from *alpha*-pinene. Although they speculated that *alpha*-pinene oxide was a likely precursor of terpene alcohols which are pheromones of certain *Dendroctonus* species, White *et al.* (1979) were unable to confirm that these terpene alcohols were produced by the microsomal fraction. No further experimentation on the role of monooxygenase enzymes in bark beetle pheromone production has been reported.

Although it has been established that *alpha*-pinene autoxidizes in the presence of air to form *trans*-verbenol, verbenone and other products (Moore *et al.* 1956), this fact appears to have been largely ignored by researchers studying bark beetle behaviour. Since many of the bark beetles which have been reported to use *alpha*-pinene as a kairomone also use *trans*- or *cis*-verbenol as an aggregation pheromone and verbenone as an antiaggregation pheromone, it is possible that a portion of the activity which has been attributed to *alpha*-pinene is actually due to products of its autoxidation. The frequent incidence of bark beetle attack on lightning-struck trees (Coulson *et al.* 1983, 1985, 1986; Krawielitzki *et al.* 1983) and wounded trees may be explained by the autoxidation of monoterpenes in wound exudate. Many experiments on the chemical ecology of bark beetles which have been conducted using *alpha*-pinene, especially under field conditions, would have to be re-evaluated if it was found that the autoxidation of this monoterpene occurs at a significant rate.

Since it was my intention to examine how pheromones are produced by bark beetles, and not to become involved in the isolation and identification of pheromones, I chose to study 2 species of scolytids in which the pheromone chemistry had already been fairly well studied: the mountain pine beetle, *Dendroctonus ponderosae* Hopkins, and the California five-spined ips, *Ips paraconfusus* (Lanier). These 2 species were also chosen because they are readily reared under laboratory conditions, and they are typical representatives of the 2 major genera of North American bark beetles. Finally, they were used because much of the research which has indicated that microorganisms are involved in the production of bark beetle pheromones has been conducted on *I. paraconfusus* (Brand

et al. 1975; Byers and Wood 1981), and *Dendroctonus* species (Brand *et al.* 1976, 1977; Brand and Barras 1977).

D. ponderosae is found throughout western North America where it is restricted to overmature and weakened *Pinus* species, except during outbreaks (Wood 1982). Females of this species convert the host tree monoterpene *alpha*-pinene to *trans*-verbenol[1] (Hughes 1973), an aggregation pheromone for this species (Pitman 1971), and a variety of other terpene alcohols. Two bicyclic ketals, *exo*-brevicomin and frontalin, which are of uncertain biosynthetic origin (Vanderwel and Oehlschlager 1987), are apparently multi-functional pheromones, acting to promote aggregation (Rudinsky *et al.* 1974a; Conn *et al.* 1983; Borden *et al.* 1983, 1987) or antiaggregation (Rudinsky *et al.* 1974a; Pitman *et al.* 1978; Ryker and Rudinsky 1982; Ryker and Libbey 1982; Borden *et al.* 1987) depending on concentration and which host is involved. *endo*-Brevicomin (Ryker and Rudinsky 1982), verbenone (Ryker and Yandell 1983; Borden *et al.* 1987), and pinocarvone (Libbey *et al.* 1985) have all been shown to have activity as antiaggregation pheromones, while the host-produced monoterpenes *alpha*-pinene (Pitman 1971), and myrcene and terpinolene (Billings *et al.* 1976; Conn *et al.* 1983; Borden *et al.* 1983) act as synergistic kairomones in promoting aggregation.

I. paraconfusus is usually found in weakened or fallen *Pinus* species in Oregon, Nevada and California (Wood 1982). It also infests the upper boles of trees killed by the western pine beetle, *Dendroctonus brevicomis* LeConte, and in some cases kills the upper crown of mature ponderosa pines, *Pinus ponderosa* Laws, or entire young trees (Struble and Hall 1955). *I. paraconfusus* converts the host monoterpene *alpha*-pinene to *cis*-verbenol (Renwick *et al.* 1976a), and the host monoterpene myrcene to ipsdienol (Byers *et al.* 1979; Hendry *et al.* 1980), and then to ipsenol (Fish *et al.* 1979; Hendry *et al.* 1980). Ipsenol, ipsdienol and *cis*-verbenol all act as aggregation pheromones in this species (Wood *et al.* 1968).

Evidently the mechanisms of pheromone production in bark beetles are poorly understood, and further research is required. In this regard my objectives were:

1. to assess the abilities of axenically-reared and streptomycin-fed *D. ponderosae* and *I. paraconfusus* to produce pheromones;

2. to assess the abilities of certain microorganisms associated with *D. ponderosae* to produce and interconvert their hosts' pheromones;

3. to determine whether the autoxidation of *alpha*-pinene into pheromones of *D. ponderosae* is biologically significant;

4. to investigate the role of monooxygenase enzymes in pheromone production in *D. ponderosae*; and

5. to investigate the behavioural significance for *D. ponderosae* of ipsdienol, identified in the course of this study as a terpene alcohol produced by male *D. ponderosae.*

II. GENERAL MATERIALS AND METHODS

Collection Of Insects and Host Material

Lodgepole pines, *Pinus contorta* var. *latifolia* Engelmann, infested with *D. ponderosae* were collected from various sites in southern British Columbia as indicated in the individual sections that follow. Adult *I. paraconfusus* were obtained from Dr. D. L. Wood[2], and a colony was maintained on ponderosa pine, *P. ponderosa*, bolts (60-70 cm long, approximately 20 cm diameter) at S.F.U. Infested and uninfested lodgepole and ponderosa pine logs were obtained near Princeton in the southern interior of British Columbia. The cut ends of the logs were sealed with hot paraffin wax to prevent drying, and the logs were either used while fresh or stored at 4°C until use.

Adult *D. ponderosae* and *I. paraconfusus* were collected daily after they emerged from caged logs which were kept at 27°C. Emerged beetles were stored on moistened paper towels at 2-4°C in loosely-capped, screw-top jars. When beetles were needed for experiments, jars which had been stored a maximum of 2 weeks were rewarmed to room temperature, and normal and healthy-appearing individuals were selected.

Pheromone Production and Extraction Techniques

Pheromone production was induced in test insects using one of 2 methods:

1. Beetles were placed in gelatin capsule halves which were taped over entrance holes drilled in the bark of pine logs. Beetles were allowed to bore in the inner bark for 24 h. Those beetles which entered the inner bark were chipped out and held over dry ice until a sufficient number had been accumulated for dissection.
2. Individual beetles were placed in upright, open-ended glass cylinders (1.4 cm ID) inside a 500 ml glass jar. Open vials (2 ml screw-cap) containing 25 µl of the pheromone precursors *alpha*-pinene and/or myrcene were also placed in the jar, and the jar was sealed and held in the dark for 24 h, after

which the beetles were immediately dissected.

Beetle abdomens were removed using fine forceps under a compound microscope, and immediately immersed individually in 100 µl of double distilled pentane in individual 2 ml glass vials over dry ice. The excised abdomens included the hindgut, Malpighian tubules, a large portion of the midgut, and other tissues, but did not include the wings. Each abdomen was macerated with the tip of a spatula in the pentane over the dry ice, and the vial was then sealed and allowed to sit at room temperature for approximately 15 min. The pentane extract was then drawn off with a syringe and put into a clean vial. The macerated tissue was rinsed twice each with 25 µl of double distilled pentane at room temperature, and this was added to the clean vial, which was then closed with a teflon-lined screw-cap, and stored at -20°C.

Gas Liquid Chromatographic (GLC) Analyses

Single beetle extracts were analysed on a Hewlett Packard 5880A gas chromatograph equipped with a capillary inlet system and a flame ionization detector. A glass capillary column (30 m x 0.66 mm ID) coated with SP-1000 (Supelco, Inc., Bellefonte, PA) was used with the following temperature program: 120°C for 2 min, then increased by 4°C/min to 180°C. The injection port temperature was 260°C, the flame ionization detector temperature was 275°C, and helium was used as the carrier gas. A known quantity of 3-octanol was added to the distilled pentane used to extract the abdomens, and the same quantity of 2-octanol was added following extraction. The area under the 3-octanol peak was used as a reference for calculating the quantities of each compound present in each sample, and the ratio of 3- to 2-octanol recovered was used to monitor the loss of volatile compounds during the extraction process.

Each day that samples were analysed by GLC a standard sample made up of 2- and 3-octanol, *alpha*-pinene, myrcene, *cis*- and *trans*-verbenol, verbenone, myrtenol, ipsenol, ipsdienol, myrcenol and any other compounds of interest for particular experiments was also analysed to aid in the identification of unknown compounds in the samples by comparison of retention times. In addition these compounds were periodically added to an abdominal extract and that mixture was analysed to

ensure correct identification by co-chromatography with unknown compounds in the extract. Selected extracts were analysed using gas chromatography/mass spectroscopy to ensure proper identification of compounds. For most samples the detection limit for GLC analysis of compounds of interest was set at 5 ng/abdomen.

III. PRODUCTION OF PHEROMONES BY MICROBE-REDUCED BEETLES

Introduction

The role of symbiotic microorganisms in pheromone production by bark beetles has previously been studied by isolating and culturing these microbes, and examining their capacities to produce terpene alcohol pheromones *in vitro.* As discussed in Section IV these studies have frequently disclosed that, when any one of many microorganisms found in association with scolytids are exposed to monoterpene precursors of pheromones, they can produce some of the same compounds which are utilized as terpene alcohol pheromones by their hosts (Brand *et al.* 1975; Chararas *et al.* 1980). This finding is not surprising since these microorganisms are exposed in nature to the same host tree monoterpene pheromone precursors as are the host beetles, and it is well documented that many species of microorganisms are capable of oxidizing monoterpene hydrocarbons (Bhattacharyya *et al.* 1960; Prema and Bhattacharyya 1962; Shukla *et al.* 1968; Fonken and Johnson 1972; Kieslich 1976). However, there is no evidence that microbe-produced terpene alcohols or ketones are being used in nature as pheromones by the host beetles, or that these compounds are even being produced by the symbionts in quantities which are biologically significant to the host beetles.

Individual monoterpenes or extracts of resin have been found to be toxic to microorganisms associated with bark beetles, such as several *Ceratocystis* species (Cobb *et al.* 1968; DeGroot 1972), *Trichosporium symbioticum* Wright (Raffa *et al.* 1985) and fungal symbionts of *D. ponderosae* (Shrimpton and Whitney 1968), as well as being toxic to *Dendroctonus* species (Smith 1965; Reid and Gates 1970; Coyne and Lott 1976; Raffa and Berryman 1983b). Therefore, it is of adaptive advantage for the microorganisms to be capable of allylic oxidations of monoterpenes to detoxify these compounds and secondarily to use them as energy sources.

In order to assess the importance of the microbial contribution to overall pheromone production it is essential to ascertain the metabolic capabilities of beetles which are relatively free of microbial symbionts. However, the production of pheromones by microbe-free or microbe-reduced bark beetles

apparently has been studied only by Chararas (1980) and Byers and Wood (1981)[3]. Chararas (1980) claimed that after *Ips sexdentatus* Boerner, *Ips typographus* (L.) and *Ips acuminatus* Gyllenhal, had been fed a wide spectrum antibiotic, either ceporoxine or ampicillin, their frass showed slightly reduced attractiveness to other beetles of the same species. Chararas also reported that the frass of antibiotic-fed beetles had more of the monoterpenes *alpha*-pinene, ß-pinene and Δ_3-carene, and less of the conversion products of these compounds, than did frass from normal beetles. However, he presented no methodological details and no data to support any of these claims. Byers and Wood (1981), found that *I. paraconfusus* which were fed the antibiotic streptomycin were unable to convert the host tree monoterpene myrcene to the pheromones ipsenol and ipsdienol, while the synthesis of the pheromone *cis*-verbenol from the host tree monoterpene *alpha*-pinene was not inhibited. This suggested that the *in vitro* conversion of *alpha*-pinene to *cis*-verbenol by a strain of *Bacillus cereus* isolated from the guts of *I. paraconfusus* (Brand *et al.* 1975) is not the only method of production of this pheromone. Byers and Wood (1981) also reported that penicillin G, which is an effective antibiotic against many Gram-positive bacteria such as *B. cereus*, did not affect the synthesis of *cis*-verbenol, ipsdienol or ipsenol. They interpreted these results to indicate that symbiotic bacteria sensitive to streptomycin are involved in the synthesis of ipsenol and ipsdienol, and that *cis*-verbenol is either synthesized by insect cells or by microorganisms which are unaffected by the concentrations of antibiotics used. However, while this selective effect of antibiotics is supportive of their hypothesis, the treatment of insects with dietary antibiotics can produce adverse physiological effects in the insects (Greenberg 1970), as well as creating a microbial imbalance by eliminating only certain types of microbes, or affecting microbial physiology without actually killing them (H. S. Whitney[4], pers. comm.). It is possible that the elimination of ipsdienol and ipsenol production through streptomycin-feeding was the result of such effects.

The usefullness of axenic-rearing for the study of symbiosis in insects has been reviewed by Rodriguez (1966), and Vanderzant (1974). The development of a technique for rearing bark beetles axenically (Bedard 1966; Whitney and Spanier 1982) has provided an opportunity to study the

production of pheromones by bark beetles with reduced levels of microorganisms without the use of antibiotics. The capabilities of axenically-reared beetles to oxidize host tree monoterpenes can then be compared with those of wild and antibiotic-fed beetles.

Materials and Methods

Axenically-reared Beetles

The axenic-rearing technique developed by Whitney and Spanier (1982) was used to obtain beetles which were devoid of readily culturable microorganisms. The outer bark was shaved off fresh pine bolts, and the exposed phloem was then removed and cut into 1 cm^2 pieces. Lodgepole pine, *P. contorta*, was used for rearing *D. ponderosae*, and ponderosa pine, *P. ponderosa*, for *I. paraconfusus*. The phloem was frozen at -25°C, ground in a pre-chilled Waring^R blender, and passed through #12 mesh screen. Grinding and sieving were conducted in a -25°C walk-in cold-room, and ground phloem was stored in plastic bags in a -20°C freezer. When diet was required for insect rearing, the phloem was thawed, and water and dehydrated Brewer's yeast were added at 50% and 10%, respectively, of the weight of the phloem. The mixed diet was dispensed into 19x55 mm shell vials at approximately 0.75 g/vial using a 10 ml plastic syringe with the bottom removed. The vials were capped with Morton^R stainless steel culture closures (18 mm), pasteurized in an Amsco^R isothermal sterilizer for 1.0 h at 85°C, held at room temperature for 4 days, and then re-pasteurized. This double treatment of the vials in the isothermal sterilizer yielded sterile diet.

Adult female *D. ponderosae* or male *I. paraconfusus* were individually caged onto fresh, waxed bolts of lodgepole or ponderosa pine, respectively, using gelatin capsules (Lanier and Wood 1968). After 24 h, a mate was introduced to each capsule and the bolts were kept at 26-28°C. After 10 days for *D. ponderosae* or 7 days for *I. paraconfusus*, the bark was removed and using a blunt probe the eggs were collected from the parent galleries. The eggs were transferred to moistened filter paper in a petri dish, sealed in a plastic bag to prevent moisture loss, and incubated at 22-24°C in the dark. Incubating eggs were examined twice daily for *D. ponderosae* and 3 times daily for *I. paraconfusus*. Fourth stage eggs (Reid and Gates 1970) were surface-sterilized in 0.1% mercuric chloride (Fisher Scientific Co., Fair Lawn, NJ), rinsed 6 times in sterile distilled water, and placed individually in the vials of sterilized diet. Sterilized Pasteur pipettes were used to transfer the eggs and the procedure was

performed aseptically on a sterile air bench. Emergent axenically-reared adults were allowed to remain in the rearing vials until used.

Checks for aerobic microorganisms in the sterilized diet, as well as externally and internally from all stages of the developing axenically-reared insects, were done on malt extract agar, potatoe dextrose agar and plate count agar. Further culture checks for aerobic and anaerobic microorganisms were performed by Whitney and Spanier (1982). Axenically-reared adult beetles were also examined for microorganisms on their exposed cuticle using scanning electron microscopy.

Antibiotic-feeding

An antibiotic-feeding technique similar to that developed by Byers and Wood (1981) was used to obtain beetles with reduced levels of symbiotic microorganisms. A mixture of 35 g of powdered cellulose (alpha-cellulose, Sigma Chemical Co., St. Louis, MO), 8 g of sucrose and 22 g of ground pine phloem was added to 62 ml of distilled water containing 10 mg of streptomycin sulfate (Sigma Chemical Co., St. Louis, MO)/ml of water. Lodgepole pine phloem was used when the diet was intended for *D. ponderosae* and ponderosa pine phloem for *I. paraconfusus*. The ingredients were thoroughly mixed and then dispensed into 19x55 mm shell vials at approximately 0.75 g/vial using a 10 ml plastic syringe with the bottom removed for a dispenser. An adult beetle was added to each vial and the insects were allowed to feed for 96 h at 22-24^{o}C. Petri dishes were not used as in Byers and Wood (1981), as the use of individual vials made it possible to eliminate from the experiment beetles which did not feed on the antibiotic diet.

Maturation Experiments

When wild[5] adult beetles of a known age were required for experiments, the bark was removed from infested bolts and pupae were collected. The pupae and bark were placed in petri dishes containing moistened filter paper and the insects were examined daily until eclosion[6]. Callow adults

were removed from the petri dishes and allowed to mature in individual vials containing moistened bark until reaching the desired age. Since fungi found in the pupal chamber are thought to be important in the maturation feeding of callow scolytids (Whitney 1971; Barras 1973), bark from around the pupal chambers in the infested bolt was included in each vial.

Vials containing axenically-reared individuals were examined daily near the time of eclosion so that the maturity of adult beetles used in experiments was known.

Recontamination With Associated Microorganisms

Attempts were made to re-expose axenically-reared and antibiotic-fed beetles to their natural complement of microorganisms by allowing them to crawl over and bore into bark and phloem which had just been removed from bolts containing apparently healthy pupae and callow adults. Beetles were recontaminated for various lengths of time in 150x25 mm petri dishes. To distinguish between the effects of recontamination and any diminishing or delayed effects of axenic-rearing or streptomycin-feeding, groups of control beetles were held in petri dishes containing ground pine phloem and distilled water without antibiotics for the same time periods as for recontamination.

Pheromone Analysis

The methods used for monoterpene exposure, dissection and extraction of insects, and GLC analysis are described in the Section II, while the technique for steam distilling the axenic diet is described in Section V.

The pheromone data were tested for homogeneity of variances using Cochran's C test as well as the Bartlett-Box F test using SPSS^{X} (SPSS 1983). The data were heteroscedastic; therefore they were analysed using the Kruskal-Wallis test (Sokal and Rohlf 1981) followed by a non-parametric multiple comparisons test (Conover 1980, p. 231), $P < 0.05$.

Experiments Conducted

Twenty-five experiments were conducted with *D. ponderosae* and *I. paraconfusus* adults of both sexes, with objectives as outlined below. Details of experimental treatments and replicates are given in Tables 1-5, and Figs. 1-10.

Female *D. ponderosae*

Experiment No.	Objectives
I	To assess the effect of axenic-rearing or streptomycin-feeding on the conversion of *alpha*-pinene into terpene alcohols.
II	To assess the effect of further maturation on the ability of axenically-reared beetles to produce terpene alcohols.
III	To assess geographic variation in terpene alcohol production by wild beetles.
IV	To compare the effect of various degrees of maturation on the ability of axenically-reared and wild beetles to produce terpene alcohols.
V	To compare the levels of terpene alcohol production by mature[7] axenically-reared, mature wild beetles, and emerged wild beetles.
VI and VII	To compare the effects of streptomycin-feeding on terpene alcohol production in beetles exposed to *alpha*-pinene either as vapours or through feeding.
VIII	To determine the effects of axenic-rearing on the conversion of ingested *alpha*-pinene into terpene alcohols.
IX	To determine the ability of females to convert myrcene into terpene alcohols.

Male *I. paraconfusus*

Experiment No.	Objective
X and XI	To compare the effect of various degrees of maturation on the ability of axenically-reared and wild beetles to produce *alpha*-pinene-derived (Exp. X) and myrcene-derived (Exp. XI) terpene alcohols.
XII	To compare the levels of myrcene-derived terpene alcohol production by mature axenically-reared, mature wild beetles and emerged wild beetles.
XIII	To compare the levels of terpene alcohol production by mature axenically-reared, mature wild beetles, and emerged wild beetles.
XIV	To assess the effects of streptomycin-feeding on terpene alcohol production in wild beetles exposed to *alpha*-pinene vapours.
XV and XVII	To determine the effects of streptomycin-feeding (Exp. XV) or axenic-rearing (Exp. XVII) on terpene alcohol production in beetles exposed to *alpha*-pinene through feeding.
XVI	To compare the efficacy of 2 different batches of streptomycin in reducing the production of myrcene-derived terpene alcohols.
XVIII and XIX	To examine the effect on terpene alcohol production of recontamination of axenically-reared or axenically-reared and streptomycin-fed beetles with their natural complex of microorganisms.
XX	To examine the effect on terpene alcohol production of recontaminating, or holding without recontamination, streptomycin-fed beetles.
XXI	To compare the levels of terpene alcohol production in beetles

exposed to myrcene vapours, myrcene and *alpha*-pinene vapours simultaneously, or the myrcene and *alpha*-pinene encountered while feeding on bolts of *P. ponderosa.*

XXII	To determine the effects of distending the guts of *I. paraconfusus* with powdered cellulose on the conversion of myrcene vapours to ipsdienol and ipsenol.

Male *D. ponderosae*

Experiment No.	Objective
XXIII and XXIV	To determine the effects of axenic-rearing and streptomycin-feeding on the conversion of *alpha*-pinene (Exp. XXIII) and myrcene (Exp. XXIV) vapours into terpene alcohols.

Female *I. paraconfusus*

Experiment No.	Objectives
XXV	To determine the effects of axenic-rearing and streptomycin-feeding on the conversion of *alpha*-pinene vapours into terpene alcohols.

Axenically-reared beetles

During axenic-rearing most batches contained only a small number of vials with visible signs of contamination; these were discarded. One batch of axenically-reared *D. ponderosae* contained large numbers of contaminated vials, and closer examination revealed that the vials also contained large populations of mites. These mites, which are frequently associated with wild *D. ponderosae*, are small enough that they can enter the shell vials after capping with the steel culture closures. Contamination with mites, and the fungi that they carry, was avoided in subsequent batches of axenically-reared beetles by ensuring that the capped vials were not handled or stored in areas of the laboratory where wild beetles were handled.

Checks for aerobic microorganisms in the vials of sterilized diet, as well as externally and internally from all stages of axenically-reared *D. ponderosae* and *I. paraconfusus*, did not reveal the presence of any microorganisms. Further culture checks for aerobic as well as anaerobic microorganisms which were performed on *D. ponderosae* obtained using the same axenic-rearing technique also did not show the presence of microorganisms (Whitney and Spanier 1982). In addition, no microorganisms other than Brewer's yeast cells were observed on the external cuticular surface of axenically-reared adults which were examined by scanning electron microscopy. Similar examinations of beetles which were reared on a yeast-free axenic diet did not reveal the presence of any microorganisms.

Although all of the above checks and precautions indicate that beetles obtained using the axenic-rearing technique are likely to be truely axenic, it is not possible to entirely rule-out the possibility that trans-ovarially transmitted, obligate symbionts may still be associated with these beetles. As a result I refer to beetles obtained using this technique as "axenically-reared" or "microbe-reduced", and not "axenic".

Of the *D. ponderosae* eggs which were surface sterilized and placed in vials of sterilized diet

generally 60-80% survived to become apparently axenic, mature adults. Only 40-60% of axenically-reared *I. paraconfusus* survived, probably because the eggs of this species are more fragile, and were easily damaged during handling. Attempts were made to use diet which was autoclaved at conventional temperatures instead of being double-pasteurized at 85°C in the isothermal sterilizer, and to use lodgepole instead of ponderosa pine phloem for axenically-reared *I. paraconfusus*; in both cases there was no beetle survival.

Initial Experiments on Pheromone Production by Axenically-reared Beetles

The first experiment on the capabilities of axenically-reared female *D. ponderosae* to convert *alpha*-pinene vapours into *trans*-verbenol was conducted at S. F. U. by J. E. Conn. Results from this experiment indicated that female *D. ponderosae* which were free of readily culturable microorganisms contained a mean of 9900 ng of *trans*-verbenol/abdomen, a level which far exceeded *trans*-verbenol production in wild control beetles (Conn *et al.* 1984). My initial attempt to replicate this experiment was unsuccesful, as axenically-reared beetles were found to contain *trans*- and *cis*-verbenol and myrtenol at levels which were too low to be quantified (Table 1, Exp. I). Streptomycin-fed beetles and wild controls also contained unusually low levels of these terpene alcohols (Table 1, Exp. I).

In a subsequent experiment beetles from the same batch of axenically-reared, female *D. ponderosae*, which were allowed to mature for a further 14 days, showed increased levels of conversion of *alpha*-pinene vapours (Table 1, Exp. II). In this experiment the levels of terpene alcohol production by axenically-reared, female *D. ponderosae* were somewhat higher than in Exp. I, and normal levels of production were found in wild, control beetles (Table 1, Exp. II). The quantities of *trans*- and *cis*-verbenol produced by axenically-reared beetles in this experiment were not significantly different from those produced by wild emerged beetles, although the production of myrtenol was significantly reduced in the axenically-reared individuals (Table 1, Exp. II).

Table 1. Oxygenated monoterpene production in axenically-reared, streptomycin-fed and wild *Dendroctonus ponderosae* females.

Exp. no.	Treatment	No. beetles	Mean amount of volatiles in abdominal extract (ng/beetle)[a]		
			trans-verbenol	*cis*-verbenol	myrtenol
I	Wild/air	7	<5 a	<5 a	<5 a
	Wild/α-pinene vapours	8	46 b	20 a	7 a
	Wild/streptomycin/air	7	20 ab	8 a	<5 a
	Wild/streptomycin/α-pinene vapours	8	44 b	17 a	7 a
	Axenically-reared/air	8	<5 a	<5 a	<5 a
	Axenically-reared/α-pinene vapours	7	<5 a	<5 a	<5 a
	Axenically-reared/streptomycin/air	8	8 a	<5 a	<5 a
	Axenically-reared/streptomycin/ α-pinene vapours	7	<5 a	<5 a	<5 a
			trans-verbenol	*cis*-verbenol	myrtenol
II	Wild/air	10	<5 a	<5 a	<5 a
	Wild/α-pinene vapours	8	446 b	70 b	79 b
	Axenically-reared/air	9	7 a	<5 a	<5 a
	Axenically-reared/α-pinene vapours	8	117 b	26 b	28 c
			trans-verbenol	*cis*-verbenol	myrtenol
III	Lillooet/air	9	<5 a	<5 a	<5 a
	Lillooet/α-pinene vapours	11	219 b	47 b	46 c
	Riske Cr./air	5	15 a	<5 a	<5 a
	Riske Cr./α-pinene vapours	7	245 b	19 b	10 ab
	Manning Pk./air	6	<5 a	<5 a	<5 a
	Manning Pk./α-pinene vapours	11	76 b	31 b	32 bc

Table 1. continued

Exp. no.	Treatment	No. beetles	Mean amount of volatiles in abdominal extract (ng/beetle)[a]			
			trans-verbenol	*cis*-verbenol	myrtenol	verbenone
V	Wild/emerged/air	10	<5 a	<5 a	<5 a	<5 a
	Wild/emerged/α-pinene vapours	11	194 b	37 b	37 b	<5 a
	Wild/mature/air	6	<5 a	<5 a	<5 a	<5 a
	Wild/mature/α-pinene vapours	6	637 bc	49 bc	127 c	<5 a
	Axenically-reared/mature/air	10	<5 a	<5 a	<5 a	<5 a
	Axenically-reared/mature/ α-pinene vapours	10	1216 c	87 c	112 c	<5 a
			trans-verbenol	*cis*-verbenol	myrtenol	
VI	Wild/air	7	<5 a	<5 a	<5 a	
	Wild/α-pinene vapours	7	730 b	133 b	252 b	
	Wild/streptomycin/air	7	<5 a	<5 a	<5 a	
	Wild/streptomycin/α-pinene vapours	7	2056 b	104 b	172 b	
			trans-verbenol	*cis*-verbenol	myrtenol	
VII	Wild/air	6	<5 a	<5 a	<5 a	
	Wild/α-pinene vapours	9	261 b	30 b	39 b	
	Wild/*P. contorta*	6	17 a	<5 a	<5 a	
	Wild/streptomycin/air	7	<5 a	<5 a	<5 a	
	Wild/streptomycin/α-pinene vapours	10	3375 c	155 c	264 c	
	Wild/streptomycin/*P. contorta*	5	17 a	<5 a	<5 a	

Table 1. continued

Exp. no.	Treatment	No. beetles	Mean amount of volatiles in abdominal extract (ng/beetle)[a]			
			trans-verbenol	verbenone		
VIII	Wild/air	12	<3 a	<3 a		
	Wild/*P. contorta*	12	241 b	30 b		
	Axenically-reared/air	11	<3 a	<3 a		
	Axenically-reared/*P. contorta*	12	<3 a	<3 a		
			ipsdienol	*Z*-myrcenol	*E*-myrcenol	unknown (RT 14.74)
IX	Wild/air	7	<10 a	<5 a	<5 a	<5 a
	Wild/myrcene	9	<10 a	11 a	415 b	55 b
	Wild/streptomycin/air	9	<10 a	<5 a	<5 a	<5 a
	Wild/streptomycin/myrcene vapours	8	<10 a	10 a	641 b	103 b

[a]Data analysed using the Kruskal-Wallis test followed by a non-parametric multiple comparisons test (Conover 1980, p. 231), $P < 0.05$. Means within a column for each experiment followed by the same letter are not significantly different.

Geographic Variation in Pheromone Production

The beetles used in Exp. I were all from naturally-infested material obtained near Manning Park, while in Exp. II the axenically-reared beetles were from Manning Park and the wild beetles were from near Pavilion. In the experiment conducted by J. E. Conn the wild beetles were from near Pavilion and the axenically-reared beetles had been produced by Mr. O. J. Spanier[4] from beetles from Riske Creek. To test whether the disparity in results between these 3 experiments was partially due to geographic variation in terpene alcohol production, wild beetles from the Riske Creek, Pavilion and Manning Park areas of southern B. C. were tested for their abilities to convert *alpha*-pinene vapours into *cis*- and *trans*-verbenol and myrtenol. After 24 h exposure to *alpha*-pinene vapours the Manning Park beetles produced only 76 ng of *trans*-verbenol/beetle, while Riske Creek and Pavilion beetles produced 245 and 219 ng/beetle, respectively (Table 1, Exp. III). The Manning Park population of *D. ponderosae* is known to have sustained heavy parasitism by a nematode, *Sphaerulariopsis hastata* Kahn (identified by D. N. Kinn[8]), a possible cause of reduced vigour (Khan 1957a, b; Thong and Webster 1975; MacGuidwin *et al.* 1980). Parasitism by this nematode may have been the cause of the low levels of terpene alcohol production noted in Table I, Exps. I-III. Although the terpene alcohol levels found in beetles from Manning Park were not significantly lower than those found in Pavilion and Riske Creek beetles, Manning Park beetles were not used in subsequent experiments.

To standardize experiments and avoid any effects of geographic variation an effort was made to use *D. ponderosae* from the same infestation for all subsequent research. Lodgepole pine infested with *D. ponderosae* was collected from sites near Pavilion for experiments conducted in 1982-83. However, the infested sites near Pavilion were logged extensively in the fall of 1983 to prevent the spread of the beetles. In 1984-1986 infested material was collected from about 30 km northeast of Princeton, primarily in the Shinish Creek Valley. This area was chosen because the beetles appeared to be healthy, while beetles in other areas of the province were undergoing a decline due to overwintering mortality and other undetermined factors. In addition, this area was suitable because the terpene alcohol producing capabilities of *D. ponderosae* from this area did not appear to differ from that

of Pavilion beetles.

Effect of Maturation on Terpene Alcohol Production in Female *D. ponderosae*

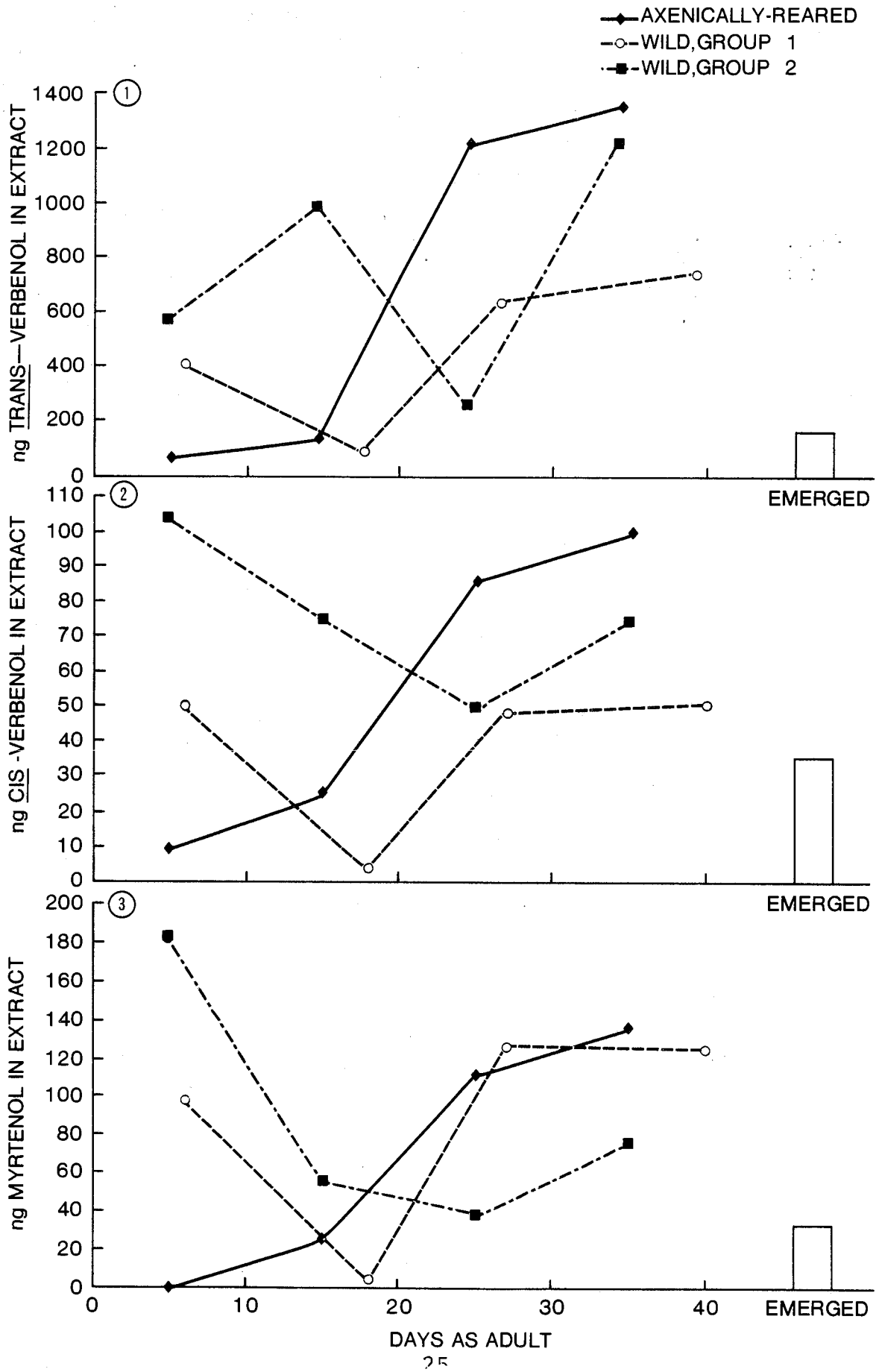
The axenically-reared female *D. ponderosae* used for Exp. I (Table 1) were fully darkened adults, and it was initially assumed that callow adults which had darkened completely had reached physiological maturity. However, since individuals from this batch of axenically-reared beetles which were allowed to mature for a further 14 days after Exp. I produced terpene alcohols at higher levels, and because no record was made of the age of the axenically-reared female *D. ponderosae* used by Conn *et al.* (1984), I hypothesized that maturation beyond the point of darkening of axenically-reared *D. ponderosae* was required before peak terpene alcohol production occurred.

To test whether the physiological maturity of axenically-reared *D. ponderosae* affected terpene alcohol production, females of varying posteclosion ages were exposed to *alpha*-pinene vapours. Axenically-reared individuals of 5 and 15 days posteclosion produced *trans*- and *cis*-verbenol and myrtenol at relatively low levels, while levels in beetles of 25 and 35 days posteclosion were generally higher than those in wild emerged beetles (Figs. 1-3). Thus a 3- to 4-week maturation period is apparently required before adult axenically-reared *D. ponderose* develop maximal enzymatic capacity for the oxidation of *alpha*-pinene into terpene alcohols.

Since the posteclosion age of *D. ponderosae* is evidently important in determining the physiological maturity of these beetles, much of the data reported in Conn *et al.* (1984) must be questioned. Although Conn *et al.* (1984) certainly did establish that axenically-reared *D. ponderosae* are capable of producing terpene alcohol pheromones, it is likely that the generally low levels of production of these compounds reported by Conn *et al.* (1984) simply reflect the use of axenically-reared beetles which were not yet mature. The production of very high levels of *trans*-verbenol by axenically-reared female *D. ponderosae* in one experiment (9900 ng/beetle) probably indicates the fortuitous use of mature beetles in that one experiment.

The pattern of terpene alcohol production in relation to age for wild female *D. ponderosae* was

Figures 1-3. Quantities of *trans*-verbenol (Fig. 1), *cis*-verbenol (Fig. 2), and myrtenol (Fig. 3), extracted in Exp. IV from individual female *D. ponderosae* of various posteclosion ages which were exposed to *alpha*-pinene vapours for 24 h. Numbers of beetles used for each treatment were as follows. Axenically-reared: 5 days-9, 15 days-10, 25 days-10, 35 days-8. Wild, Group #1: 6 days-6, 18 days-6, 27 days-6, 40 days-9. Wild, Group #2: 5 days-11, 15 days-8, 25 days-10, 35 days-10.


AXENICALLY-REARED
WILD, GROUP 1
WILD, GROUP 2
1
ng TRANS—VERBENOL IN EXTRACT
1400
1200
1000
800
600
400
200
0
EMERGED
2
ng CIS -VERBENOL IN EXTRACT
110
100
90
80
70
60
50
40
30
20
10
0
EMERGED
3
ng MYRTENOL IN EXTRACT
200
180
160
140
120
100
80
60
40
20
0
0
10
20
30
40
EMERGED
DAYS AS ADULT

different than that for axenically-reared individuals (Figs. 1-3). In 2 separate experiments (Figs. 1-3, groups 1 and 2) wild beetles produced *trans*- and *cis*-verbenol and myrtenol at moderate levels at 5 days posteclosion. Production in wild beetles declined at 18-25 days posteclosion, and then increased again in beetles of 35-40 days posteclosion (Figs. 1-3). The terpene alcohol production in wild callow adults of 5 days posteclosion is not likely to be due to production by microorganisms since wild beetles of 18-25 days posteclosion, which should have the same complement of symbiotic microorganisms, produced terpene alcohols at generally lower levels (Figs. 1-3). Possibly the *cis*- and *trans*-verbenol and myrtenol produced by wild beetles of 5 days posteclosion is from derivatized *alpha*-pinene sequestered by the insects as larvae or pupae (Hughes 1975), and this sequestered precursor is metabolized by 18-25 days posteclosion. Axenically-reared callow adults could not produce significant quantities of terpene alcohols from sequestered precursors since the monoterpenes in the ground phloem in the axenic diet are virtually eliminated during the sterilization process, as shown by the analysis of steam distillates of axenic diet (Table 2). The increase in terpene alcohol production in wild beetles after 25 days posteclosion would then be due to the maturation of the enzyme systems responsible for oxidizing monoterpenes which are encountered as the mature adult attacks a new host.

Terpene alcohol production was generally higher in wild beetles of 27 and 40 days posteclosion than in wild emerged beetles (Figs. 1-3). This difference apparently indicates that wild emerged beetles are not yet capable of peak pheromone production. There is evidence that certain species of bark beetles undergo a period of obligatory dispersal before they become responsive to olfactory signals associated with their hosts or other beetles. Flight exercise was found to increase positive responses to attractive semiochemicals in *Dendroctonus frontalis* Zimmerman (Andryszak *et al.* 1982), *Dendroctonus pseudotsugae* Hopkins (Bennett and Borden 1971), *Trypodendron lineatum* (Olivier) (Graham 1959, 1962; Bennett and Borden 1971), and *Scolytus multistriatus* (Marsham) (Choudhury and Kennedy 1980). It is thought that beetles which are not yet responsive to attractive semiochemicals contain higher levels of lipids, and that these individuals become responsive once lipid reserves have been reduced below a threshold level through flight exercise (Atkins 1966, 1969). It is my hypothesis that a period of lipid metabolism through dispersal and flight exercise may also be

Table 2. Effect of pasteurization on monoterpene content of ground phloem in axenic diet.

Phloem source	% Reduction	
	α-pinene	myrcene
Pinus contorta var. *latifolia*	99%	98%
Pinus ponderosa	78%	90%

necessary before pheromone production can reach its maximum. In this way healthy, lipid-rich beetles could disperse farther from trees in which they developed before being able to produce large quantities of attractive pheromones. This longer dispersal would be of adaptive advantage as these individuals would be more likely to encounter suitable hosts, and obtain an adequate supply of high quality phloem in which to feed and breed. A long dispersal flight would also enable individuals to interbreed with beetles from other populations. Older, wild beetles (collected as pupae and held 35-40 days posteclosion) which show somewhat higher levels of pheromone production than wild emerged beetles (Figs. 1-3) have probably lost some of their lipid reserves through walking exercise in the petri dishes, and also may have faced lower quality diet due to drying out of the pieces of phloem on which they were allowed to feed. Sanders (1983) showed that starvation or walking activity released host-positive resposes in host-negative *Pityogenes chalcographus* (L.). Similarly, Gries (1984) showed that, on the average, *I. typographus* were not host-responsive until having incurred a mean weight loss that was equivalent to weight lost during a 7 km flight. Therefore, I hypothesize that the older wild beetles shown in Figs. 1-3 have foregone a dispersal flight due to decreasing lipid reserves, and have become physiologically ready to attack new hosts, detoxify monoterpenes present in those hosts, and release aggregation pheromones at high levels.

It would be of interest to know how long adult *D. ponderosae* normally spend maturing in the phloem of a host tree before emerging and attacking new hosts. This information could help to explain the adaptive significance of the pattern of terpene alcohol production in relation to posteclosion age of wild beetles (Figs. 1-3). Unfortunately such data are largely unavailable. It is well known that fully darkened adult *D. ponderosae* frequently do not emerge for long periods of time, but this is not well documented, and the environmental and physiological factors responsible for this delay in emergence are unknown.

When pupae were collected from infested bolts for use in the wild beetle maturation experiments, the beetles from 2 additional infested bolts from the same tree were allowed to emerge naturally in cages. In this way it was possible to estimate how long the average adult *D. ponderosae* spends maturing in the phloem before emerging. The average period of time between eclosion to

adults in the petri dishes of collected pupae and emergence from infested bolts was approximately 30 days. This is a very rough estimate of the time which adults wait before emerging since the infested bolts were held in cages at approximately 27°C, and this elevated, constant temperature, in combination with the accelerated drying of the phloem in the bolts, probably resulted in earlier emergence than would occur in nature. Nonetheless this result indicates that the 35-40 days posteclosion adults in Figs. 1-3 would have emerged and dispersed before this time, which may explain why they produced terpene alcohols at higher levels than emerged beetles.

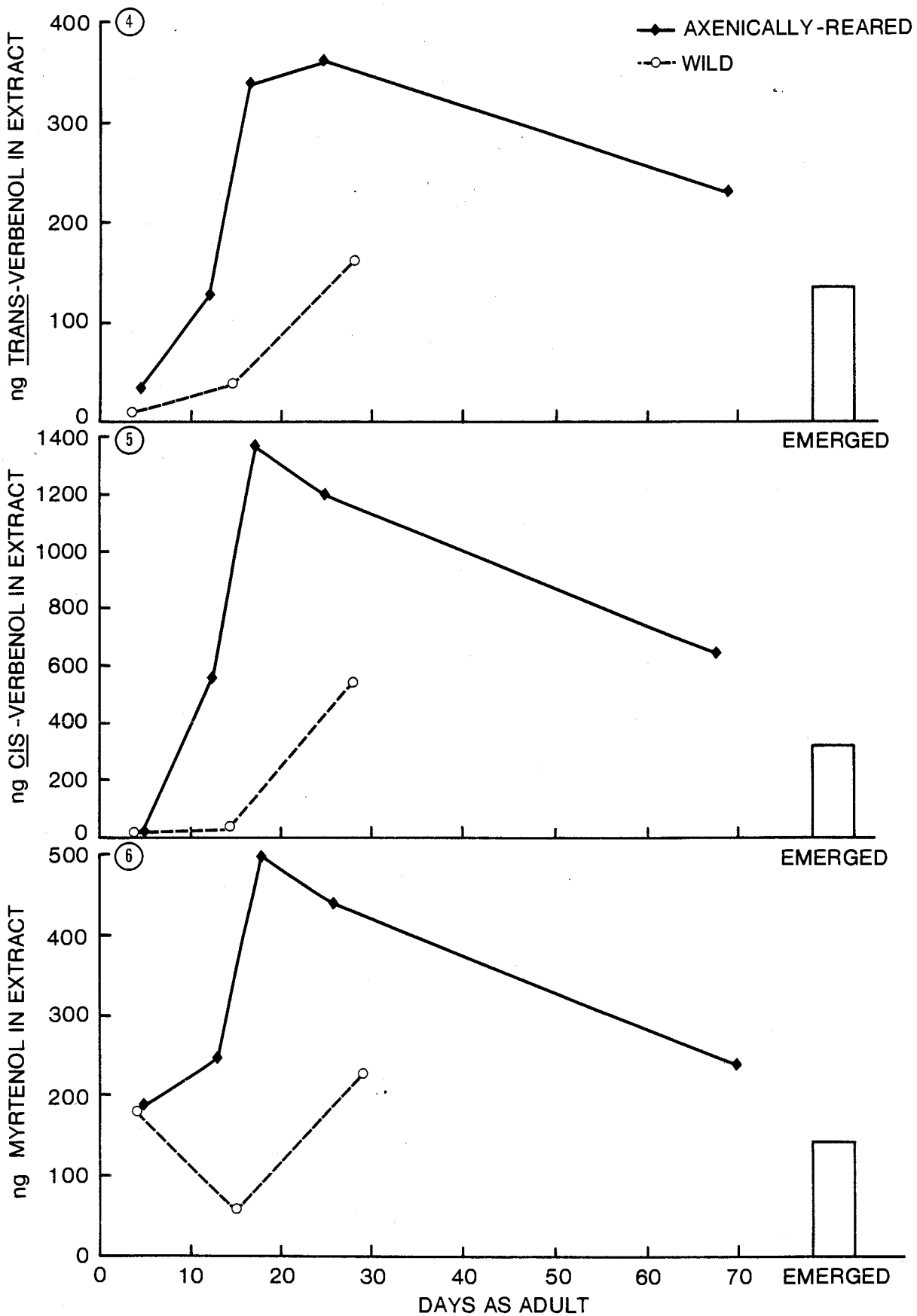
Although the results reported in Figs. 1-3 indicate that wild emerged beetles are not yet capable of maximal terpene alcohol production, it was decided that wild emerged beetles would be used as a standard for comparison in all experiments with axenically-reared and streptomycin-fed beetles. Wild emerged insects are not ideal as controls in these experiments since they may differ from the axenically-reared and streptomycin-fed individuals in terms of age, genetics and diet, but they do offer a rough guide as to the normal levels of production in natural beetles.

Effect of Maturation on Terpene Alcohol Production in Male *I. paraconfusus*

To test whether the physiological maturity of axenically-reared *I. paraconfusus* also affects the beetle's ability to oxidize monoterpenes, males of varying posteclosion ages were exposed to *alpha*-pinene and myrcene vapours. Axenically-reared individuals of 5 and 13 days posteclosion produced *trans*- and *cis*-verbenol and myrtenol at relatively low levels, while beetles of 18 and 26 days posteclosion produced these compounds at levels much higher than those in wild emerged beetles (Figs. 4-6). Adults which were allowed to mature to 69 days posteclosion showed declining levels of terpene alcohol production. Wild beetles also showed a maturation in their ability to oxidize *alpha*-pinene (Figs. 4-6), but, with the exception of myrtenol (Fig. 6), did not exhibit the early peak in terpene alcohol production noted in young *D. ponderosae* adults (Figs. 1-3). High levels of mortality prevented the testing of wild beetles beyond 28 days posteclosion.

The peak levels of terpene alcohols produced by wild *I. paraconfusus* were only half that of

Figures 4-6. Quantities of *trans*-verbenol (Fig. 4), *cis*-verbenol (Fig. 5), and myrtenol (Fig. 6), produced in Exp. X by individual male *I. paraconfusus* of various posteclosion ages which were exposed to *alpha*-pinene vapours. Numbers of beetles used for each treatment were as follows. Axenically-reared: 5 days-10, 13 days-11, 18 days-12, 26 days-11, 70 days-10. Wild: 4 days-7, 15 days-5, 29 days-5.


AXENICALLY-REARED
WILD
4
ng TRANS-VERBENOL IN EXTRACT
400
300
200
100
0
EMERGED
5
ng CIS-VERBENOL IN EXTRACT
1400
1200
1000
800
600
400
200
0
EMERGED
6
ng MYRTENOL IN EXTRACT
500
400
300
200
100
0
0
10
20
30
40
50
60
70
EMERGED
DAYS AS ADULT

those produced by axenically-reared beetles (Figs. 4-6). In contrast to *D. ponderosae* the peak levels of terpene alcohols produced by mature, wild *I. paraconfusus* did not exceed the production by wild emerged beetles (Figs. 4-6). Oxidation products of myrcene, such as ipsdienol and ipsenol, were generally detectable only at low levels in axenically-reared beetles and wild controls which were exposed to myrcene vapours.

Axenically-reared male *I. paraconfusus* of 8 days posteclosion which were exposed to myrcene through feeding on bolts of *P. ponderosa* were unable to produce ipsenol and ipsdienol at measurable levels, while beetles of 24 and 32 days posteclosion produced these pheromones at levels which were similar to those in wild emerged beetles (Figs. 7-8). When mature (24 days posteclosion), axenically-reared individuals were fed on ponderosa pine bolts they were found to contain levels of ipsenol and ipsdienol which were not significantly different from those in mature wild beetles or wild emerged beetles (Table 3, Exp. XII).

Evidently, as with *D. ponderosae*, the posteclosion age of *I. paraconfusus* is important in determining the physiological maturity of these beetles, so much of the data on *I. paraconfusus* reported in Conn *et al.* (1984) also must be questioned. Conn *et al.* (1984) did establish that axenically-reared *I. paraconfusus* are capable of producing terpene alcohol pheromones, but it is likely that the generally low levels of production which they report simply reflect the use of axenically-reared beetles which were not yet mature.

The maturation period which is required before adult *I. paraconfusus* can convert myrcene into ipsenol and ipsdienol (Figs. 7-8), and before adult *I. paraconfusus* and *D. ponderosae* can convert *alpha*-pinene into *cis*- and *trans*-verbenol and myrtenol (Figs. 1-6) may explain why immature *I. paraconfusus* were unattractive to other beetles when introduced into new hosts (Wood and Vité 1961; Vité and Gara 1962; Borden 1967). It also may explain why immature female *D. frontalis* (Bridges 1982) and *D. brevicomis* (Byers 1983) were less capable of converting *alpha*-pinene to *trans*-verbenol than older females. Byers (1983) also found that immature male *I. paraconfusus* were not able to produce ipsenol and ipsdienol, although he reported that immature beetles were capable of producing normal amounts of *cis*- and *trans*-verbenol and myrtenol from *alpha*-pinene.

Figures 7-8. Quantities of ipsenol (Fig. 7), and ipsdienol (Fig. 8), extracted in Exp. XI from individual male *I. paraconfusus* of various posteclosion ages which were fed on bolts of ponderosa pine for 24 h. Numbers of beetles used for each treatment were as follows. Axenically-reared: 8 days-7, 24 days-10, 32 days-12. Wild: 1 day-9, 5 days-9, 15 days-13, 30 days-8.

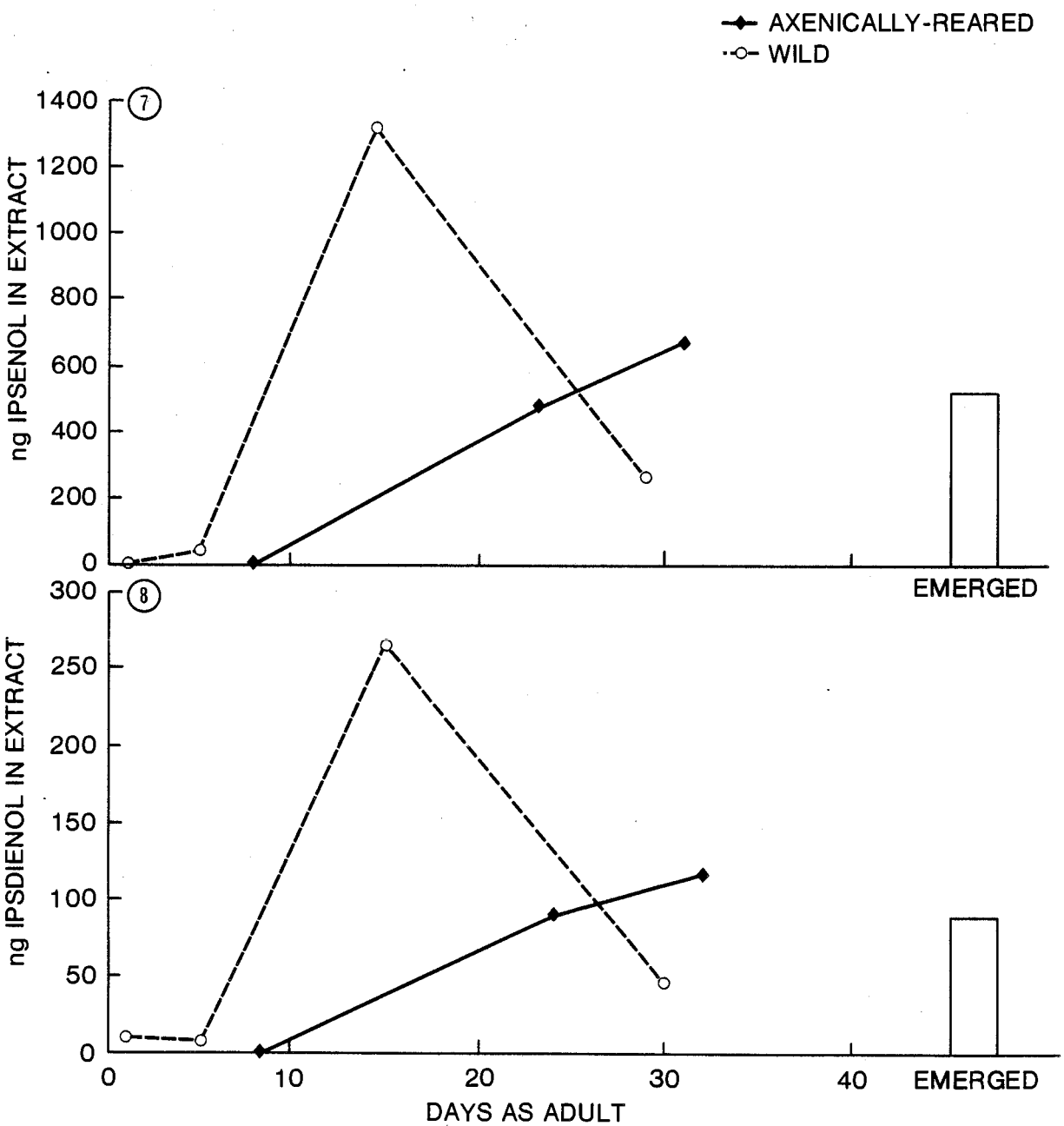

AXENICALLY-REARED
WILD
7
ng IPSENOL IN EXTRACT
1400
1200
1000
800
600
400
200
0
EMERGED
8
ng IPSDIENOL IN EXTRACT
300
250
200
150
100
50
0
0
10
20
30
40
EMERGED
DAYS AS ADULT

Table 3. Oxygenated monoterpene production in axenically-reared, streptomycin-fed and wild *Ips paraconfusus* males.

Exp. no.	Treatment	No. beetles	Mean amount of volatiles in abdominal extract (ng/beetle)[a]		
			ipsdienol	ipsenol	
XII	Wild/emerged/air	9	<5 a	<5 a	
	Wild/emerged/*P. ponderosa*	9	89 b	510 b	
	Wild/mature/air	10	<5 a	<5 a	
	Wild/mature/*P. ponderosa*	13	264 b	1320 b	
	Axenically-reared/mature/air	10	<5 a	<5 a	
	Axenically-reared/mature/*P. ponderosa*	10	92 b	478 b	
			trans-verbenol	*cis*-verbenol	myrtenol
XIII	Wild/emerged/air	10	<5 a	<5 a	<5 a
	Wild/emerged/α-pinene and myrcene vapours	10	123 b	298 b	137 b
	Wild/mature/air	5	<5 a	<5 a	<5 a
	Wild/mature/α-pinene and myrcene vapours	5	159 bc	536 bc	226 bc
	Axenically-reared/mature/air	11	<5 a	<5 a	<5 a
	Axenically-reared/mature/α-pinene and myrcene vapours	12	336 c	1359 c	498 c
			trans-verbenol	*cis*-verbenol	myrtenol
XIV	Wild/air	10	<5 a	<5 a	<5 a
	Wild/α-pinene vapours	11	160 b	320 b	150 b
	Wild/streptomycin/air	9	<5 a	<5 a	<5 a
	Wild/streptomycin/α-pinene vapours	10	209 b	413 b	195 b

Table 3. continued

Exp. no.	Treatment	No. beetles	Mean amount of volatiles in abdominal extract (ng/beetle)[a]				
			trans-verbenol	*cis*-verbenol	myrtenol	ipsdienol	ipsenol
XV	Wild/air	12	<5 a	<5 a	<5 a	<5 a	<5 a
	Wild/*P. ponderosa*	6	8 a	12 a	7 a	151 b	765 b
	Wild/streptomycin/air	11	<5 a	<5 a	<5 a	<5 a	<5 a
	Wild/streptomycin/*P. ponderosa*	12	6 a	17 a	9 a	5 a	16 a
			ipsdienol	ipsenol			
XVI	Wild/*P. ponderosa*	8	281 a	1529 a			
	Wild/new streptomycin/air	8	<5 b	<5 b			
	Wild/new streptomycin/*P. ponderosa*	8	59 c	305 c			
	Wild/old streptomycin/air	8	<5 b	<5 b			
	Wild/old streptomycin/*P. ponderosa*	8	38 c	220 c			
			trans-verbenol	*cis*-verbenol	myrtenol	ipsdienol	ipsenol
XVII	Wild/air	10	<5 a	<5 a	<5 a	<5 a	<5 a
	Wild/*P. ponderosa*	11	9 b	40 b	21 b	195 b	1097 b
	Axenically-reared/air	6	<5 a	<5 a	<5 a	<5 a	<5 a
	Axenically-reared/*P. ponderosa*	11	13 b	29 b	15 c	107 b	548 b

Table 3. continued

Exp. no.	Treatment	No. beetles	Mean amount of volatiles in abdominal extract (ng/beetle)[a]				
			ipsdienol	ipsenol			
XVIII	Axenically-reared/*P. ponderosa*	10	110 a	601 a			
	Axenically-reared/recontaminated/ *P. ponderosa*	6	52 a	302 a			
	Axenically-reared/held/ *P. ponderosa*	11	68 a	376 a			
			ipsdienol	ipsenol			
XIX	Axenically-reared/ *P. ponderosa*	10	110 a	601 a			
	Axenically-reared/streptomycin/ *P. ponderosa*	7	10 b	45 c			
	Axenically-reared/streptomycin/ recontaminated/*P. ponderosa*	6	13 b	77 bc			
	Axenically-reared/streptomycin/ held/*P. ponderosa*	6	22 b	128 b			
			trans-verbenol	*cis*-verbenol	myrtenol	ipsdienol	ipsenol
XXI	Wild/air	10	<5 a	<5 a	<5 a	<5 a	<5 a
	Wild/myrcene vapours	10	22 b	7 a	17 a	10 a	29 a
	Wild/α-pinene and myrcene vapours	8	123 c	298 b	137 b	<5 a	<5 a
	Wild/*P. ponderosa*	10	11 ab	9 a	19 a	54 b	299 b

Table 3. continued

Exp. no.	Treatment	No. beetles	Mean amount of volatiles in abdominal extract (ng/beetle)[a]	
			ipsdienol	ipsenol
XXII	Wild/myrcene vapours	10	<5 a	11 a
	Wild/cellulose-fed/myrcene vapours	11	6 a	37 a

[a]Data analysed using the Kruskal-Wallis test followed by a non-parametric multiple comparisons test (Conover 1980, p. 231), $P < 0.05$. Means within a column for each experiment followed by the same letter are not significantly different.

Polysubstrate monooxygenases, which have been implicated in terpene alcohol pheromone production in bark beetles as a by-product of monoterpene detoxication (White *et al.* 1980), generally show higher activity levels in more active life stages of insects (Brattsten 1979). Thus the increase in levels of terpene alcohol production in *D. ponderosae* and *I. paraconfusus* of 3- to 4-weeks posteclosion may represent an increase in polysubstrate monooxygenase activity in insects which are ready to emerge and find new hosts. Alternatively, the increase in levels of terpene alcohol production may be due to an increase in population levels of symbiotic microorganisms which produce these compounds.

Microbial Involvement in *alpha*-pinene Vapour Oxidation

Mature, axenically-reared, female (Table 1, Exp. V) and male (Table 4, Exp. XXIII) *D. ponderosae* produced significantly more *trans*- and *cis*-verbenol and myrtenol upon exposure to *alpha*-pinene vapours than wild, emerged beetles. In addition, streptomycin-fed *D. ponderosae* females produced significantly more of these terpene alcohols than wild, emerged beetles in one experiment (Table 1, Exp. VII), while in a second there was no significant difference (Table 1, Exp. VI). Streptomycin-fed male *D. ponderosae* also produced significantly more of these terpene alcohols than wild, emerged males (Table 4, Exp. XXIII). Similarly, mature, axenically-reared, male *I. paraconfusus* also produced significantly more *trans*- and *cis*-verbenol and myrtenol upon exposure to *alpha*-pinene vapours than wild, emerged beetles (Table 3, Exp. XIII). Mature, axenically-reared, female *I. paraconfusus* produced these terpene alcohols at levels which were not significantly different from those produced by wild emerged females (Table 5, Exp. XXV). Streptomycin-fed female (Table 3, Exp. XIII) and male (Table 5, Exp. XXV) *I. paraconfusus* also produced these terpene alcohols at levels which were not significantly different than wild, emerged beetles.

Evidently both male and female *D. ponderosae* and *I. paraconfusus* which have had their natural levels of symbiotic microorganisms reduced through axenic-rearing or streptomycin-feeding are capable of converting *alpha*-pinene vapours into the terpene alcohols *trans*- and *cis*-verbenol and

Table 4. Oxygenated monoterpene production in axenically-reared, streptomycin-fed and wild *Dendroctonus ponderosae* males.

Exp. no.	Treatment	No. beetles	Mean amount of volatiles in abdominal extract (ng/beetle)[a]			
			trans-verbenol	*cis*-verbenol	myrtenol	
XXIII	Wild/air	7	<5 a	<5 a	<5 a	
	Wild/α-pinene vapours	7	169 b	98 b	91 b	
	Wild/streptomycin/air	7	<5 a	<5 a	<5 a	
	Wild/streptomycin/α-pinene vapours	11	1534 c	482 c	180 c	
	Axenically-reared/air	9	<5 a	<5 a	<5 a	
	Axenically-reared/α-pinene vapours	10	1735 c	571 c	207 c	
			ipsdienol	Z-myrcenol	*E*-myrcenol	unknown (RT 14.74)
XXIV	Wild/air	10	<10 a	<5 a	<5 a	<5 a
	Wild/myrcene vapours	11	1906 b	156 b	1341 b	167 b
	Wild/streptomycin/air	10	<10 a	<5 a	<5 a	<5 a
	Wild/streptomycin/myrcene vapours	10	1867 b	57 c	277 c	36 c
	Axenically-reared/air	10	<10 a	<5 a	<5 a	<5 a
	Axenically-reared/myrcene vapours	12	3122 b	110 bc	222 c	62 c

[a]Data analysed using the Kruskal-Wallis test followed by a non-parametric multiple comparisons test (Conover 1980, p. 231), $P < 0.05$. Means within a column for each experiment followed by the same letter are not significantly different.

Table 5. Oxygenated monoterpene production in axenically-reared, streptomycin-fed and wild *Ips paraconfusus* females.

Exp. no.	Treatment	No. beetles	Mean amount of volatiles in abdominal extract (ng/beetle)[a]		
			trans-verbenol	*cis*-verbenol	myrtenol
XXV	Wild/air	10	<5 a	<5 a	<5 a
	Wild/α-pinene vapours	10	602 b	152 b	316 b
	Axenically-reared/air	10	<5 a	<5 a	<5 a
	Axenically-reared/α-pinene vapours	11	211 b	79 b	322 b
	Wild/air	10	<5 a	<5 a	<5 a
	Wild/α-pinene vapours	10	307 b	129 b	301 b

[a]Data analysed using the Kruskal-Wallis test followed by a non-parametric multiple comparisons test (Conover 1980, p. 231), $P < 0.05$. Means within a column for each experiment followed by the same letter are not significantly different.

myrtenol at levels equal to or significantly greater than those found in wild control beetles. These results confirm that readily culturable microorganisms are not solely responsible for the production of these terpene alcohols in these beetles. The fact that terpene alcohol levels were significantly higher in microbe-reduced than wild beetles in several experiments indicates that certain microorganisms may metabolize the available *alpha*-pinene precursor into other products, so that the *alpha*-pinene is unavailable to the wild beetles, or that they may utilize the terpene alcohols as substrates, metabolizing them into other products. In these ways the microorganisms present in wild bark beetles could regulate the levels of the aggregation pheromones *trans*-verbenol in *D. ponderosae* and *cis*-verbenol in *I. paraconfusus*, preventing them from reaching excessive levels and disposing of them once the aggregation phase is complete. Alternatively it is possible that reducing the levels of microorganisms in the beetles through axenic-rearing or streptomycin-feeding may remove microorganisms which are mildly pathogenic to the beetles, and that the resultant healthier beetles simply produce higher levels of terpene alcohols. Results which support this hypothesis were reported by Gueldner *et al.* (1977) who found that boll weevils, *Anthonomus grandis* Boheman, which were artifically contaminated with *Streptococcus* sp., *Micrococcus varians* Migula, and *Enterobacter aerogenes* Hormaeche and Edwards, 3 bacteria which were isolated from apparently normal, insectary-reared weevils, showed significantly reduced levels of pheromone production.

Two Sites and Two Methods of *alpha*-pinene Oxidation

While axenically-reared and streptomycin-fed *D. ponderosae* which were exposed to *alpha*-pinene vapours produced *trans*- and *cis*-verbenol and myrtenol at levels which were at least as high as those produced by wild beetles, the production of *trans*-verbenol from *alpha*-pinene obtained through feeding was virtually eliminated in axenically-reared beetles (Table 1, Exp. VIII). This result suggests that there are two production systems for this pheromone, one by the beetle's own enzymes and one by symbiotic microorganisms. Since axenically-reared *D. ponderosae* are unable to convert *alpha*-pinene obtained through the alimentary system into *trans*-verbenol, it appears likely that the low

levels of *trans*-verbenol present in extracts of wild beetles which have been fed on lodgepole pine are the result of the conversion of *alpha*-pinene by microorganisms present in the gut. The high levels of *trans*-verbenol, as well as *cis*-verbenol and myrtenol, found in extracts of axenically-reared and streptomycin-fed *D. ponderosae* which had been exposed to *alpha*-pinene vapours would then be due to the beetle's own enzymes oxidizing *alpha*-pinene vapours which had entered through the spiracles.

Since axenically-reared *D. ponderosae* produce *trans*-verbenol primarily from inhaled *alpha*-pinene (Table 1, Exp. V), not from ingested *alpha*-pinene (Table 1, Exp. VIII), it is likely that the conversion does not occur in the gut as has previously been suggested for other scolytids (Pitman *et al.* 1965; Zenther-Møller and Rudinsky 1967). *trans*-Verbenol is probably produced in the hemolymph; it would then have to be transported into the hindgut, probably through the Malpighian tubules, before it left the beetle in the frass. This explanation is consistent with Hughes' (1973) finding of *trans*-verbenol and other monoterpene oxidation products in the hemolymph of *D. ponderosae*, which he interpreted as indicating that the metabolism of terpenes occurs outside the gut.

Wild female *D. ponderosae* which had fed in lodgepole pine bolts for 24 h contained significant amounts of verbenone while axenically-reared individuals contained only trace quantities (Table 1, Exp. VIII). However, neither wild nor axenically-reared female *D. ponderosae* which had been exposed to *alpha*-pinene vapours contained quantifiable levels of verbenone (Table 1, Exp. V), even when the extracts were concentrated to approximately 5 µl over a stream of nitrogen before GLC analysis. It has previously been shown that *alpha*-pinene vapour exposure also does not result in verbenone production in *D. frontalis* (Renwick *et al.* 1973), or *D. brevicomis* (Byers 1983). This result suggests that the production of verbenone in female *D. ponderosae* is solely due to the conversion of beetle-ingested precursor by gut microorganisms. This hypothesis has interesting implications for the chemical ecology of *D. ponderosae* since it has been shown that verbenone functions as an antiaggregation pheromone in this species (Ryker and Yandell 1983; Libbey *et al.* 1985; Borden *et al.* 1987). The possible role of symbiotic microorganisms in the production of verbenone is discussed further in Section IV.

Microbial Involvement in Myrcene Oxidation

Although *cis*- and *trans*-verbenol and myrtenol are produced at least as efficiently by microbe-reduced *D. ponderosae* as they are by wild beetles with their full complement of microorganisms, there is evidence that the production of certain oxidation products of monoterpenes are produced less efficiently by microbe-reduced beetles. Both axenic-rearing and streptomycin-feeding significantly reduced the levels of *E*-myrcenol and an unknown product found in male *D. ponderosae* exposed to myrcene vapours. The levels of *Z*-myrcenol were significantly reduced by streptomycin-feeding, while the reduction caused by axenic-rearing was not significant (Table 4, Exp. XXIV). These results indicate that microorganisms are responsible for a significant portion of the production of certain conversion products of monoterpenes. However, streptomycin-fed female *D. ponderosae* which were exposed to myrcene vapours for 24 h produced *Z*- and *E*-myrcenol and a third unknown product at levels which were not significantly different from levels produced by wild, control beetles (Table 1, Exp. IX).

Axenically-reared or streptomycin-fed male *D. ponderosae* which were subsequently exposed to myrcene vapours contained ipsdienol at levels which were not significantly different from those found in wild, emerged beetles (Table 4, Exp. XXIV). None of the females tested produced detectable levels of ipsdienol (Table 1, Exp. IX).

Two Pheromone Production Systems

The hypothesis that both the beetle and its symbiotic microorganisms can produce the pheromones *cis*- and *trans*-verbenol, as well as other terpene alcohols, is consistent with the work of Chararas *et al.* (1980) who reported that the frass of either *I. sexdentatus*, *I. typographus* or *I. acuminatus* which had been fed a wide spectrum antibiotic showed reduced attractiveness to other beetles of the same species. Chararas *et al.* (1980) also reported that the frass of antibiotic-fed beetles

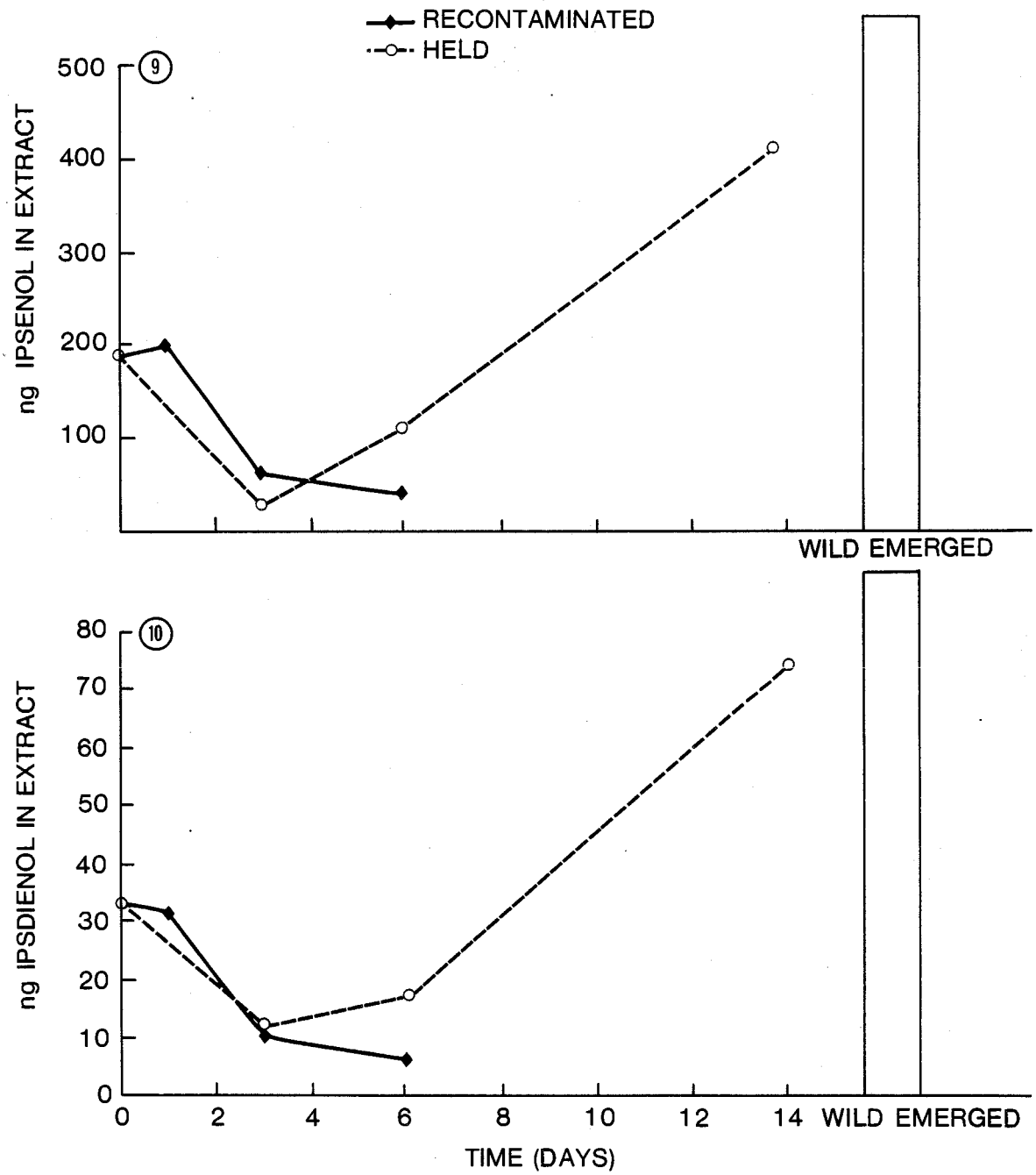
had more of the monoterpenes *alpha*-pinene, ß-pinene and Δ_3-carene and less of the conversion products of these compounds than normal beetles. This finding was interpreted to indicate that microbial conversion was responsible for a portion of total pheromone production. However, Chararas *et al.* (1980) did not present any data to support these claims, and details on the methodology used were very sketchy.

The existence of two pheromone production systems, beetle and microbial, is also consistent with what is known about polysubstrate monooxygenases (PSMO's). PSMO's, also known as mixed function oxidases (mfo's), are enzymes which catalyze a number of oxidation reactions that render lipophilic compounds, such as monoterpenes, hydrophilic, so that they can more easily be excreted. It is likely that the most important enzymes involved in the metabolism of foreign substances, such as monoterpenes, are the PSMO's (Sturgeon and Robertson 1985). Since PSMO's are believed to be present in all aerobic organisms (Brattsten 1979), and since bark beetles and their microorganisms are constantly being exposed to monoterpenes which are toxic to both the insects (Smith 1965; Reid and Gates 1970; Coyne and Lott 1976; Raffa and Berryman 1983b) and their symbiotic microorganisms (Cobb *et al.* 1968; Shrimpton and Whitney 1968; DeGroot 1972; Raffa *et al.* 1985), it is logical that both would use PSMO's to oxidize monoterpenes. The involvement of PSMO's in terpene alcohol pheromone production in *D. ponderosae* is investigated in Section VI.

Effect of Streptomycin on *I. paraconfusus*

It is evident that the effect of streptomycin-feeding on the conversion of myrcene by male *I. paraconfusus* varied considerably between experiments. In Exp. XV (Table 3) streptomycin-feeding resulted in virtual elimination of the production of ipsdienol and ipsenol, which is in agreement with the results obtained by Byers and Wood (1981). However, in a subsequent experiment streptomycin-feeding caused a significant reduction in ipsdienol and ipsenol production, but production was certainly not eliminated (Figs. 9-10). Since the streptomycin that was used in these two

Figures 9-10. Quantities of ipsenol (Fig. 9), and ipsdienol (Fig. 10), extracted from streptomycin-fed, male *I. paraconfusus* which had been recontaminated for various time periods in Exp. XX with their normal association of microorganisms or held in a ground phloem diet. Recontamination started on day zero and continued until the insects were fed on bolts of ponderosa pine. Numbers of beetles used for each treatment as follows. Recontaminated: 1 day-12, 3 days-6, 6 days-3. Held: 3 day-9, 6 days-6, 14 days-9.


RECONTAMINATED
HELD
9
500
400
300
200
100
ng IPSENOL IN EXTRACT
WILD EMERGED
10
80
70
60
50
40
30
20
10
0
ng IPSDIENOL IN EXTRACT
0
2
4
6
8
10
12
14
WILD EMERGED
TIME (DAYS)

experiments was from the same batch, newly-purchased streptomycin was used to determine whether the difference between these results was due to the streptomycin losing some of its activity during the several months between the experiments. However, it was found that the levels of ipsenol and ipsdienol found in beetles fed the old or the new streptomycin were not significantly different (Table 3, Exp. XVI).

The *I. paraconfusus* colony used for all of my experiments was initiated using beetles which had been collected from the Sierra Nevada by D. L. Wood[2], and thus these beetles should have been very similar to the beetles used by Byers and Wood (1981). I had reared these beetles through several generations at Simon Fraser University by the time Exp. XVI (Table 3) was done, and it appears that this process resulted in a shift of the population of insects or their symbiotic microorganisms towards some characteristic which promoted resistance to the effects of streptomycin.

Streptomycin-feeding vs. Axenic-rearing

There was only one discrepancy between the results obtained in experiments using axenically-reared beetles and experiments using streptomycin-fed beetles. The levels of ipsenol and ipsdienol found in axenically-reared male *I. paraconfusus* were not significantly different from the levels found in wild beetles (Table 3, Exps. XII, XVII). However, streptomycin-feeding of male *I. paraconfusus* virtually eliminated the production of these terpene alcohols (Table 3, Exp. XV), as had previously been shown by Byers and Wood (1981). This anomaly may indicate that the reduction in ipsenol and ipsdienol production due to streptomycin-feeding is not due to the elimination of certain microorganisms but is actually caused by toxic effects of this antibiotic on insect cells. As suggested by Byers and Wood (1981), this hypothesis appears to be somewhat unlikely since the production of *cis*- and *trans*-verbenol and myrtenol is not affected by streptomycin-feeding. Alternatively it is possible that the conversion of myrcene to ipsenol and ipsdienol is performed by symbiotic microorganisms which are eliminated by streptomycin-feeding but are not eliminated by axenic-rearing. These microorganisms would have to be trans-ovarially transmitted to avoid elimination through axenic-rearing, and would also

probably have to be obligate symbionts to have avoided detection during the culturing of axenically-reared beetles. Some evidence for trans-ovarial transmission of bacteria in bark beetles has previously been reported (Buchner 1965).

Recontamination Experiments

Once ipsenol and ipsdienol production had been studied in wild male *I. paraconfusus*, and compared to production by microbe-reduced beetles, recontamination experiments were performed with axenically-reared and streptomycin-fed male *I. paraconfusus* (Table 3, Exps. XVIII, XIX; Figs. 9, 10). Attempts to recontaminate microbe-reduced *I. paraconfusus* with its normal association of microorganisms were intended as a means of "reconstituting" wild beetles.

Although axenically-reared beetles, which were subsequently recontaminated for 4 days and then fed for 24 h on ponderosa pine, produced ipsenol and ipsdienol at levels which were somewhat lower than those found in axenically-reared beetles the difference was not significant (Table 3, Exp. XVIII). A similar reduction in ipsenol and ipsdienol levels in axenically-reared beetles which were held for 4 days in a non-sterilized diet composed of ground pine phloem and distilled water was also not signficant. Axenically-reared beetles, which were subsequently fed for 4 days on a diet containing streptomycin, produced ipsenol and ipsdienol at levels which were significantly lower than for axenics (Table 3, Exp. XIX). These levels increased slightly in beetles which were then recontaminated or held.

Beetles which were fed on a diet containing streptomycin contained ipsenol and ipsdienol at levels which were lower than those found in wild control beetles (Figs. 9, 10), although the reduction in the production of these terpene alcohols was not as complete as in Exp. XV (Table 3). When streptomycin-fed beetles were subsequently recontaminated the production of ipsenol and ipsdienol decreased further, with the greatest decrease found in the beetles which were recontaminated for the longest time (Figs. 9, 10). When streptomycin-fed beetles were subsequently held in a diet composed of ground pine phloem and distilled water, the production of ipsenol and ipsdienol dropped initially. However, beetles which were held for the longest time (14 days) showed increased levels of ipsenol

and ipsdienol which were nearly as high as those found in wild control beetles (Figs. 9, 10). Pheromone production could not be measured in beetles which were recontaminated for 14 days due to the high levels of mortality in this group.

These recontamination experiments indicated that recontaminating microbe-reduced male *I. paraconfusus* did not re-establish normal levels of production of ipsenol and ipsdienol. This result could be interpreted to mean that microorganisms are not significantly involved in the production of these 2 terpene alcohols. However, it would not be wise to attribute too much significance to the results obtained in these experiments. Microbe-reduced beetles which are exposed to their normal complement of microorganisms would likely be more susceptible to the pathogenic effects of certain microorganisms without the protective effects of an established gut micro-fauna. Thus, any re-establishment of pheromone production could be over-shadowed by pathogenic effects. In addition it is not known how long streptomycin would remain in the insects, and it may not be possible to re-establish the normal association of microorganisms until the residual effect of streptomycin has dissipated. Culture checks would be needed to verify that microbes had been successfully re-established.

Vapours vs Feeding - "Contact" and "Frass" Pheromones

In most bark beetles myrcene is converted to ipsdienol and ipsenol more efficiently if the exposure is through feeding, while *alpha*-pinene is converted to *cis*- and *trans*-verbenol and myrtenol more efficiently through exposure to vapours (Vité *et al.* 1972). Exp. XXI (Table 3) confirms that this trend is also true for *I. paraconfusus.* When males were exposed to *alpha*-pinene and myrcene vapours simultaneously, conversion to *trans*- and *cis*-verbenol and myrtenol, which are oxidation products of *alpha*-pinene, was much more efficient than conversion of myrcene to ipsenol and ipsdienol. When beetles were exposed to myrcene vapours in the absence of *alpha*-pinene vapours slightly more ipsenol and ipsdienol were produced, but the effect was not significant. However, when beetles were exposed by feeding to the myrcene and *alpha*-pinene present in ponderosa pine bolts the conversion

of myrcene to ipsenol and ipsdienol was much more efficient than with vapour exposure, while the conversion of *alpha*-pinene was significantly less efficient (Table 3, Exp. XXI). As a result, experiments with *I. paraconfusus* generally used vapour exposure to study the conversion of *alpha*-pinene and feeding to study the conversion of myrcene.

Hughes and Renwick (1977a) proposed that the production of ipsenol and ipsdienol by newly emerged *I. paraconfusus* is prevented by neural inhibition that is removed by distension of the gut during natural feeding. Hughes and Renwick (1977a), working with *I. paraconfusus*, and Harring (1978), working with *Pityokteines curvidens* Germ. and *Pityokteines spinidens* Reit., were able to induce the production of ipsenol and ipsdienol by distending the guts of insects with an injection of air before exposing them to myrcene vapours. This approach was criticised by Byers (1981) who proposed that the distension of the gut with injected air would enhance the diffusion of myrcene vapours in the gut, thus promoting pheromone synthesis artificially by increasing precursor diffusion to the site of synthesis. My approach to this problem was to distend the guts of *I. paraconfusus* by feeding them with powdered cellulose, and then expose the beetles to myrcene vapours. Gut distension with powdered cellulose resulted in a 3.5-fold increase in ipsenol content and a slight increase in ipsdienol content compared to controls (Table 3, Exp XXII). However, these differences were not found to be significant due to the large numbers of zeros in the data.

D. ponderosae also convert *alpha*-pinene more efficiently through vapour exposure than through feeding (Table 1). Female beetles which were fed on lodgepole pine phloem produced very small quantities of *cis*- and *trans*-verbenol and myrtenol (Table 1, Exps. VII), while beetles which were exposed to *alpha*-pinene vapours produced much larger quantities of these terpene alcohols (Table 1, Exp. VII). However, in contrast to *I. paraconfusus*, male *D. ponderosae* converted myrcene more efficiently through vapour exposure than feeding. Male beetles which were exposed to myrcene vapours produced large quantities of ipsdienol, as well as *Z*- and *E*-myrcenol (Table 4, Exp. XXIV), while males which were fed on bolts of lodgepole pine did not produce any conversion products of myrcene in quantifiable amounts.

Since myrcene is generally converted more efficiently through feeding and *alpha*-pinene is

converted more efficiently through vapour exposure ipsdienol has been termed a "frass" pheromone while *cis*- and *trans*-verbenol are termed "contact" pheromones (Vité *et al.* 1972). My data, as well as those presented by Byers (1982) and Hughes (1974), indicate that in *Dendroctonus* species myrcene is actually oxidized much more efficiently with vapour exposure than with feeding. Apparently this oxidation is not performed in the same way as in *Ips* species. This conclusion is in agreement with the hypothesis presented by Vité *et al.* (1972) suggesting that aggressive bark beetles, such as *D. ponderosae*, begin oxidizing monoterpenes upon initial contact with a new host, while less aggressive species, such as many *Ips* species, depend on feeding for the conversion of monoterpenes. Aggressive bark beetle species, which attack healthy, resinous trees, must use rapid aggregation in mass-attacking their hosts to overcome host tree resistance. If aggressive species relied on frass pheromones for aggregation then pioneer individuals would generally be killed by the defences of the host tree before feeding and defecation produced the aggregation pheromones. It is therefore logical that aggressive species would depend on contact pheromones, since they can be produced much more quickly than frass pheromones, which require feeding and defecation. Hughes (1973) reported that topical application of *alpha*-pinene to *D. ponderosae* resulted in substantial quantities of *trans*-verbenol appearing in the hindguts within 3 h, which was the shortest time period tested. However, less aggressive bark beetle species generally attack unhealthy trees with weakened or non-existent defences. Therefore, these beetles have the option of postponing the mass attack of a host until pioneer individuals have established, through feeding and defecation, that the host phloem is of suitable quality.

Summary

Since the reduction in levels of microorganisms in *I. paraconfusus* through antibiotic-feeding reduces the production of ipsenol and ipsdienol, while axenic-rearing or antibiotic-feeding of *D. ponderosae* does not reduce the production of terpene alcohols, it appears that *I. paraconfusus* may be more dependent on its symbiotic microorganisms than *D. ponderosae*. *D. ponderosae* is a very

aggressive bark beetle which attacks healthy, resinous trees. It requires a very active enzyme system to rapidly detoxify the monoterpenes encountered in the hosts, as well as to produce large quantities of aggregation pheromones rapidly in order to attract large numbers of individuals which can overcome host resistance. However, *I. paraconfusus* generally breeds in fallen or cut trees (Wood 1982), which have reduced levels of monoterpene-rich resin. These beetles do not require as efficient an enzyme system because the host environment is less toxic than that for *D. ponderosae*, and also because a less rapid and efficient production of aggregation pheromones is adequate in overcomong host resistance. Thus it is possible for *I. paraconfusus* to rely partially on microorganisms for the production of terpene alcohols, while *D. ponderosae* is apparently capable of performing this conversion without microorganisms.

Although the production of normal or elevated levels of pheromones and other terpene alcohols by axenically-reared *D. ponderosae* and *I. paraconfusus* does not rule out the possibility that symbiotic microorganisms are involved in the production of these compounds, it does indicate that readily culturable microorganisms are not required for this process. Since the reports in the literature which describe the production of coleopteran pheromones *in vitro* by their symbionts (Hoyt *et al.* 1971; Brand *et al.* 1975, 1976; Chararas *et al.* 1980) involve readily culturable microorganisms, my conclusion is that there is no direct evidence that symbiotic microorganisms are required for pheromone production in the Coleoptera, with the exception of the antibiotic-feeding studies conducted herein and by Byers and Wood (1981). My data add support to the assertion that certain microorganisms are capable of oxidizing monoterpenes, but establishes that *D. ponderosae* and, to a certain extent, *I. paraconfusus* are capable of producing large quantities of terpene alcohol pheromones in the absence of readily culturable microorganisms.

IV. INVOLVEMENT OF MICROORGANISMS IN PHEROMONE PRODUCTION

Introduction

The hypothesis that microorganisms are involved in the production or modification of bark beetle pheromones has been debated for over half a century. Person (1931) hypothesized that the western pine beetle, *D. brevicomis*, introduced a yeast into the inner bark of host trees which produced fermentation products responsible for secondary attraction. Callaham and Shifrine (1960) reported on a number of previously unpublished studies which indicated that yeast-inoculated phloem was attractive to various species of *Dendroctonus*.

More recently it has been confirmed that a variety of symbionts associated with bark beetles and other coleoptera are capable of producing the same compounds which are used as pheromones by their hosts. Bacteria isolated from female grass grub beetles, *Costelytra zealandica* (White), produced an unidentified compound which attracted male *C. zealandica* under field conditions (Hoyt *et al.* 1971). A *Serratia* species isolated from the bark beetle, *Phloeosinus armathus*, converted sabinene into terpinene-4-ol and *alpha*-terpineol, both of which were attractive to male and female *P. armathus* (Chararas *et al.* 1980). A strain of *B. cereus* which was isolated from the guts of *I. paraconfusus* converted *alpha*-pinene to the host's pheromone *cis*-verbenol (Brand *et al.* 1975). Brand *et al.* (1976) demonstrated that fungi from the mycangia of *D. frontalis* were capable of oxidizing *trans*-verbenol to the antiaggregation pheromone verbenone. The yeasts *Hansenula holstii* Wickerham and *Pichia pinus* (Holst) Phaff, also isolated from *D. frontalis*, produced metabolites which enhanced attraction of these beetles to a (1:1:12) mixture of frontalin: *trans*-verbenol: turpentine (Brand *et al.* 1977). French *et al.* (1984) reported that flying *S. multistriatus* were attracted to agar cultures of *Bacillus subtilis* (Ehrenberg) Cohn, *Bacillus pumilis* Meyer and Gottheil, and *Enterobacter cloacae* (Jordan) Hormaeche and Edwards, isolated from elm trees. Several species of yeasts isolated from *I. typographus* were capable of interconverting *cis*-verbenol and verbenone, both of which function as pheromones for their hosts, as well as *trans*-verbenol (Leufvén *et al.* 1984). Other more general examples of fungi and bacteria

which are capable of oxidizing *alpha*-pinene and other monoterpenes are found in Bhattacharyya *et al.* (1960), Prema and Bhattacharyya (1962), Shukla *et al.* (1968), Fonken and Johnson (1972), and Kieslich (1976).

My objective was to determine whether microorganisms associated with *D. ponderosae* were also capable of the production or interconversion of pheromones of the host. I choose to conduct my study on 2 yeasts frequently associated with *D. ponderosae*, *Hansenula capsulata* Wickerham and *P. pinus*, since yeasts were originally implicated in the production of attractants for bark beetles (Person 1931; Callaham and Shifrine 1960), and many of the microorganisms which have been found to produce pheromones of other bark beetles have been yeasts (Brand *et al.* 1977; Leufvén *et al.* 1984).

Microorganisms

Cultures of two species of yeasts which are frequently associated with *D. ponderosae* were obtained on Sabouraud dextrose agar (SDA) (Difco Laboratories, Detroit, MI) from H. S. Whitney[4], and were maintained at S.F.U. on SDA. These 2 species were identified by L. J. Wickerham[9] as *Pichia pinus*, and *Hansenula capsulata.*

Chemical Conversion Experiments

Conversion experiments were conducted using a procedure similar to that employed by Leufvén *et al.* (1984). *alpha*-Pinene, *trans*- and *cis*-verbenol, and verbenone were dissolved individually at 3 mg/ml in 95% ethanol; 250 µl of either ethanolic solution was added to 250 ml Erlenmeyer flasks containing 50 ml of Sabouraud dextrose broth (Difco Laboratories, Detroit, MI) which had just been inoculated by aseptically transferring a small amount of cells from a fresh SDA culture of one of the yeasts with a sterile inoculating loop. After 24 h at 21-23^{0}C with slight shaking, another 250 µl of the ethanolic solution was added, yielding a final ethanol concentration in the medium of 1% (v/v). All of the flasks which contained broth which had been inoculated with one of the yeasts appeared cloudy after 24 h, indicating growth of the yeasts. After a further 24 h of incubation, 5 ml of the medium was extracted 3 times with 1 ml each of distilled pentane containing 2 µl of 2-octanol/ml. Each treatment for each yeast was replicated 3 times, as were control treatments in which a sterile inoculating loop was dipped into the medium. The extracts were analysed by gas chromatography as outlined in Section II.

The *trans*-verbenol used in this study, which was 75%(-):25%(+), and was contaminated with approximately 12% *cis*-verbenol and 0.8% verbenone, was obtained from Phero Tech, Vancouver, B.C. Racemic *cis*-verbenol, which was contaminated with approximately 14% *trans*-verbenol, was obtained from Borregaard, A. S., Sarpsborg, Norway, and racemic *alpha*-pinene (>99% pure)was

obtained from Aldrich Chemical Co., Milwaukee, WI. Racemic verbenone (97% pure, as determined by GLC) was obtained from Indukern, Barcelona, Spain.

Results

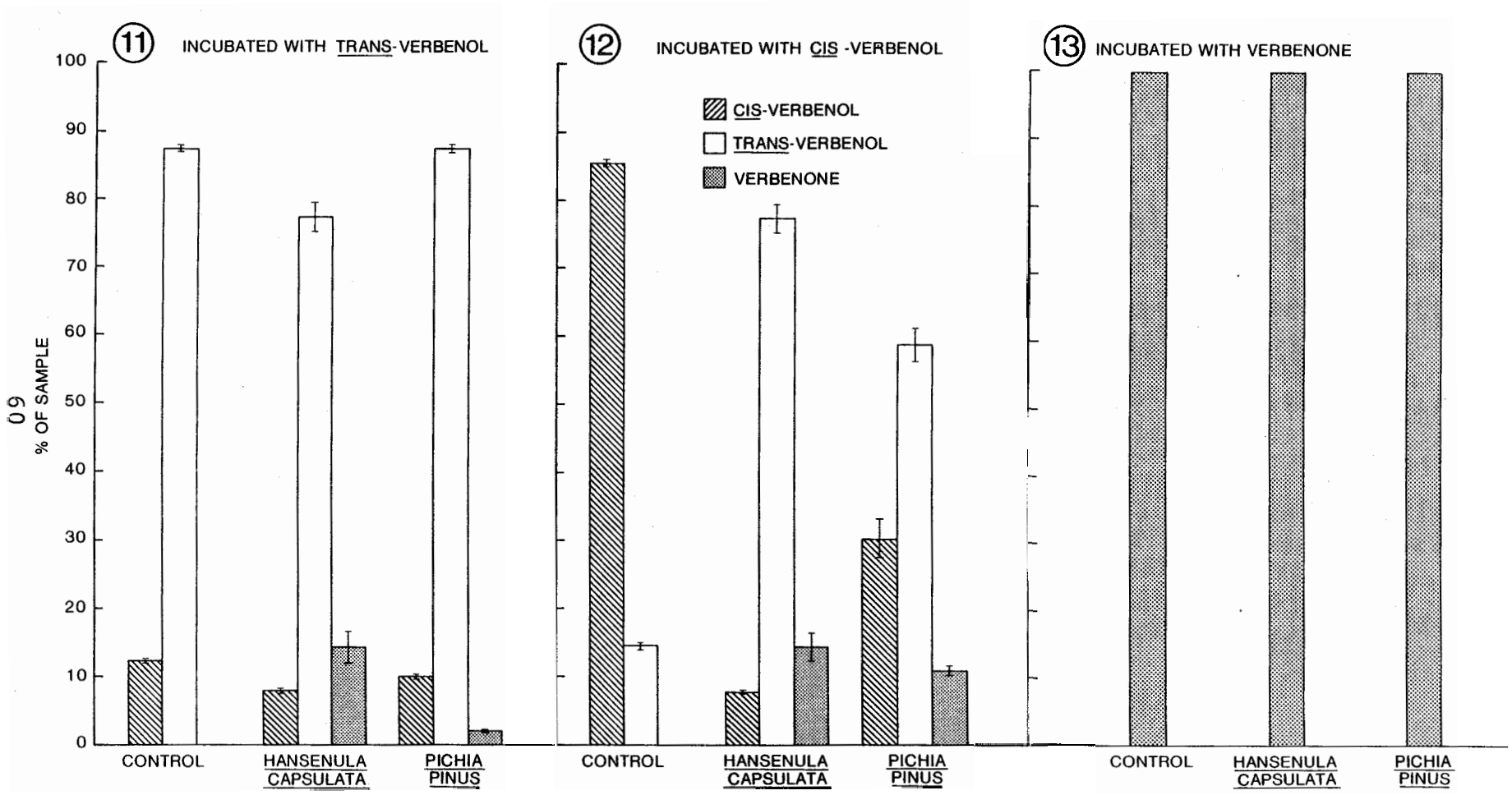
Due to difficulties with the extraction procedure the 2-octanol internal standard was not used to calculate absolute amounts of the detected compounds. Instead the relative proportions of the gas chromatographic peak areas were compared as in Leufvén *et al.* (1984). The sum of the gas chromatographic peak areas of *trans*- and *cis*-verbenol and verbenone was approximately the same after all incubations, indicating that the compounds were primarily interconverted, and not metabolized into other compounds.

Incubation of either yeast with *trans*-verbenol resulted in accumulations of verbenone, while the *trans*-verbenol control flasks, which were incubated without yeasts, contained no verbenone (Fig. 11). Incubation of *H. capsulata* with *trans*-verbenol caused an accumulation of 14.5±2.1% verbenone, while incubation with *P. pinus* caused an accumulation of 2.2±0.1% verbenone (Fig. 11).

Incubation of either yeast with *cis*-verbenol resulted in accumulations of *trans*-verbenol and verbenone, while the *cis*-verbenol control flasks, which were incubated without yeasts, contained both verbenols at the original levels (Fig. 12).

Extracts of *alpha*-pinene incubations did not contain detectable levels of *alpha*-pinene or terpene alcohols, even when the medium was extracted immediately following the addition of *alpha*-pinene. Thus the *alpha*-pinene was apparently bound very rapidly to an unidentified constituent of the medium. Neither of the yeasts produced *trans*- or *cis*-verbenol from verbenone (Fig. 13).

Figures 11-13. Mean percent content (±SE) of *trans*- and *cis*-verbenol and verbenone in extracts of cultures of *H. capsulata* or *P. pinus* which had been incubated with *trans*-verbenol (Fig. 11), *cis*-verbenol (Fig. 12), or verbenone (Fig. 13) for 48 h. N=3 for each treatment in each experiment.


11
INCUBATED WITH TRANS-VERBENOL
12
INCUBATED WITH CIS -VERBENOL
13
INCUBATED WITH VERBENONE
CIS-VERBENOL
TRANS-VERBENOL
VERBENONE
% OF SAMPLE
100
90
80
70
60
50
40
30
20
10
0
CONTROL
HANSENULA CAPSULATA
PICHIA PINUS

Discussion

The aggregation of *D. ponderosae* on host trees is partially due to the beetle-produced terpene alcohol *trans*-verbenol, in combination with host tree monoterpenes (Pitman *et al.* 1968), although low concentrations of the male-produced, multifunctional pheromones, *exo*-brevicomin and frontalin may also be involved (Rudinsky *et al.* 1974a; McKnight 1979; Conn *et al.* 1983; Borden *et al.* 1983, 1987; Chatelain and Schenk 1984). Once a certain attack density is reached the attack switches to adjacent trees (McCambridge 1967; Geiszler and Gara 1978; Geiszler *et al.* 1980), preventing the increased competition within hosts and reduced brood survival which occur at overly high attack densities (Reid 1963). This switching appears to be due to a number of factors, including a decrease in resin flow and *trans*-verbenol production (Renwick and Vité 1970), and the production of antiaggregation pheromones (Rudinsky *et al.* 1974a). Although several compounds which are produced by *D. ponderosae* have proven to be inhibitory to these beetles in laboratory and field tests (Ryker and Libbey 1982; Ryker and Rudinsky 1982; Libbey *et al.* 1985; Section VII), leading to speculation that they may act as antiaggregation pheromones, the antiaggregative activity of verbenone (Ryker and Yandell 1983) is generally thought to be the most significant.

In lodgepole pine trees attacked by *D. ponderosae* aggregation generally peaks on approximately the second day of attack, and then declines to zero within 4-7 days of the initiation of attack (Raffa and Berryman 1983a). The conversion of *trans*-verbenol into verbenone (Fig. 11), particularly by *H. capsulata*, suggests that this termination of aggregation on trees attacked by *D. ponderosae* is partially the result of verbenone production by yeasts introduced into the trees by the attacking beetles. It is my hypothesis that the yeasts which are introduced into the attacked trees by *D. ponderosae* require a few days to reach a population size in the galleries which is large enough to enable them to convert significant quantities of *trans*-verbenol, which has been produced in large quantities by the beetles for the first few days, into verbenone. The switching of the attack to adjacent trees (McCambridge 1967; Geiszler and Gara 1978; Geiszler *et al.* 1980) is then due largely to the production of verbenone by yeasts (Figs. 11, 12).

The hypothesis that fungi introduced into attacked trees by bark beetles are responsible for the termination of aggregation has been proposed for other species. Brand *et al.* (1976) hypothesized that fungi which are introduced into the galleries of *D. frontalis* may convert *trans*-verbenol, which is involved in the aggregation of this bark beetle (Renwick and Vité 1969; Payne *et al.* 1978), into verbenone, which can function as an antiaggregation pheromone (Rudinsky 1973; Rudinsky *et al.* 1974a). Also, Leufvén *et al.* (1984) hypothesized that yeasts introduced into the galleries of *I. typographus* may convert the aggregation pheromone *cis*-verbenol (Bakke *et al.* 1977) into verbenone, an antiaggregation pheromone (Bakke 1981).

The quantification of compounds produced by yeasts in broth culture is complicated by difficulties with the extraction technique (Leufvén *et al.* 1984). The recovery of pentane after extraction is very poor, often as low as 10%, probably due to extensive foaming. Also the partitioning of compounds between the water and pentane phases was so variable between extractions that it was difficult to measure the extraction efficiency for the different compounds and to establish correction factors. Therefore, the 2-octanol internal standard was not used to calculate absolute amounts of the compounds. However, tests indicated that the extraction efficiencies for *cis*- and *trans*-verbenol and verbenone within any one extraction were relatively similar; thus comparisons of the relative proportions of the gas chromatographic peak areas for different compounds within an extraction appeared to be justified. The only exception to this is *alpha*-pinene, which was not extractable with pentane. It may have been possible to extract the *alpha*-pinene from the broth medium with a different solvent, or combination of solvents. However, it appears that the *alpha*-pinene was bound in some way within the water phase, so it may not have been available to be metabolized by the yeasts. Therefore, additional research is required to establish the capacity of these yeasts to metabolize *alpha*-pinene.

Although under laboratory conditions many microorganisms are capable of producing or interconverting compounds which are used as pheromones by bark beetles (Bhattacharyya *et al.* 1960; Prema and Bhattacharyya 1962; Fonken and Johnson 1972), and some of these microorganisms are found in association with bark beetles (Brand *et al.* 1975, 1976, 1977; Chararas *et al.* 1980; Leufvén *et al.* 1984), there is little evidence to suggest that these conversions are of any significance to the

chemical ecology of bark beetles under natural conditions. There are, however, 4 pertinent observations in support of the hypothesis that the conversion of *trans*-verbenol into verbenone by yeasts associated with *D. ponderosae* is of genuine significance to the chemical ecology of this beetle:

Firstly, although certain microorganisms such as *B. cereus* (Brand *et al.* 1975), and *Aspergillus niger* van Tieghem (Bhattacharyya *et al.* 1960; Prema and Bhattacharyya 1962) are capable of the production of verbenols, my data indicate that *D. ponderosae* which are free of these readily culturable microorganisms are capable of producing verbenols at normal or above normal levels (Section III). In contrast, microbe-reduced *D. ponderosae* did not produce quantifiable levels of verbenone, while beetles with their normal complement of microorganisms did (Section III), as did yeasts associated with *D. ponderosae* (Figs. 11, 12).

Verbenone is apparently not metabolized further by *H. capsulata* or *P. pinus* (Fig. 13), so it would probably build up at significant levels in attacked trees. Thus it would be of adaptive advantage for individual *D. ponderosae* to exploit verbenone as a signal of an established attack in host trees that offer limited resources.

Candida nitratophila, the only species of yeast isolated by Leufvén *et al.* (1984) that converted *trans*-verbenol into verbenone, has also been isolated from *D. ponderosae* (Shifrine and Phaff 1956).

Finally, there is a highly persistent association between *D. ponderosae* and its associated yeasts, suggesting a mutualistic association (Whitney 1971). Whitney (1971) reported that *H. capsulata* and *P. pinus* are closely associated with *D. ponderosae* during brood development in lodgepole pine, and he was unable to find *D. ponderosae* populations free of these yeasts in an extensive survey in B. C. and Idaho. Farmer (1965) also found that these yeasts were closely associated with *D. ponderosae* in lodgepole pine. Finally, these yeasts were frequently isolated from the maxillary mycangium of *D. ponderosae* (Whitney and Farris 1970), which is compelling evidence of the importance of this symbiotic relationship.

When various yeasts associated with *I. typographus* during attack on spruce trees were quantified it was found that those yeasts which were capable of converting the aggregation pheromone *cis*-verbenol into the antiaggregation pheromone verbenone (Leufvén *et al.* 1984) were

present on the beetles and in the galleries in much higher numbers during those phases of the attack at which verbenone was known to be present in much higher levels than *cis*-verbenol (Leufvén and Nehls 1986). During early attack phases, when aggregation is occurring on the trees and the beetles are producing large quantities of *cis*-verbenol these yeasts were present on the insects in very low numbers (Leufvén and Nehls 1986). I hypothesize that a similar temporal relationship exists between gallery development in *D. ponderosae* and the population of yeasts therein.

H. capsulata, *P. pinus*, and other yeasts capable of converting verbenols to verbenone (Leufvén *et al.* 1984), have frequently been isolated from *Dendroctonus* and *Ips* species (Callaham and Shifrine 1960; Shifrine and Phaff 1956). Many of these bark beetles use *cis*- or *trans*-verbenol as an aggregation pheromone, and verbenone as an antiaggregation pheromone, suggesting that microbial involvement in the termination of bark beetle aggregation may be widespread. Of particular interest are insects such as *D. pseudotsugae* and *D. frontalis* for which verbenone is a multifunctional pheromone, attractive at low concentrations and inhibitory at high concentrations (Rudinsky 1973; Rudinsky *et al.* 1974a, b). In this situation the low population levels of yeasts which are present in beetle galleries soon after the initiation of attack could contribute toward beetle aggregation by producing low levels of verbenone. In more advanced stages of attack the higher levels of verbenone produced by larger yeast populations would contribute toward terminating the attack on individual trees, and directing it toward other trees.

Although it was my original intention to culture microorganisms from large numbers of *D. ponderosae* and to examine the abilities of the isolated microorganisms to produce and interconvert compounds which function as semiochemicals for their hosts, I now believe that such a survey would add little to what is already known about microbial involvement in bark beetle pheromone production and regulation. Considering the number of microorganisms which have already been found to be capable of producing or interconverting bark beetle pheromones in laboratory experiments it is evident that the metabolic capacities for these conversions are common. It would be much more edifying to establish whether or not these metabolic capacities are relevant in the chemical ecology of bark beetles under natural conditions. This question could be addressed by quantifying the production of

metabolites by microorganisms exposed to realistic levels of precursors under natural conditions and at population levels which are similar to those found naturally in association with bark beetles.

V. THE ROLE OF AUTOXIDATION OF *alpha*-PINENE IN THE PRODUCTION OF *D. ponderosae* PHEROMONES

Introduction

alpha-Pinene is a monoterpene which is a relatively major and constant component of the resin of *Pinus* species, as well as many other conifers. In *P. contorta* and *P. ponderosa*, both major hosts of *D. ponderosae*, *alpha*-pinene constitutes approximately 5-7% of the monoterpenes present in xylem resin (Smith 1967, 1977, 1983). Female *D. ponderosae* convert *alpha*-pinene into *trans*-verbenol (Hughes 1973), an aggregation pheromone for this species (Pitman 1971), as well as other oxygenated products. A variety of other bark beetle species are also known to produce verbenols, which they use as pheromones, on exposure to *alpha*-pinene (Hughes 1973, 1975; Renwick *et al.* 1973; Renwick *et al.* 1976a; Klimetzek and Francke 1980). In addition *alpha*-pinene is of further behavioral significance since it has been reported to act as a host-produced kairomone for a variety of bark beetle species, including *D. ponderosae* (Pitman 1971), *D. pseudotsugae* (Furniss and Schmitz 1971), *D. frontalis* (Renwick and Vité 1970), *Dendroctonus rufipennis* (Kirby) (Furniss *et al.* 1976), *Gnathotrichus retusus* (LeConte) (Borden *et al.* 1980), *Gnathotrichus sulcatus* (LeConte) (Borden *et al.* 1980), and *T. lineatum* (Vité and Bakke 1979).

It has been established that *alpha*-pinene autoxidizes at elevated temperatures in the presence of oxygen to form *trans*-verbenol, verbenone and other products (Moore *et al.* 1956). The possibility that *alpha*-pinene could autoxidize under ambient conditions in nature appears to have been largely overlooked by researchers studying bark beetle behaviour. Since many of the bark beetles which have been reported to use *alpha*-pinene as a kairomone also use *trans*- or *cis*-verbenol as an aggregation pheromone, and verbenone as an antiaggregation pheromone, it seems likely that a portion of the activity which has been attributed to *alpha*-pinene is actually due to products of its autoxidation. Moreover, through autoxidation of *alpha*-pinene some of the reported bark beetle pheromones may be wholly or in part products of the host tree. Many experiments on the chemical ecology of bark beetles

which have involved *alpha*-pinene, especially under field conditions, would have to be re-evaluated if it was found that the autoxidation of this monoterpene occurs at a biologically significant rate in the field. In addition, certain phenomena such as the increased incidence of bark beetle attack on lightning-struck trees (Coulson *et al.* 1983, 1985, 1986; Krawielitzki *et al.* 1983) and wounded trees, may be attributed in part to attractants produced by autoxidation of *alpha*-pinene.

It was my objective to elucidate in a qualitative and quantitative manner the autoxidation of *alpha*-pinene to *cis*- and *trans*-verbenol and verbenone under normal temperature conditions, and to evaluate whether or not such products of autoxidation could be of behavioural significance for *D. ponderosae* in nature.

Material and Methods

alpha-Pinene (>99% pure, Aldrich Chemical Co., Milwaukee, WI) was distilled with lithium aluminum hydride at reduced pressure such that no oxygenated products were detectable using GLC, and this material was stored well sealed at -9 or -20°C. To assess the production of autoxidation products in *alpha*-pinene, aliquots of this recently distilled *alpha*-pinene (0.2 ml each) were added to empty 2 ml vials or 2 ml vials containing powdered cellulose (alpha-cellulose, Sigma Chemical Co., St. Louis, MO), and exposed to air at room temperature. The powdered cellulose was used to simulate the autoxidation and evaporation of *alpha*-pinene on the frass present in bark beetle galleries. After various time periods, samples of the contents in the vials were extracted in distilled pentane and the products of *alpha*-pinene autoxidation were quantified using GLC.

Since containers of *alpha*-pinene are routinely stored in laboratories at S.F.U. for prolonged periods, an experiment was conducted to determine whether the production of autoxidation products within containers of *alpha*-pinene could result in a significant accumulation of bark beetle pheromones. Samples were taken from 3, 1 kg jars of *alpha*-pinene (>99% pure, Aldrich Chemical Co., Milwaukee, WI) which had been stored in laboratories at S.F.U. for 2.5 years, and these were analysed by GLC to quantify the presence of oxygenated products. Two of the jars had been opened periodically and some of the *alpha*-pinene removed. Storage temperatures are listed in Table 6.

A series of experiments was conducted to assess the relative role of autoxidation in the formation of oxygenated products of *alpha*-pinene by *D. ponderosae*. To do this specially prepared jars were constructed to simulate the phloem tissue in which bark beetle galleries are constructed, and the production of oxygenated products of *alpha*-pinene by female *D. ponderosae* over time was compared with production through autoxidation. A sheet of fiberglass screen (16 mesh) was stapled onto a sheet of Saran Fabric (30 mesh; Chicopee Manufacturing Co., Cornelia, GA), which was then shaped into cylinders of the same height as 500 ml jars, and fitted inside the jars to allow approximately 4 mm of clearance around the full circumference of the screen. In some jars 40 g of a medium composed of powdered cellulose and distilled water, or powdered cellulose, distilled water, and ground lodgepole

pine phloem, was packed into the 4 mm space between the screen and the inside wall of the jars so that no large air spaces remained. This cellulose-based medium was used to simulate the phloem tissue in which bark beetle galleries are constructed. In some jars 20 or 50 wild or axenically-reared, female *D. ponderosae* were allowed to bore in the medium, while in other jars no beetles were added. A hole was drilled in the lid of each jar, and each hole was sealed with a rubber septum. A 2 ml vial containing 0.2 ml of recently distilled *alpha*-pinene was placed in each jar, and the jars were tightly sealed. One group of jars containing medium were autoclaved once at approximately 115^{o}C for 30 min.

The atmosphere in the jars was sampled at 24 or 48 h intervals by inserting the needle on a 10 ml gas-tight syringe (Hamilton Co., Reno, NA) through the rubber septum in the lid. Each time a sample was taken, the syringe was pumped 4 times to coat the plunger and the interior surface of the syringe with the contents of the atmosphere in the jar, and then 3 ml was injected directly into the GLC. To quantify compounds which were produced by the beetles and/or by *alpha*-pinene autoxidation, and which adhered to the medium in the jars, medium from each jar was steam distilled at the end of an experiment. This was done using a continuous steam distillation/continuous liquid-liquid extraction, micro apparatus similar to that described by Godefroot *et al.* (1981, 1982). Samples of medium with known amounts of *alpha*-pinene added were also steam distilled to assess any oxidation due to the heat and aeration involved in this process.

Five each of the wild and axenically-reared *D. ponderosae* which had been boring in the autoclaved medium contained in the jars were individually extracted at the end of the experiment (168 h).

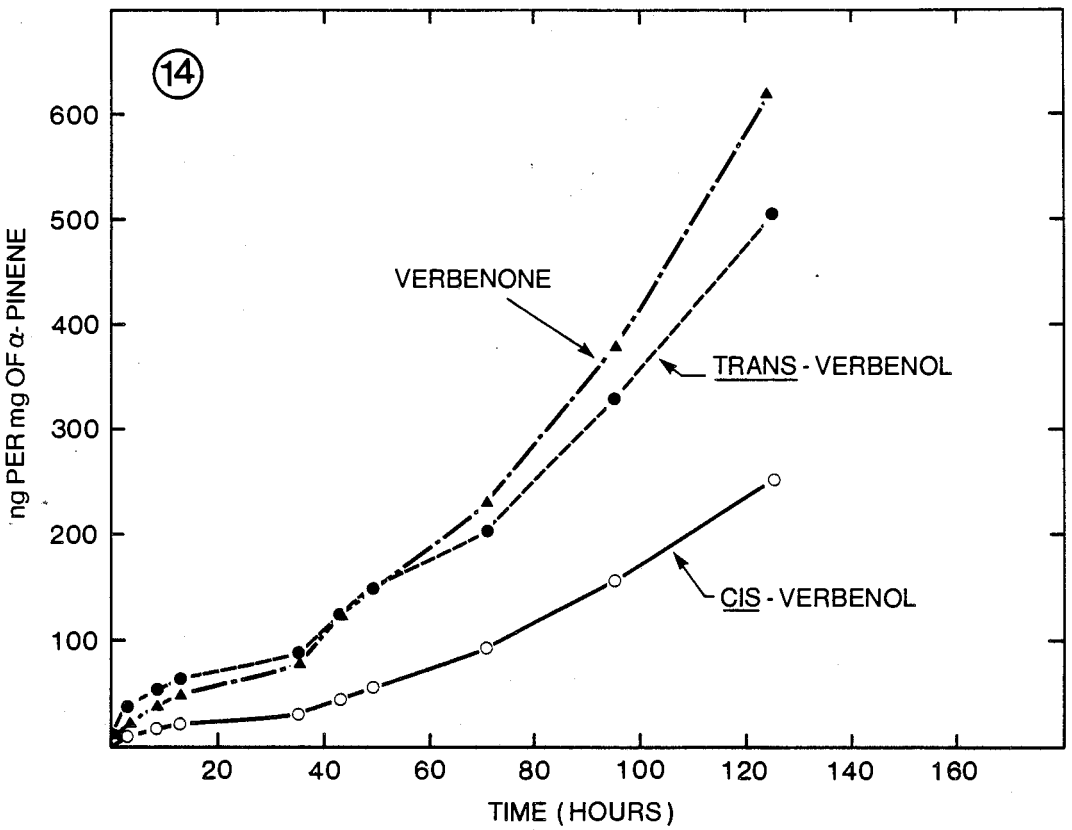
Figs. 14 and 15 clearly show that *alpha*-pinene autoxidizes to *cis*- and *trans*-verbenol and verbenone when exposed to air at room temperature. Several other products were also formed but these compounds are not reported here. For the first 1 to 2 days *trans*-verbenol was found in larger amounts than verbenone, while verbenone predominated thereafter. Although the *alpha*-pinene used in these experiments had been distilled several weeks previous to its use, and stored tightly sealed at -9°C, it was found to contain small amounts of oxygenated products before being exposed to air (Figs. 14, 15). As a result subsequent experiments used more recently distilled *alpha*-pinene which was stored at -20°C.

Table 6 shows the quantities of *cis*- and *trans*-verbenol, myrtenol and verbenone which were found in 1 kg jars of *alpha*-pinene stored under various conditions. These data indicate that the *alpha*-pinene used in various experiments on the role of *alpha*-pinene in the chemical ecology of bark beetles could have contained 0.2% *trans*-verbenol, or even more.

trans-Verbenol and verbenone were among the predominant autoxidation products of *alpha*-pinene in the atmosphere in a 500 ml jar containing 0.2 ml of distilled *alpha*-pinene (Fig. 16), while *cis*-verbenol and myrtenol were present at levels too low to be quantified. *trans*-Verbenol accumulated at approximately 2.7 ng/mg of *alpha*-pinene/day over 8 days, while verbenone accumulated at approximately 1.0 ng/mg of *alpha*-pinene/day. These values are equivalent to 0.67 and 0.25 ng/ml of air, respectively, after 8 days. The slower accumulation of oxygenated products in the atmosphere during the first few days of the experiment was probably due to these compounds adhering to the surface of the glass jar. During the later stages of the experiment oxygenated compounds accumulated more quickly in the atmosphere, indicating that the glass surface of the jar had been saturated. When the cellulose medium was added to the jar the accumulation of products in the atmosphere was reduced (Table 7, Exps. II, III), particularly when beetles were not added (Table 7, Exp. II). The products were probably adhering to the medium.

Steam distillations of the medium confirmed that large quantities of oxygenated products of

Figures 14, 15. Accumulation of *alpha*-pinene oxidation products in 0.2 ml samples of distilled *alpha*-pinene exposed to air in 2 ml vials.


14
600
500
400
300
200
100
ng PER mg OF α-PINENE
VERBENONE
TRANS - VERBENOL
CIS - VERBENOL
20
40
60
80
100
120
140
160
TIME (HOURS)

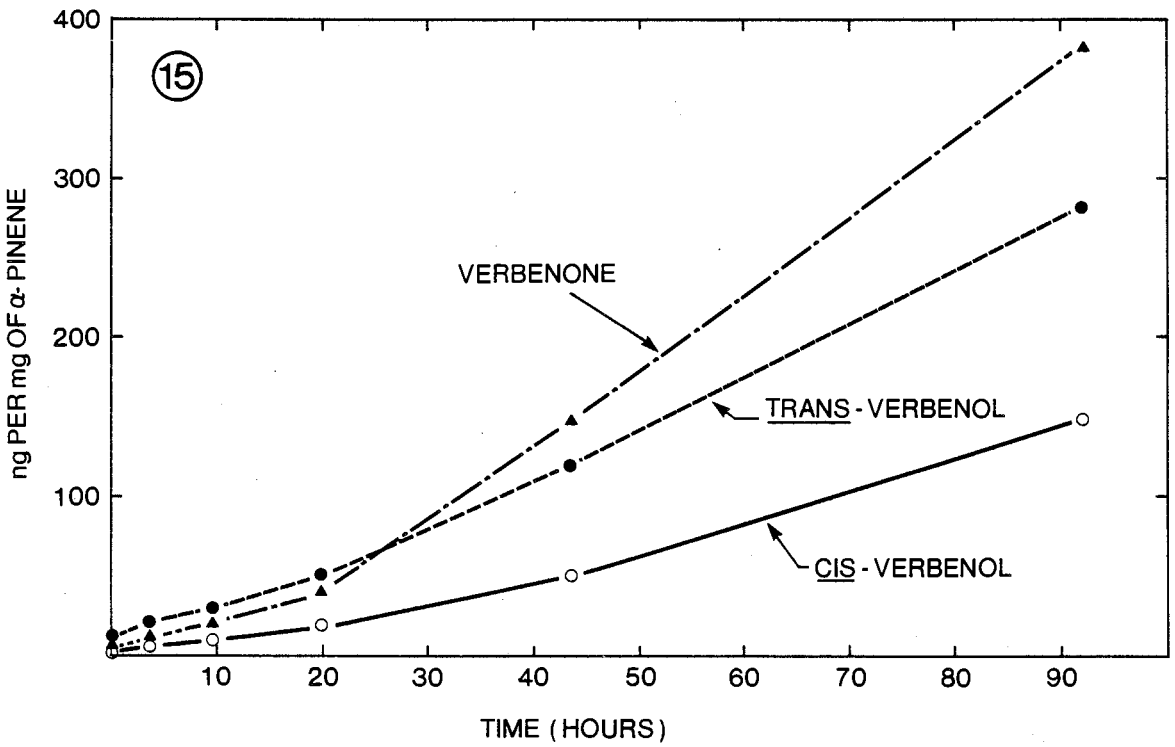

15
400
300
200
100
ng PER mg OF α-PINENE
VERBENONE
TRANS - VERBENOL
CIS - VERBENOL
10
20
30
40
50
60
70
80
90
TIME (HOURS)

Table 6. Accumulation of α-pinene oxidation products in 1 kg jars of α-pinene stored under various conditions for 2 1/2 years.

Storage conditions	Percent composition			
	trans-verbenol	verbenone	*cis*-verbenol	myrtenol
Unopened, 0-4°C	0.08	0.05	0.02	0.02
Opened, 0-4°C	0.10	0.09	0.04	0.02
Opened, room temp.	0.19	0.16	0.05	0.04

Figure 16. Accumulation of *alpha*-pinene oxidation products in the atmosphere contained in a 500 ml jar containing a 0.2 ml sample of distilled *alpha*-pinene.

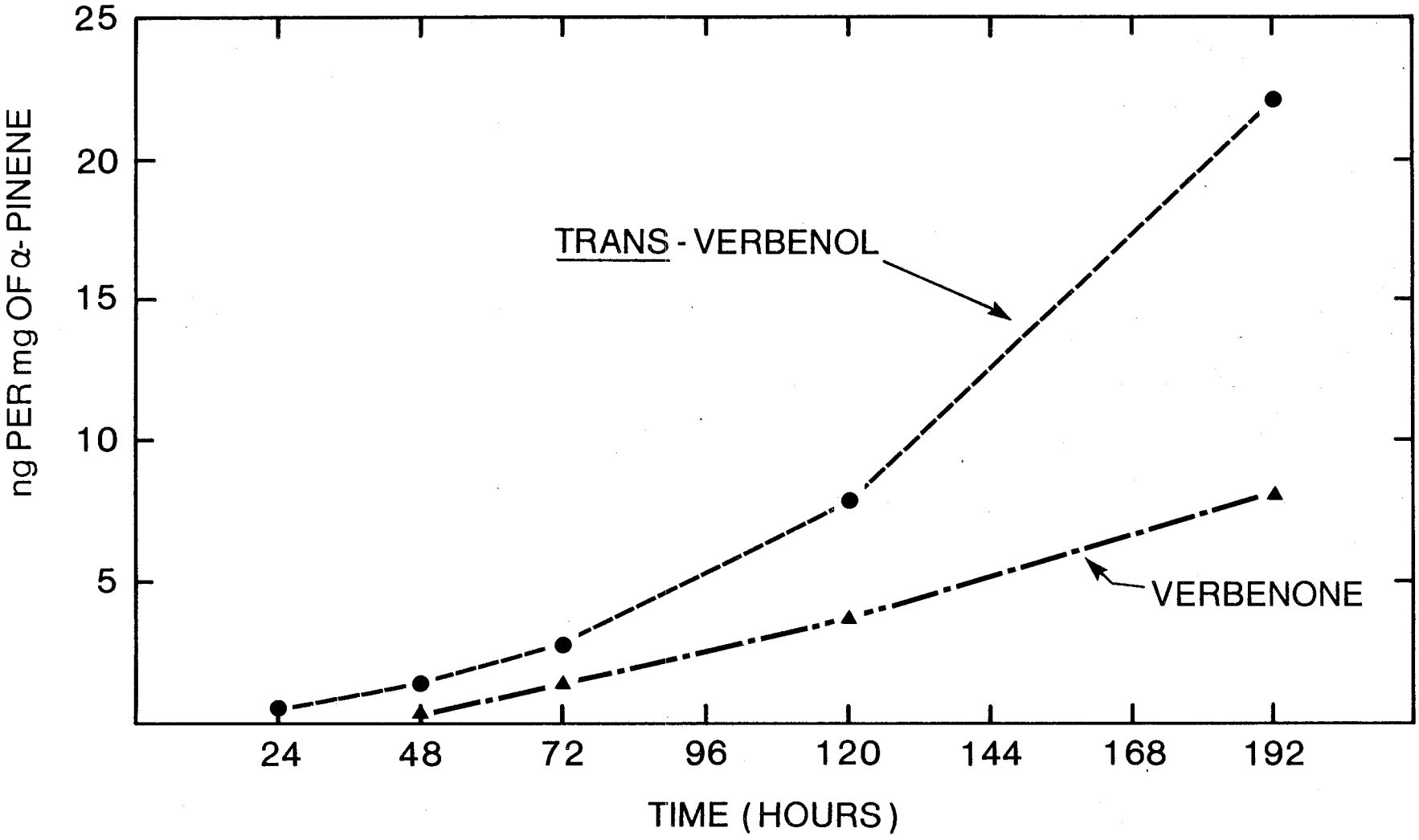

25
20
15
10
5
ng PER mg OF α-PINENE
TRANS - VERBENOL
VERBENONE
24
48
72
96
120
144
168
192
TIME (HOURS)

Table 7. Accumulation of α-pinene oxidation products in the atmosphere within a 500 ml jar containing a 0.2 ml sample of distilled α-pinene in an open 2 ml vial.

Exp. no.	Treatment	Time after exposure to air (h)	Amount of oxidation products (ng/mg of α-pinene)	
			trans-verbenol	verbenone
I	α-pinene	24	0.4	<0.2
		48	1.4	0.3
		72	2.8	1.4
		120	7.9	3.8
		192	21.9	8.1
II	α-pinene 40 g of cellulose-based medium[a]	24	<0.2	<0.2
		48	<0.2	<0.2
		96	0.3	0.3
		168	0.6	0.6
III	α-pinene 40 g of cellulose-based medium[a] 50 ♀ *D. ponderosae*	24	2.2	0.2
		48	2.6	0.7
		96	14.1	3.8
		168	14.0	2.7

[a]Medium composed of powdered cellulose, ground lodgepole pine phloem, and distilled water.

alpha-pinene were adhering to the medium lining the 500 ml glass jars (Table 8). Verbenone predominated when the jars of medium were not autoclaved, with *cis*- and *trans*-verbenol only present at trace levels (Table 8, Exp. I). When 50 wild female *D. ponderosae* were added to the non-autoclaved medium the level of verbenone in the medium was approximately 7-fold higher than in the jars without beetles. Due to the presence of ground phloem in the medium in Exp. I it was not known how much of the verbenone was formed from vapours from the vial of *alpha*-pinene, and how much was formed from *alpha*-pinene present in the phloem.

When ground phloem was eliminated from the medium, and the jars of medium were autoclaved before the experiment, moderate quantities of *cis*- and *trans*-verbenol, as well as verbenone, were found in the steam distillations of the medium (Table 8, Exp. II). Medium in the jar containing wild *D. ponderosae* contained 23 and 74 times the amounts of *trans*-verbenol and verbenone, respectively, than in the medium with no beetles. Medium in the jar containing axenically-reared *D. ponderosae* contained approximately 2.6 times as much *trans*-verbenol and only half as much verbenone as the medium with wild *D. ponderosae*. The 147 ng of *trans*-verbenol and 35 ng of verbenone/mg of *alpha*-pinene in the autoclaved medium without added beetles (Table 8, Exp. II) should be entirely due to autoxidation of the *alpha*-pinene in the vial.

A distillation of a sample of medium which was spiked with a known amount of distilled *alpha*-pinene confirmed that the heat and aeration involved in steam distilling samples of the medium did not oxidize detectable amounts of the *alpha*-pinene present. This absence of autoxidation during distillation was probably due to the atmosphere inside the apparatus being saturated with pentane vapour.

The 5 each of axenically-reared and wild beetles which were removed from the autoclaved medium at the termination of the experiment contained normal levels of *trans*- and *cis*-verbenol, indicating that the beetles in these jars were producing these compounds at normal levels during the experiment.

Table 8. Accumulation of α-pinene oxidation products in 40 g of medium contained in a 500 ml jar containing a 0.2 ml sample of distilled α-pinene for 192 h.

Exp. no.	Treatment	Amount of oxidation products (ng/mg of α-pinene)			
		cis-verbenol	*trans*-verbenol	verbenone	myrtenol
I[a]	Cellulose-based medium, not autoclaved	<7	<7	7,920	7,400
	50 wild ♀ *D. ponderosae* in medium, not autoclaved	<7	<7	55,320	19,293
II[b]	Cellulose-based autoclaved medium	16	147	35	10
	20 wild ♀ *D. ponderosae* in autoclaved medium	57	3,442	2,603	40
	20 axenic ♀ *D. ponderosae* in autoclaved medium	1,481	8,893	1,526	1,456

[a]Medium composed of powdered cellulose, ground lodgepole pine phloem, and distilled water.

[b]Medium composed of powdered cellulose and distilled water.

Discussion

While the accumulations of *cis*- and *trans*-verbenol and verbenone in vials of distilled *alpha*-pinene exposed to air (Figs. 14, 15) demonstrate conclusively that *alpha*-pinene can autoxidize at room temperature to form these compounds, it is difficult to interpret these data without examining the quantities being given off from the liquid. However, these data and those from Table 6 indicate that containers of *alpha*-pinene which are stored at room temperature for extended periods before being used in experiments would actually contain significant quantities of *cis*- and *trans*-verbenol, and verbenone. Even a jar of *alpha*-pinene which had not previously been opened contained low levels of these compounds (Table 6).

The more rapid accumulation of *trans*-verbenol than verbenone in the atmosphere above a source of *alpha*-pinene (Fig. 16) is in opposition to the more rapid accumulation of verbenone relative to *trans*-verbenol within samples of *alpha*-pinene (Figs. 14, 15). This apparent anomaly may reflect differences in the rates of evaporation of *trans*-verbenol and verbenone formed in the *alpha*-pinene.

Although *D. ponderosae* produce large quantities of *trans*-verbenol and other oxygenated compounds when exposed to *alpha*-pinene vapours (Section III) these products are presumably not released from their guts unless the insects are feeding. Thus, it was necessary to include the powdered cellulose medium in the jars in order to induce the release of oxygenated compounds which had been formed from *alpha*-pinene vapours presumably taken-in through the insects' spiracles.

The minimal accumulation of products of *alpha*-pinene autoxidation in the atmosphere in jars containing the powdered cellulose medium, particularly when beetles were not present (Table 7), was taken to indicate that the oxygenated products were adhering to the medium. However, GLC analysis of the steam distillate of the medium revealed virtually no verbenols, but large quantities of verbenone (Table 8, Exp. I). Since these results contradicted the finding that there was more *trans*-verbenol than verbenone present in the atmosphere in these jars (Table 7), I initially hypothesized that the heat and aeration involved in steam distilling the medium had caused the *trans*-verbenol to oxidize into verbenone. This hypothesis was rejected when a *trans*-verbenol-spiked sample of medium, which was

steam distilled and subject to GLC analysis, was found to contain only *trans*-verbenol.

To test the hypothesis that verbenols were being converted to verbenone in the jars containing medium, Exp. I (Table 8) was repeated using a vial containing 0.2 ml of *trans*-verbenol instead of *alpha*-pinene. After 16 days GLC analysis revealed that the vial contained approximately 85% *trans*-verbenol, 14% *cis*-verbenol and 1% verbenone, while the steam distillate of the powdered cellulose medium contained 79% *trans*-verbenol, 6% *cis*-verbenol and 15% verbenone. The degree of conversion of both *cis*- and *trans*-verbenol to verbenone in this experiment is similar to that for the conversion of verbenols by yeasts (Section IV).

I then hypothesized that microbial contaminants introduced into the medium by the wild *D. ponderosae* or through the air were responsible for converting the *trans*- and *cis*-verbenol into verbenone. To test this hypothesis in a subsequent experiment, the jars of medium were autoclaved in an attempt to eliminate microbial contamination, and axenically-reared beetles were added to one of the jars instead of wild beetles. In addition the ground phloem was eliminated from the medium in this experiment so that the only source of *alpha*-pinene present in the jars was the 0.2 ml of distilled *alpha*-pinene in the vials. The steam distillate of the autoclaved medium contained large quantities of *trans*-verbenol (Table 8, Exp. II), suggesting that in the non-autoclaved medium (Table 8, Exp. I) *trans*-verbenol was probably converted to verbenone by microbial contaminants.

The complete conversion of verbenols to verbenone in non-autoclaved medium in both the jar with wild female *D. ponderosae* and the jar without beetles (Table 8, Exp. I), suggests that the metabolic capacity for efficiently converting verbenols to verbenone is not only present in microorganisms associated with *D. ponderosae*, but also in microorganisms found in the air. This is supportive of my hypothesis that the ability to perform conversions such as this is common among many microorganisms (Section IV).

The much higher ratio of *trans*-verbenol to verbenone in medium from the jar with axenically-reared *D. ponderosae* than in medium from the jar with wild *D. ponderosae* (Table 8, Exp. II), is consistent with the finding that yeasts which are closely associated with *D. ponderosae* are capable of converting *trans*-verbenol into verbenone (Section IV). It is likely that yeasts such as *H. capsulata* and

P. pinus were transported into the jar by the wild beetles, particularly in their mycangia (Whitney and Farris 1970), that these yeasts became established in the medium during the experiment, and that they were partially responsible for the conversion of *trans*-verbenol into verbenone.

The higher levels of oxygenated products of *alpha*-pinene in medium from jars with axenically-reared *D. ponderosae* than in medium from jars with wild *D. ponderosae* (Table 8, Exp. II) are consistent with the observation that axenically-reared *D. ponderosae* produced higher levels of these compounds than did wild beetles (Section III). However, the presence of significant quantities of verbenone in the medium which had contained axenically-reared beetles (Table 8, Exp. II) is not consistent with the finding that axenically-reared beetles did not produce verbenone (Section III). It is possible that this verbenone was produced by microbial contaminants which were not eliminated by autoclaving, although it is more likely that this verbenone was produced by autoxidation of *trans*-verbenol produced by the beetles. Recent work by B. S. Lindgren[10] has indicated that *trans*-verbenol will autoxidize rapidly to form large quantities of verbenone under certain situations (pers. comm.). Lindgren found that semiochemical baits for *D. ponderosae* which contained 30 µl of *trans*-verbenol on cotton in open polyethylene centrifuge tubes had autoxidized to form 5.9% verbenone after 2 weeks, and 62% verbenone after 2 months.

The 147 ng of *trans*-verbenol/mg of *alpha*-pinene and 35 ng of verbenone/mg of *alpha*-pinene which were present in the autoclaved medium without added beetles (Table 8, Exp. II) is presumed to be due entirely to the autoxidation of *alpha*-pinene. When compared to the 3442 ng of *trans*-verbenol/mg of *alpha*-pinene and 2603 ng of verbenone/mg of *alpha*-pinene which were found in the medium from the autoclaved jar containing 20 wild *D. ponderosae*, it appears that autoxidation is responsible for approximately 4.3% of the *trans*-verbenol and 1.3% of the verbenone produced in these jars. This translates into *alpha*-pinene autoxidation producing approximately 0.9 female equivalents of *trans*-verbenol and 0.3 female equivalents of verbenone in this experiment.

It is also possible to relate the levels of *trans*-verbenol and verbenone in the atmosphere of jars containing *alpha*-pinene (Table 7), or in the cellulose medium in these jars (Table 8, Exp. II), with the rate of resin exudation from wounds in living *P. contorta*, and the proportion of *alpha*-pinene present in

that resin. For example, the 147 ng of *trans*-verbenol formed by autoxidation of *alpha*-pinene in Exp. II (Table 8) is equal to a rate of approximately 0.8 ng/mg of *alpha*-pinene/h. If an average female *D. ponderosae* produces 200-300 ng of *trans*-verbenol in 24 h of exposure to *alpha*-pinene (Section III), the hourly production rate is approximately 10 ng/h. Thus, about 12.5 mg of *alpha*-pinene exudation/h would produce *trans*-verbenol at approximately the same rate as an average female beetle. At a content of 5-7% *alpha*-pinene in the monoterpenes of lodgepole pine xylem resin (Smith 1967, 1983), and with approximately 14% of lodgepole pine xylem resin collected in B. C. being composed of turpentine (Mirov 1961), then approximately 1360 mg/h of exuded resin would be required to produce one female equivalent of *trans*-verbenol from autoxidation. Raffa and Berryman (1983a) reported that lodgepole pines which sustained high *D. ponderosae* attack densities exhibited a mean pre-attack resin flow of 0.719 ml/h from a single 11 mm diameter hole drilled into the cambium. This rate of resin exudation should produce approximately 0.5 female equivalents of *trans*-verbenol from *alpha*-pinene autoxidation. Thus, the *trans*-verbenol formed from autoxidation of *alpha*-pinene present in resin may be responsible for the increased incidence of *D. ponderosae* attack on wounded trees, as well as contributing to aggregation on already attacked trees.

Although *alpha*-pinene is only present as 5-7% of the monoterpenes in resin from *P. contorta* and *P. ponderosa*, other hosts of *D. ponderosae* contain much higher levels of *alpha*-pinene. In *Pinus edulis* Engelm., *Pinus flexilis* James, *Pinus lambertiana* Dougl., *Pinus monophylla* Torr. and Frém., and *Pinus monticola* Dougl. *alpha*-pinene is present as 60-85% of the monoterpenes (Mirov 1961). Other *Pinus* species which are hosts of bark beetles which use verbenols as pheromones also contain high levels of *alpha*-pinene. For example, of the 10 hosts of *D. frontalis* (Wood 1982) *P. ponderosa* is the only one in which *alpha*-pinene is not the major monoterpene present (Mirov 1961). This prevalence of *alpha*-pinene in *Pinus* species suggests that autoxidation of *alpha*-pinene in wound exudate may be a significant factor in attracting bark beetles to host pines.

One key to the role of *alpha*-pinene autoxidation in modifying the behavior of *D. ponderosae* under natural attack conditions, as well as instances in which *alpha*-pinene is being used by man to attract beetles, is the ratio of production of *trans*-verbenol to verbenone. Ryker and Yandell (1983)

found that verbenone significantly reduced the number of *D. ponderosae* caught in traps baited with *trans*-verbenol when the release rate of the verbenone was 15% of that of the *trans*-verbenol. In contrast, J. H. Borden[11] (pers. comm.) found that semiochemical baits for *D. ponderosae* which contained *trans*-verbenol on cotton in open polyethylene centrifuge tubes, which had probably autoxidized to about 2/3 verbenone by the time the beetles flew (B. S. Lindgren[10], pers. comm.), were still somewhat attractive to flying beetles in the field. My data indicate that in the atmosphere above a source of *alpha*-pinene, verbenone is being produced by autoxidation at approximately 25-30% of the level of *trans*-verbenol (Fig. 16). Thus the net effect of the autoxidation of *alpha*-pinene may be partially antiaggregative for *D. ponderosae* under these conditions, although further research is needed to determine how much verbenone can be present in a sample of *trans*-verbenol before the aggregative effect is significantly reduced. In contrast, bark beetles such as *D. frontalis*, for which verbenone is a multifunctional pheromone (Rudinsky 1973; Rudinsky *et al.* 1974a, b) and *trans*-verbenol is attractive (Renwick and Vité 1969), would probably be attracted to the products of autoxidation unless the concentration of verbenone reached a very high level.

While my experiments indicate that the autoxidation of *alpha*-pinene may produce behaviourally-significant levels of *D. ponderosae* pheromones, more rigorous experiments are required. To evaluate adequately the relative contribution of *alpha*-pinene autoxidation toward pheromone production in bark beetles it would be necessary to compare the levels of production through autoxidation with production by beetles, all under natural conditions. Several factors present under natural conditions could drastically modify my results. In my experiments autoxidation and evaporation are occurring from the very small surface area of the liquid/air interface. Under natural conditions beetles which bore in the phloem of host trees produce large amounts of frass which is soaked with oleoresin, and the large surface area of the frass/air interface should result in very rapid autoxidation and evaporation of the *alpha*-pinene present in the frass.

The effect of the large surface area of frass on the rate of *alpha*-pinene autoxidation and evaporation was simulated when 0.2 ml samples of distilled *alpha*-pinene were added to small quantities of powdered cellulose. The cellulose was extracted with pentane at the same time intervals

used for the experiments reported in Figs. 14 and 15. Not surprisingly the rate of evaporation of *alpha*-pinene from the powdered cellulose was very high, and virtually all of the sample had evaporated within 3.5 h of its exposure to air. Of the sample which remained at 3.5 h, 4.3% was *trans*-verbenol and 1.8% was verbenone. The rapid autoxidation of *trans*-verbenol to verbenone on cotton in semiochemical baits for *D. ponderosae* (B. S. Lindgren[10], pers. comm.) is also likely to be due to the large surface area of the cotton.

The use of pure *alpha*-pinene in these experiments is somewhat artificial since *alpha*-pinene is seldom present as more than 5-7% of the monoterpenes of xylem resin of major hosts of *D. ponderosae* such as *P. contorta* and *P. ponderosa* (Smith 1967, 1977, 1983). However, the volume of *alpha*-pinene used in my experiments was extremely small in relation to the large volume of resin exuded when *Pinus* species are wounded or attacked by bark beetles. Thus the actual quantity of *alpha*-pinene which is exposed to air would probably be much larger in attacked pines than it was in my experiments.

Recent work by G. Gries[11] has indicated that the quantities of *trans*-verbenol released from the autoxidation of *alpha*-pinene in phloem may be significant in relation to the rate of release of *trans*-verbenol from bark beetles galleries (pers. comm.). Gries compared the volatiles trapped on Porapak Q (Applied Science Laboratories Inc., State College, PA) aerations of strips of Engelmann spruce, *Picea engelmannii* Parry, phloem with those from aerations of individual female *D. rufipennis* boring in spruce phloem sandwiched between sheets of plexiglass. The ratios of *alpha*-pinene to *trans*-verbenol in the trapped volatiles indicated that the aerations of strips of phloem without beetles contained approximately 15% of the *trans*-verbenol found in aerations of phloem with beetles. This result suggests that approximately 15% of the *trans*-verbenol released from *D. rufipennis* galleries is due to the autoxidation of *alpha*-pinene and/or the release of oxygenated compounds found in the phloem before bark beetle attack. Gries was unable to repeat this experiment with *P. contorta*, probably due to the lower levels of *alpha*-pinene present in lodgepole pine relative to Engelmann spruce (Drew and Pylant 1966).

When the composition of resin from *Pinus* species is analysed, oxgenated compounds are

seldom reported (e.g. Smith 1967, 1977, 1983). It is not clear if this is because oxygenated compounds are not present, or simply that they are not noticed. Lu *et al.* (1975) reported that 2.4% of the neutral fraction of the oleoresin of *Pinus taiwanensis* Hayata was composed of oxygenated compounds, with *trans*-verbenol and verbenone prominant among these. Assuming that these oxygenated terpenes were not formed from autoxidation after the resin was collected, then the resin which exudes from wounded trees may contain significant quantities of verbenols and verbenone before it is even exposed to air. It should be noted that the *alpha*-pinene content in *P. taiwanensis* turpentine is approximately 73% (Mirov 1961), which is much higher than in hosts of *D. ponderosae* such as *P. contorta* and *P. ponderosa* (Smith 1967, 1977, 1983).

From surveys which were carried out over a 20 year period it has been determined that 23-77% of more than 2000 *D. frontalis* infestations surveyed were associated with lightning-struck trees (Hodges and Pickard 1971; Lorio and Bennett 1974; Hicks 1980). Although it is generally thought that this correlation is due to lightning-strikes causing trees to be stressed (Coulson *et al.* 1983), which makes them more susceptible to beetle attack, it is not known how the beetles locate the lightning-struck trees. Payne (1986) reported that tree odours appear to function in host selection in *D. frontalis* by arresting beetle flight. Electrophysiological studies have shown that *trans*-verbenol also suppresses pheromone-elicited muscle potentials in *D. frontalis* (Dickens and Payne 1978), suggesting that *trans*-verbenol formed through *alpha*-pinene autoxidation may function as an attractive or arrestant 'tree odour' for this species.

The autoxidation of *alpha*-pinene to *trans*- and *cis*-verbenol and verbenone at the levels reported in this Section necessitates the re-evaluation of many experiments involving *alpha*-pinene. Many bark beetle species which use *trans*- or *cis*-verbenol as a pheromone have also been reported to use *alpha*-pinene as a kairomone. It is possible that for some species this is an erroneous conclusion, and that the activity of *alpha*-pinene is partly or entirely due to the formation of verbenols or verbenone through autoxidation. The finding that *trans*-verbenol can substitute for *alpha*-pinene as a synergist for frontalin in attracting *D. frontalis* (Payne *et al.* 1978; Renwick and Vité 1969) could indicate that the activity of *alpha*-pinene for this beetle is due to the formation of *trans*-verbenol through autoxidation.

Similarly, although *alpha*-pinene is thought to be a synergist for lineatin in attracting *T. lineatum*, both *cis*- and *trans*-verbenol also act as synergists (Klimetzek 1984). The observation that day-old oleoresin from ponderosa pine becomes ineffective as a synergist for *trans*-verbenol in attracting *D. ponderosae* (Billings *et al.* 1976) could be due to a buildup of verbenone through autoxidation of *alpha*-pinene in the resin. Certain phenomena such as the increased incidence of bark beetle attack on lightning-struck trees (Coulson *et al.* 1983, 1985, 1986; Krawielitzki *et al.* 1983) and wounded trees, may be explained by *alpha*-pinene autoxidation.

VI. THE ROLE OF POLYSUBSTRATE MONOOXYGENASE ENZYMES IN TERPENE ALCOHOL PHEROMONE PRODUCTION IN *D. ponderosae* [12]

Introduction

A group of enzymes called polysubstrate monooxygenases (PSMO's), formerly called mixed function oxidases (mfo's), are involved in catalyzing reactions which cause lipophilic compounds, such as monoterpenes, to become more hydrophilic and more easily excretable (Brattsten 1979). Although it has been hypothesized that bark beetle PSMO's are involved in converting host monoterpenes into terpene alcohol pheromones (White *et al.* 1980), experimental evidence is inconclusive. Sturgeon and Robertson (1985) measured polysubstrate monooxygenase activity in midgut tissues from *D. ponderosae*, but they did not evaluate the role of these enzymes in terpene metabolism and pheromone production. White *et al.* (1979) reported that microsomal fractions of *D. terebrans* could produce *alpha*-pinene oxide, and other unidentified compounds, from *alpha*-pinene. They hypothesized that *alpha*-pinene oxide may be the first step in the *in vivo* production of certain terpene alcohol pheromones of *Dendroctonus* species. No studies have been carried out on the role of PSMO's in the oxidation of myrcene by *Dendroctonus* species.

Methylene-dioxyphenyl compounds, such as piperonyl butoxide and some of the components of sesame oil, are known to inhibit the activity of PSMO's (Metcalf *et al.* 1966). These compounds are widely used as insecticide synergists, and have become standard tools for evaluating the involvement of PSMO's in insecticide metabolism (Brattsten 1979). Thus, they would also seem to be useful in studying the role of PSMO's in terpene alcohol pheromone production.

My objective in the following experiments was to use polysubstrate monooxygenase inhibitors to study the role of these enzymes in *D. ponderosae* in converting host tree monoterpenes into pheromones and other terpene alcohols.

Material and Methods

Piperonyl butoxide (analytical grade; McLaughlin, Gormley, King Co., Minneapolis, MI) or sesame oil (Sigma Chemical Co., St. Louis, MO) was applied to the pronotum of each beetle. In the initial experiment 1 µl of sesame oil was applied without dilution, and in subsequent experiments piperonyl butoxide or sesame oil were diluted in acetone and 1 µl of a 20% solution (v/v) was used. GLC analysis of the piperonyl butoxide established that the material was 95% pure. Instead of being treated with 1 of the inhibitors 1 group of female beetles was treated with a dosage of diazinon which approximated the LD_{50} for this species. Control beetles received 1 µl of water on the pronotum in the initial experiment, and 1 µl of acetone in all subsequent experiments. Females were then exposed to the vapours from a 2 ml vial containing 25 µl of distilled *alpha*-pinene (>99% pure, Aldrich Chemical Co., Milwaukee, WI) in a 500 ml jar for 24 h. Males were exposed in an identical manner to vapours from myrcene (>99% pure, Phero Tech Inc., Vancouver, B. C.).

The levels of monoterpenes and terpene alcohols present in the beetles were quantified as outlined in Section II. Differences in the quantities of compounds present in the extracts were analyzed using the Mann-Whitney U test ($P < 0.05$), except for the data in Exp. IV (Table 9), which were analyzed using a non-parametric multiple comparisons test (Conover 1980, p. 231).

Results

Female *D. ponderosae* treated with sesame oil before exposure to *alpha*-pinene contained significantly lower levels of *trans*-verbenol than control beetles in 1 of 2 experiments (Table 9, Exp. II). A similar, but not statistically significant, effect occurred for *cis*-verbenol, and myrtenol (Table 9, Exp. I, II). Females which had been treated with piperonyl butoxide contained lower levels of *trans*- and *cis*-verbenol than control beetles, but the effect was not statistically significant (Table 9, Exp. III). Females which had been treated with sesame oil or piperonyl butoxide retained significantly higher levels of *alpha*-pinene than control beetles in all experiments (Table 9, Exp. I-III).

Females which had been treated with a dosage of diazinon which approximated the LD_{50} for this species, and which were moribund following 24 h exposure to *alpha*-pinene vapours, contained terpene alcohols at levels which were not significantly lower than those in acetone-treated controls (Table 9, Exp. IV). Those diazinon-treated females which appeared healthy following 24 h exposure to *alpha*-pinene vapours actually contained terpene alcohols at levels which were significantly higher than in acetone-treated controls (Table 9, Exp. IV). Topical application of acetone had no apparent effect on terpene alcohol production in females (Table 9, Exp. V).

Male *D. ponderosae* treated with sesame oil before exposure to myrcene contained ipsdienol at lower levels than in control beetles, but the effect was not significant (Table 10, Exp. I, II). Males which had been treated with piperonyl butoxide contained significantly lower levels of ipsdienol than acetone-treated controls (Table 10, Exp. III). A similar, but not statistically significant, effect occurred for *E*- and *Z*-myrcenol in piperonyl butoxide-treated males (Table 10, Exp. III). Males which had been treated with sesame oil or piperonyl butoxide retained significantly higher levels of myrcene than control beetles in all experiments (Table 10, Exp. I-III). Acetone-treated males contained levels of myrcene and ipsdienol which were not significantly different from those in untreated beetles (Table 10, Exp. IV).

Table 9 .. Production of pheromones and other terpene alcohols from α-pinene in female *Dendroctonus ponderosae* following topical application with monooxygenase inhibitors or an insecticide.

Exp.	Treatment	Mortality (24 h)	No. beetles extracted	Mean amount in abdomen (ng/beetle)[a] α-pinene	*cis*-verbenol	*trans*-verbenol	myrtenol
I	1 µl 100% Sesame Oil	4/12	8	1750 a	62 a	1423 a	96 a
	1 µl Water	2/12	8	281 b	107 a	2862 a	125 a
II	1 µl 20% Sesame Oil	2/15	12	1034 a	38 a	294 a	87 a
	1 µl Acetone	3/15	12	231 b	65 a	1469 b	103 a
III	1 µl 20% Piperonyl Butoxide	5/17	11	1044 a	20 a	441 a	51 a
	1 µl Acetone	0/15	11	247 b	43 a	914 a	38 a
IV	0.1 µg Diazinon	10/20	5 Healthy	304 b	363 b	8400 b	217 b
			5 Moribund	681 c	123 a	3161 a	89 a
	1 µl Acetone	0/20	10	79 a	129 a	3718 a	97 a
V	1 µl Acetone	1/20	10	223 a	69 a	2218 a	103 a
	Untreated	0/20	10	271 a	54 a	2034 a	92 a

[a]Values for treatments in each experiment followed by same letter are not significantly different (P>0.05), Mann-Whitney U test except for Exp. IV which was analysed using a non-parametric multiple comparisons test (Conover 1980, p. 231).

Table 10. Production of pheromones and other terpene alcohols from myrcene in male *Dendroctonus ponderosae* following topical application with monooxygenase inhibitors.

Exp.	Treatment	Mortality (24 h)	No. beetles extracted	Mean amount in abdomen (ng/beetle)[a]			
				myrcene	ipsdienol	*E*-myrcenol	Z-myrcenol
I	1 µl 20% Sesame Oil	1/20	10	1470 a	1637 a	-	-
	1 µl Acetone	0/20	10	232 b	2815 a	-	-
II	1 µl 20% Sesame Oil	5/20	11	320 a	1655 a	169 a	134 a
	1 µl Acetone	1/20	11	82 b	1925 a	245 a	156 a
III	1 µl 20% Piperonyl Butoxide	4/15	10	1463 a	107 a	103 a	64 a
	1 µl Acetone	1/14	10	105 b	1820 b	122 a	93 a
IV	1 µl Acetone	1/20	10	173 a	1987 a	267 a	163 a
	Untreated	1/20	10	151 a	2104 a	239 a	146 a

[a]Values for treatments in each experiment followed by same letter are not significantly different ($P>0.05$), Mann-Whitney U test.

Discussion

My findings that topical applications of piperonyl butoxide, as well as the PSMO inhibitors present in sesame oil, cause a decrease in the levels of terpene alcohols, and a concurrent buildup of their precursors, are supportive of the hypothesis that PSMO's are involved in the initial oxidation of monoterpenes by *D. ponderosae*. Thus, it appears that the mountain pine beetle is able to detoxify host monoterpenes, and produce the aggregation pheromone *trans*-verbenol, rapidly and simultaneously using the same enzyme system. In this way this insect has adapted to use the metabolic products of detoxication as pheromones (Hughes 1973). This strategy is much more energetically efficient than producing pheromones *de novo* from other precursors.

It is known that monoterpenes such as *alpha*-pinene and myrcene are potent and rapid inducers of cytochrome P-450 linked monooxygenases in certain insects (Brattsten *et al.* 1977). Such induction probably also occurs in *D. ponderosae*, and greatly facilitates the rapid detoxication of host monoterpenes, and the production of the aggregation pheromone *trans*-verbenol.

Since PSMO inhibitors such as piperonyl butoxide possess some toxicity to arthropods (Schuntner *et al.* 1974), it is possible that the reduction in pheromone levels and concurrent increase in precursor levels following topical application of these compounds could be due to sublethal toxicity. However, my dosages of piperonyl butoxide and sesame oil caused only low levels of beetle mortality (Tables 9, 10); thus it appears unlikely that the reduction in terpene alcohol production is caused by toxicity. In addition, my data indicate that topical application with an insecticide, diazinon, at a dosage which approximates the LD_{50} for female *D. ponderosae*, actually increased the production of terpene alcohols in females which remained healthy during *alpha*-pinene exposure (Table 9, Exp. IV). This finding is further evidence that the reductions in terpene alcohol production were not due to sublethal toxicity.

It is not certain why the application of diazinon resulted in increased levels of *trans*- and *cis*-verbenol and myrtenol in female *D. ponderosae* (Table 9, Exp. IV). As previously mentioned

monoterpenes such as *alpha*-pinene and myrcene are potent and rapid inducers of P-450 linked monooxygenase enzymes in mammals (Pap and Szarvas 1976), as well as certain insects (Brattsten *et al.* 1977). In *Spodoptera eridania* (Cramer) larvae a significant increase in monooxygenase activity was measured within 30 min after an oral dose of *alpha*-pinene (Brattsten *et al.* 1977). Since insecticides are also known to induce monooxygenase enzymes, the increased levels of terpene alcohols found in diazinon-treated *D. ponderosae* may be due to induction of the monooxygenase enzymes by the diazinon. Alternatively, since pheromone production is under hormonal and neural regulation (Hughes and Renwick 1977a, b), it is possible that diazinon has some effect on the central nervous system of *D. ponderosae*, which results in increased pheromone production. Increased production could also be the result of an increased respiratory rate in diazinon-treated beetles, or simply a toxic effect on symbiotic microorganisms responsible for breaking down pheromones. The hypothesis that the effect of diazinon was due to an increase in respiration and resultant increase in precursor intake through the spiracles seems likely since the diazinon-treated beetles contained increased levels of *alpha*-pinene as well as increased levels of terpene alcohols (Table 9, Exp. IV). It would be interesting to determine whether this increase in pheromone production could also be brought about by other organophosphate insecticides, as well as organochlorine or carbamate insecticides.

Data indicating that polysubstrate monooxygenase enzymes are involved in the production of pheromones and other terpene alcohols from host monoterpenes (Tables 9, 10) are not inconsistent with the hypothesis that microorganisms are involved in producing their hosts' pheromones. It is possible that these reactions are being catalyzed by monooxygenase enzymes present in the insects, in their symbiotic microorganisms, or both. This question could be addressed through PSMO inhibitor treatment of microorganisms which are capable of the *in vitro* production of their hosts' pheromones, as well as by treating axenically-reared beetles with PSMO inhibitors.

VII. THE ROLE OF IPSDIENOL IN THE CHEMICAL ECOLOGY OF *D. ponderosae*

Introduction

Although males of *Ips* species produce the terpene alcohol pheromones ipsdienol and/or ipsenol when exposed to the monoterpene myrcene (Hughes 1974), these compounds have been found only rarely in *Dendroctonus* species. Hughes (1973) reported that males of *D. brevicomis*, *D. valens*, *D. ponderosae* and *D. pseudotsugae* produced ipsdienol when exposed to myrcene vapours, but females were apparently not tested, nor was the behavioral significance of ipsdienol investigated. Renwick *et al.* (1976b) and Byers (1982) found that only male *D. brevicomis* produce ipsdienol when both sexes were exposed to myrcene vapours. Byers (1982) also discovered that this ipsdienol was predominantly the (+) enantiomer, and that, in the field, racemic ipsdienol significantly reduced the response by *D. brevicomis* of both sexes to a mixture of myrcene, *exo*-brevicomin and frontalin, suggesting that ipsdienol may be involved in regulating colonization density in this species.

The terpene alcohol myrcenol has also been found in *Dendroctonus* species after beetles were exposed to myrcene. *D. brevicomis* of both sexes contained significant amounts of myrcenol after exposure to myrcene vapours (Renwick *et al.* 1976b), and female *D. ponderosae* produced small quantities of myrcenol when fed on pine logs (Conn 1981); neither study reported whether the compound was *E*- or *Z*-myrcenol or a mixture of both isomers. Conn (1981) found myrcenol to be attractive to *D. ponderosae* of both sexes in laboratory bioassays, but in the field response to other attractants appeared to be slightly inhibited by the presence of *E*-myrcenol (Conn *et al.* 1983).

With the exception of the work by Byers (1982) on *D. brevicomis*, and Conn (1981) and Conn *et al.* (1983) on *D. ponderosae*, the oxidation of myrcene by *Dendroctonus* species, and the possible biological roles of ipsdienol in *Dendroctonus* species, have not been studied. This omission is likely due to the fact that in most pheromone isolation studies beetles are induced to produce pheromones by allowing them to bore into host phloem tissue in cut logs that no longer produce copious amounts of monoterpene-rich resin. Thus, it is not surprising that oxidation products of monoterpene vapours

have been largely overlooked. In addition, female *Dendroctonus* species, which have been studied more intensively than males because they initiate attacks on new host trees, do not appear to oxidize myrcene to the same extent as males. As a result the possible behavioral significance of ipsdienol and myrcenol for *Dendroctonus* species has been largely ignored.

My objectives were: to confirm that *D. ponderosae* can produce ipsdienol and myrcenol, to determine which sex(es) produce the compounds, to identify which geometric isomers of myrcenol or enantiomers of ipsdienol are produced, and to test whether ipsdienol is of behavioral significance for this species.

Pheromone Analysis

D. ponderosae of both sexes were exposed to myrcene by allowing the beetles to bore into lodgepole pine bolts for 24 h, or by exposing them to the vapours from a 2 ml vial containing 25 µl of myrcene (>99% pure) in a 500 ml jar for 24 h. GLC analyses were conducted as outlined in Section II. The chirality of the ipsdienol produced by individual beetles was determined using the technique developed by Slessor *et al.* (1985). All beetles were obtained from infested lodgepole pine from northeast of Princeton, B. C., except for one group of male beetles which were obtained near Cranbrook, B. C.

Laboratory Bioassay Procedures

Bioassays were performed in a draught-free, darkened room using an open arena olfactometer similar to that described by Borden *et al.* (1968). Groups of 10 beetles were released from overturned, 60x20 mm disposable petri dishes, on a 24 cm diameter filter paper. The release point was 8 cm lateral to, and 12 cm downwind of the outlet of an airstream with a flow rate of 1400 ml/min. During the summer of 1984, 40 or 50 beetles in groups of 10 were used for each treatment, while in 1985 the number was expanded to 100 beetles in groups of 10. A 6 V, 25 W microscope light was placed at the opposite edge of the filter paper to attract the photopositive beetles across the airstream. Twenty µl of a pentane dilution of each test stimulus was deposited on a rolled up filter paper (4.25 cm diameter) inside a glass tube (1 cm ID). The glass tube was placed at the outlet of the airstream, which then flowed across the centre of the arena perpendicular to the path of the beetles. Each group of 10 beetles was allowed 2 min to respond to the test material, with a positive response scored when a beetle walked to within 1 cm of the test stimulus source.

The experimental stimuli used in the bioassays were (+), (-) and (±)-ipsdienol (sources and

purities listed in Table 11), each tested at concentrations ranging between 4 and 400,000 ng/20 µl pentane. These same dilutions of (+), (-) and (±)-ipsdienol were also tested in combination with pentane extracts of frass produced by female *D. ponderosae* boring in lodgepole pine bolts for 24 h. Control stimuli were pentane and a pentane extract of the frass produced by females. The frass extract was tested at a concentration of 0.02 g equiv. when used as a standard or combined with ipsdienol dilutions. The frass standard was used whenever bioassays were being performed to verify that the test beetles were responsive. Tests were not run on days with low barometric pressure as beetles responded poorly on these days. Beetles were kept in disposable petri dishes at 2-4^{o}C between bioassays but were rewarmed to room temperature 10 min prior to use. Each time a new stimulus was tested the filter paper arena was changed and the bioassay room was aired-out.

Percent positive response data were transformed using the arcsine transformation (Sokal and Rohlf 1981) and then analysed by ANOVA and the Newman-Keuls test ($P < 0.05$) using SPSS^{X} (SPSS 1983).

(+)-Ipsdienol (95% optical purity) and (-)-ipsdienol (98% optical purity) were obtained by kinetic resolution of racemic ipsdienol (Sharpless *et al.* 1983) utilizing the Sharpless asymmetric epoxidation (Katsuki and Sharpless 1980) to the extent of 80%. The enantiomeric excesses of the isolated products were determined by capillary gas chromatography of the acetyl-(S)-lactyl derivatives as reported by Slessor *et al.* (1985).

Field Trapping Experiments

All field trapping experiments were done in 1984 and 1985 in lodgepole pine forests in the Shinish Cr. valley, approximately 30 km northeast of Princeton, B. C. All compounds tested in the field are listed in Table 11, along with the release devices and release rates.

Trapping experiments were done in a randomized block design using 8-funnel, multiple funnel traps (Lindgren 1983) placed in lines 25 m apart with 25 m between each trap. Baits were suspended from a wire attached to the third funnel from the bottom of the trap such that the bait was positioned

Table 11. Source, purity, release devices, and approximate release rates (at 22°C in the laboratory) for monoterpenes and terpene alcohols tested for attractancy to *D. ponderosae* in the field.

Compound	Abbrev-iation	Source	Purity[a]	Release Device	Approximate Release Rate mg/24 h
β-myrcene	M	Sigma Chem. Co. St. Louis, MO	98%	Traps:one 25 ml polyethylene snap cap vial(closed)	150
			85%	Trees:two 1.8 ml polyethylene centrifuge tubes (closed)	20
(±)-*exo*-brevicomin	eB	Albany Int'l Co. Columbus, OH	98%	Traps:Conrel fibre (Albany Int'l Co.) 0.2 mm ID, one end open	0.05
			98%	Trees:Glass capillary tube, 1.1-1.6 mm ID, one end open	0.5
trans-verbenol (75%(-); 25%(+))	tV	Phero Tech Vancouver, B.C. (12% *cis*)	88%	1.8 ml polyethylene centrifuge tube (open), containing 30 µl on cotton plug	1
Ipsdienol	I	(±):Borregard A.S., Sarpsborg, Norway	91%	1984 High:10 glass capillary tubes, 0.8-1.1 mm ID, one end open	0.5
		(+):chemical purity	91%	1984 Low:1 glass capillary tube, 0.8-1.1 mm ID, one end open	0.05
		optical purity[b]	95%		
		(-):chemical purity	93%	1985:five 2 µl microcaps (Drummond Scientific Co., Broomall, PA)	0.15
		optical purity[b]	98%		

[a] As determined by gas chromatography.

[b] Resolved from (±) by E. Czyewska, Dept. of Chemistry, S.F.U.

about 1 cm below that funnel. Jars at the bottom of the traps contained an aqueous solution of approximately 2.5 ml/L of Liqui-nox detergent (Alconox Inc., New York, NY) to decrease surface tension and kill trapped insects, and 40 mg/L sodium azide to inhibit microbial growth; beetles were collected from these jars and preserved in 90% ethanol prior to being counted and their sex determined.

In 1984, 8 replicates were performed of the following bait treatments:

1. myrcene + *trans*-verbenol + *exo*-brevicomin + high release rate (±)-ipsdienol;
2. myrcene + *trans*-verbenol + *exo*-brevicomin + low release rate (±)-ipsdienol;
3. myrcene + *trans*-verbenol + *exo*-brevicomin;
4. high release rate (±)-ipsdienol;
5. low release rate (±)-ipsdienol; and
6. unbaited control.

In 1985, 12 replicates were performed of the following bait treatments:

1. myrcene + *trans*-verbenol + *exo*-brevicomin + (±)-ipsdienol;
2. myrcene + *trans*-verbenol + *exo*-brevicomin + (+)-ipsdienol;
3. myrcene + *trans*-verbenol + *exo*-brevicomin + (-)-ipsdienol;
4. myrcene + *trans*-verbenol + *exo*-brevicomin;
5. (±)-ipsdienol;
6. (+)-ipsdienol;
7. (-)-ipsdienol; and
8. unbaited control.

One experiment of 2 days duration was conducted in 1984, and in 1985, 3 experiments each of 3 days duration were conducted with baits re-randomized within the block after each experiment.

Tests for homogeneity of variances were performed on the numbers of beetles caught using Cochran's C test as well as the Bartlett-Box F test using SPSS^X (SPSS 1983). The data were found to be heteroscedastic, so trap catch was analysed using the Kruskal-Wallis test (Sokal and Rohlf 1981) followed by a non-parametric multiple comparisons test (Conover 1980, p. 231), $P < 0.05$. The data on

sex ratios were homoscedastic, so they were analysed by ANOVA and the Newman-Keuls test ($P < 0.05$) using SPSS^X (SPSS 1983).

Baited-tree Experiments

Two baited-tree experiments were laid out in a randomized block design at the same location as the trapping experiment. The baits were contained in waterproofed, cardboard receptacles (Phero Tech Inc., Vancouver, B. C.) stapled onto mature lodgepole pine trees of dbh 22-34 cm, which were a minimum of 15 m apart. In 1984, 10 replicates were performed of the following bait treatments:

1. myrcene + *trans*-verbenol + *exo*-brevicomin + high release rate (±)-ipsdienol;
2. myrcene + *trans*-verbenol + *exo*-brevicomin;
3. high release rate (±)-ipsdienol; and
4. unbaited control.

In 1985, 11 replicates were performed of the following bait treatments:

1. myrcene + *trans*-verbenol + *exo*-brevicomin + (±)-ipsdienol;
2. myrcene + *trans*-verbenol + *exo*-brevicomin + (+)-ipsdienol;
3. myrcene + *trans*-verbenol + *exo*-brevicomin + (-)-ipsdienol;
4. myrcene + *trans*-verbenol + *exo*-brevicomin;
5. (±)-ipsdienol;
6. (+)-ipsdienol;
7. (-)-ipsdienol; and
8. unbaited control.

Following the flight period in each year, attack densities were assessed on all test trees by counting the entrance holes within 2, 20x40 cm frames held at eye level on opposite sides of the trees at 90^o from the bait station.

Using the Cochran's C test as well as the Bartlett-Box F test, the data were found to be homoscedastic, so mean attack densities were analysed by ANOVA and the Newman-Keuls test ($P <$

0.05) using SPSS^{X} (SPSS 1983).

Results

Only male *D. ponderosae* contained detectable levels of ipsdienol (>10 ng/beetle) after exposure to myrcene vapours (Table 12). This ipsdienol was predominantly the (+) enantiomer (Table 12), with relatively little variation between individuals in southwestern (Fig. 17) as well as southeastern B. C. (Fig. 18). However, there was considerable variability in the amount of ipsdienol produced by individual beetles; some produced none at all, while one male contained over 7 µg of the compound (Fig. 21). Neither male nor female beetles contained detectable levels of ipsdienol after feeding on bolts of *P. contorta*, either alone or with an individual of the other sex. Ipsenol was not produced at detectable levels at any time by either sex.

D. ponderosae of both sexes contained myrcenol, predominantly the *E*-isomer, after exposure to myrcene vapours, although males contained significantly more than females (Table 12). Neither males nor females contained detectable levels of myrcenol after feeding on bolts of *P. contorta*.

Table 13 shows the responses of both sexes of *D. ponderosae* in laboratory bioassays to dilutions of (±), (+) or (-)-ipsdienol. In most of these experiments (±), (+) or (-)-ipsdienol at one or more concentrations elicited a significantly higher percentage of positive responses than pentane controls, particularly at higher concentrations of ipsdienol. Ipsdienol at the 2 highest concentrations (400,000 ng/20 µl pentane and 40,000 ng/20 µl pentane) often elicited positive responses which were not significantly different from responses to female frass extracts.

Table 14 shows the responses of both sexes of *D. ponderosae* in laboratory bioassays to female frass extracts combined with either (+), (-) or (±)-ipsdienol at various concentrations. In most of these experiments the ipsdienol inhibited responses to the attractive frass extract, although the effect was significant only at low concentrations of ipsdienol.

Field trapping studies conducted in 1984 (Fig. 22) indicated that traps baited with (±)-ipsdienol at high or low release rates were not significantly more attractive than unbaited control traps. When the attractive bait composed of myrcene + *trans*-verbenol + *exo*-brevicomin was combined with (±)-ipsdienol at high or low release rates, trap catch was significantly lower than for myrcene +

Table 12. Quantities and isomeric composition of ipsdienol and myrcenol produced by male and female *D. ponderosae* when exposed to myrcene through feeding or vapours.

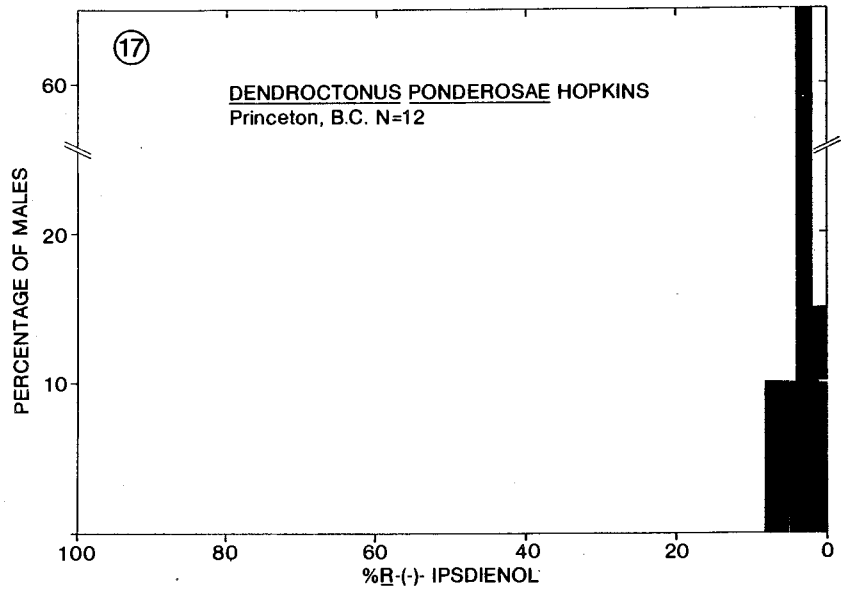
Method of exposure to myrcene	Sex	Ipsdienol			Myrcenol		
		No. exposed	ng/beetle $\bar{x}$±SE	%(+) $\bar{x}$±SE	No. exposed	ng/beetle $\bar{x}$±SE	%*E* $\bar{x}$±SE
Vapours, 24 h	Male[a]	20	1946±497	97.0±0.3	9	1497±215[c]	90.3±4.0
	Male[b]	9	1820±673	94.5±1.1			
	Female	17	<10	-	9	427±119	98.0±0.7
Fed alone for 24 h on lodgepole pine logs	Male	12	<10	-	12	<10	-
	Female	12	<10	-	12	<10	-
Fed for 24 h with an individual of the other sex	Male	12	<10	-	12	<10	-
	Female	12	<10	-	12	<10	-
No exposure; held for 24 h at room temperature	Male	12	<10	-	12	<10	-
	Female	12	<10	-	12	<10	-

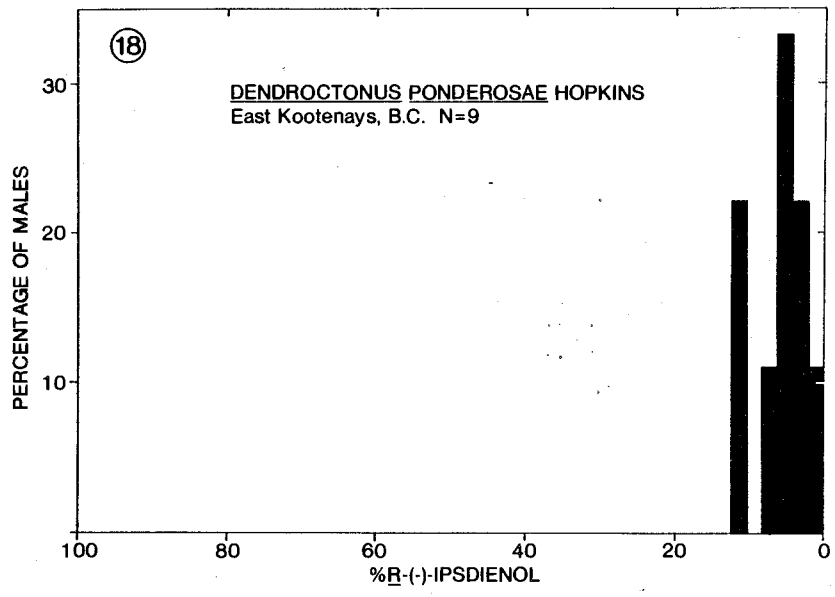
[a]Beetles from near Princeton in southwestern B.C.

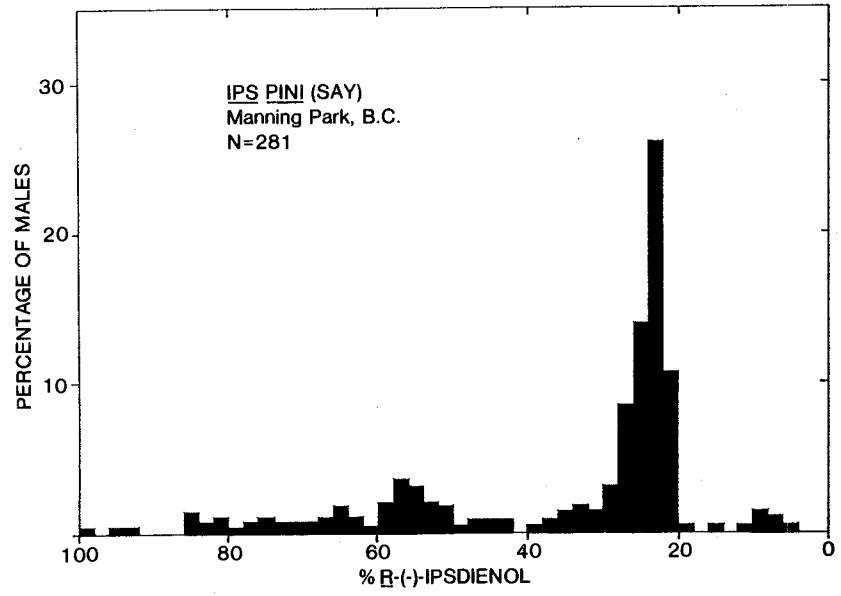
[b]Beetles from near Cranbrook in southeastern B.C.

[c]Myrcenol content significantly different between sexes, Mann-Whitney U test, $P < 0.01$.

Figures 17-20. Chirality of ipsdienol produced by individual male *D. ponderosae* and *I. pini* from southwestern (Figs. 17, 19), and southeastern B. C. (Fig. 18, 20). *D. ponderosae* exposed to myrcene vapours for 24 h, and *I. pini* fed on ponderosa pine bolts for 24 h. *I. pini* data adapted from Miller *et al.* (1987).


17
DENDROCTONUS PONDEROSAE HOPKINS
Princeton, B.C. N=12
PERCENTAGE OF MALES
60
20
10
100
80
60
40
20
0
%R-(-)- IPSDIENOL


18
DENDROCTONUS PONDEROSAE HOPKINS
East Kootenays, B.C. N=9
PERCENTAGE OF MALES
30
20
10
100
80
60
40
20
0
%R-(-)-IPSDIENOL


IPS PINI (SAY)
Manning Park, B.C.
N=281
PERCENTAGE OF MALES
30
20
10
100
80
60
40
20
0
% R-(-)-IPSDIENOL

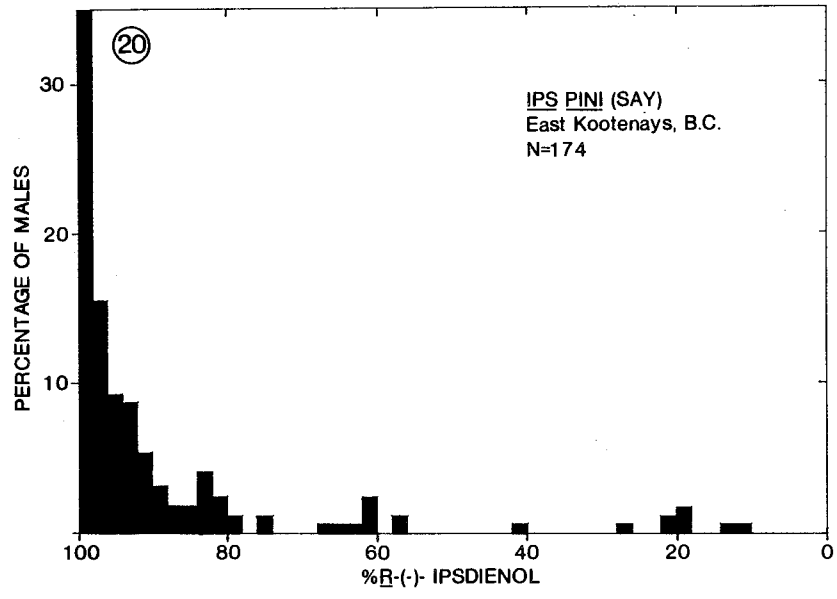

20
IPS PINI (SAY)
East Kootenays, B.C.
N=174
PERCENTAGE OF MALES
30
20
10
100
80
60
40
20
0
%R-(-)- IPSDIENOL

Table 13. Positive responses of walking *D. ponderosae* to (+)-, (-)-, and (±)-ipsdienol.

Exp. no.	Stimulus	Percent response ($\bar{x}$±SE)[ab]					
		Females			Males		
		A	B	C	D	E	F
1	pentane, 20 µl	4±2 a	5±2 a	4±2 a	4±2 a		
	(+)-ipsdienol, 4 ng	2±2 a	10±3 a	2±1 a	6±4 a		
	" , 40 ng	12±4 a	8±3 a	3±2 a	8±4 a		
	" , 400 ng	28±2 b	22±4 b	3±2 a	20±4 ab		
	" , 4,000 ng	30±3 b	27±4 b	4±2 a	26±2 bc		
	" , 40,000 ng	30±4 b	26±4 b	9±3 a	30±3 bc		
	" , 400,000 ng		40±5 c				
	♀ frass extract, 0.02 g equiv.	38±4 b	38±5 c	30±4 b	38±7 c		
2	pentane, 20 µl	4±2 a	5±2 a		4±2 a		
	(-)-ipsdienol, 4 ng	4±2 a	5±2 a		6±2 a		
	" , 40 ng	8±2 ab	11±3 a		8±2 a		
	" , 400 ng	12±2 ab	5±2 a		10±3 a		
	" , 4,000 ng	8±2 ab	7±2 a		8±4 a		
	" , 40,000 ng	16±5 ab	9±2 a		14±2 ab		
	" , 400,000 ng	22±4 b	27±3 b		26±7 bc		
	♀ frass extract, 0.02 g equiv.	38±7 c	37±4 c		38±7 c		
3	pentane, 20 µl	5±3 a	8±3 a	6±4 a	5±3 a	10±4 a	10±4 ab
	(±)-ipsdienol, 4 ng	3±3 a	23±6 ab	10±4 ab	3±3 a	28±6 ab	20±4 ab
	" , 40 ng	13±3 a	18±5 ab	16±3 ab	10±0 a	25±3 ab	10±0 ab
	" , 400 ng	13±3 a	18±9 ab	8±4 a	13±3 a	25±6 ab	14±2 ab
	" , 4,000 ng	15±3 a	8±8 a	8±4 a	15±3 a	18±3 a	4±4 a
	" , 40,000 ng	13±3 a	33±5 b	6±3 a	5±3 a	40±8 b	10±4 ab
	" , 400,000 ng	65±3 b		22±4 b	45±3 b		20±3 b
	♀ frass extract, 0.02 g equiv.	45±6 b	40±4 b	36±4 c	40±0 b	43±3 b	36±4 c

[a] Letters A-F refer to separate series of bioassays. Means in a column within an experiment followed by the same letter are not significantly different ($P > 0.05$), Newman-Keuls test.

[b] Forty beetles tested/treatment in experiments 3A, 3B, 3D, and 3E; 50 beetles tested/treatment in experiments 1A, 1D, 2A, 3C, and 3F; 100 beetles tested/treatment in experiments 1B, 1C, and 2B.

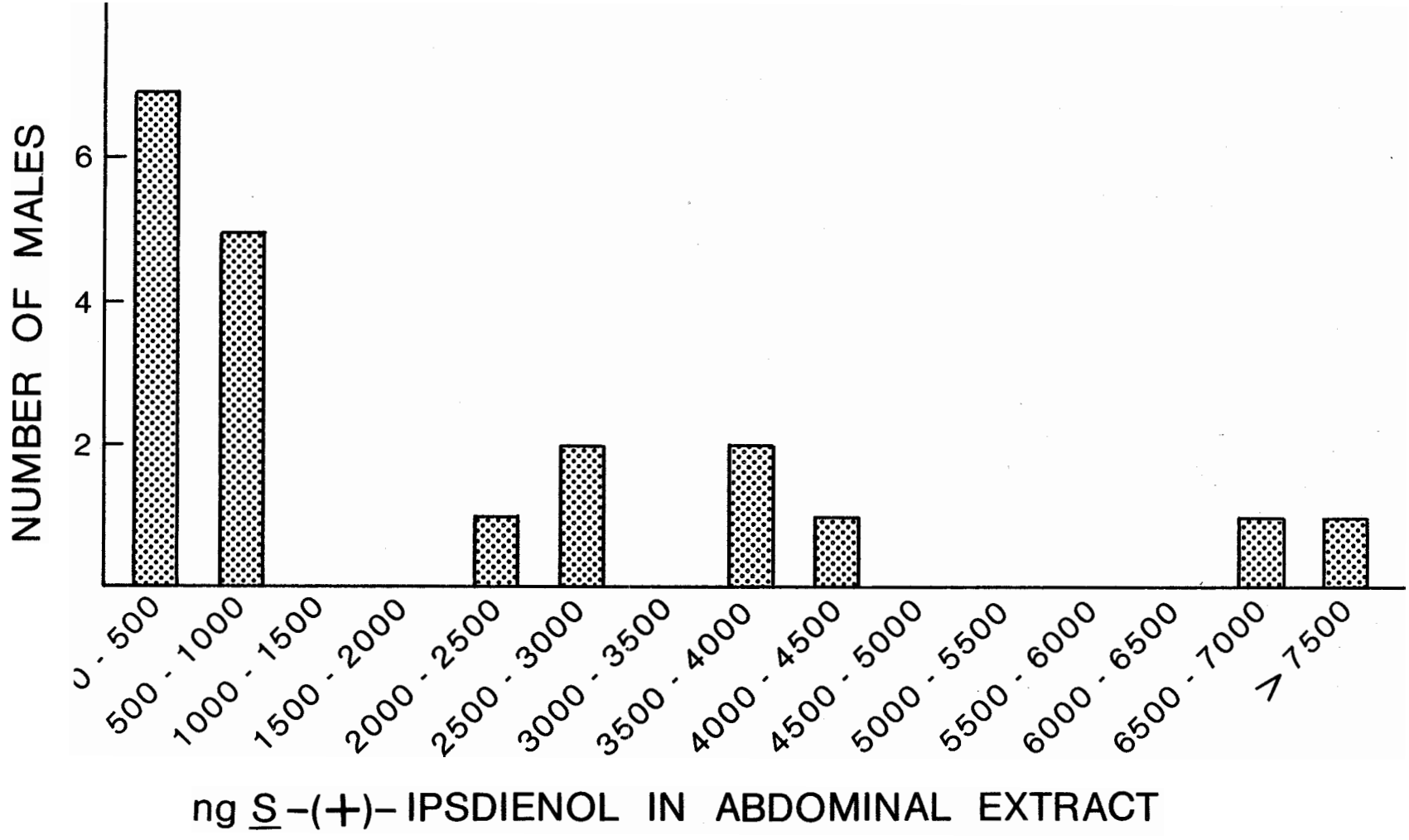
Table 14. Positive responses of walking *D. ponderosae* to (+)-, (-)-, and (±)-ipsdienol in combination with female frass extracts.

Exp. no.	Stimulus	Percent response ($\bar{x}$±SE)[ab] Females A	Females B	Males C
4	pentane, 20 µl	4±2 a	10±3 a	10±7 a
	♀ frass extract, 0.02 g equiv., with (+)-ipsdienol, 4 ng	13±5 ab	40±3 b	30±4 b
	" " " , 40 ng	20±6 abc	41±5 b	40±4 bc
	" " " , 400 ng	19±3 abc	36±5 b	46±4 c
	" " " , 4,000 ng	17±4 abc	42±4 b	53±3 c
	" " " , 40,000 ng	18±3 abc	49±5 b	56±6 c
	" " " , 400,000 ng	24±3 bc	45±5 b	
	♀ frass extract, 0.02 g equiv.	30±4 c	51±6 b	73±2 d
5	pentane, 20 µl	4±2 a		9±3 a
	♀ frass extract, 0.02 g equiv., with (-)-ipsdienol, 4 ng	11±3 ab		29±4 b
	" " " , 40 ng	20±4 bc		35±3 b
	" " " , 400 ng	25±5 c		34±5 b
	" " " , 4,000 ng	28±5 c		41±6 b
	" " " , 40,000 ng	34±4 c		48±3 b
	" " " , 400,000 ng	30±4 c		
	frass extract, 0.02 g equiv.	30±4 c		39±7 b
6	pentane, 20 µl	6±2 a		10±3 a
	♀ frass extract, 0.02 g equiv., with (±)-ipsdienol, 4 ng	14±2 ab		16±2 a
	" " " , 40 ng	20±4 b		22±4 abc
	" " " , 400 ng	20±4 b		20±3 ab
	" " " , 4,000 ng	22±4 b		30±3 bc
	" " " , 40,000 ng	28±4 bc		34±5 bc
	" " " , 400,000 ng	40±3 c		34±2 c
	♀ frass extract, 0.02 g equiv.	36±2 c		36±4 c

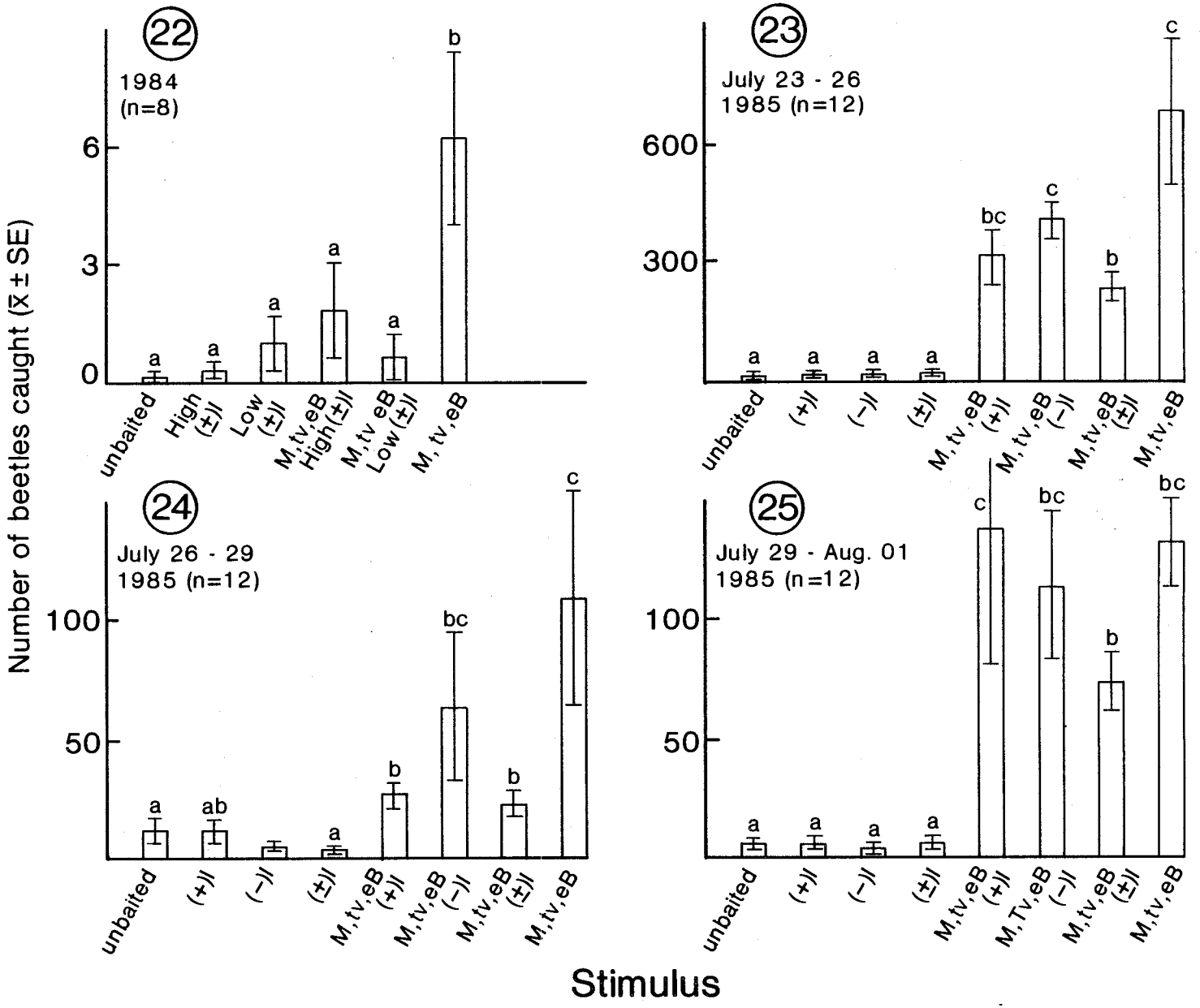
[a] Letters A-C refer to separate series of bioassays. Means in a column within an experiment followed by same letter are not significantly different ($P > 0.05$), Newman-Keuls test.

[b] Fifty beetles tested/treatment in experiments 4A and 4C; 100 beetles tested/treatment in experiments 5A, 5C, 6A, and 6C.

Figure 21. Quantity of ipsdienol produced by 20 individual male *D. ponderosae* from southwestern B.C. exposed to myrcene vapours.


NUMBER OF MALES
6
4
2
0 - 500
500 - 1000
1000 - 1500
1500 - 2000
2000 - 2500
2500 - 3000
3000 - 3500
3500 - 4000
4000 - 4500
4500 - 5000
5000 - 5500
5500 - 6000
6000 - 6500
6500 - 7000
> 7500
ng S-(+)- IPSDIENOL IN ABDOMINAL EXTRACT

Figures 22-25. Average numbers of *D. ponderosae* caught (±SE) in multiple funnel traps baited with beetle-produced volatiles and host monoterpenes. Experiments conducted in 1984 and 1985 in the Shinish Cr. Valley, 30 km northeast of Princeton, B. C. In each experiment values with the same letter are not significantly different ($P > 0.05$), non-parametric multiple multiple comparisons test (Conover, 1980, p. 231).


22
1984
(n=8)
23
July 23 - 26
1985 (n=12)
24
July 26 - 29
1985 (n=12)
25
July 29 - Aug. 01
1985 (n=12)
Number of beetles caught (x̄ ± SE)
Stimulus
unbaited
High (±)
Low (±)
M,tv,eB High(±)
M,tv eB Low(±)
M, tv, eB
(+)
(−)
(±)
M,tv,eB (+)
M,tv,eB (−)
M,tv,eB (±)
M,Tv,eB (−)

trans-verbenol + *exo*-brevicomin alone, indicating that (±)-ipsdienol inhibited response of *D. ponderosae* to these attractive semiochemicals.

Field trapping studies conducted in 1985 (Figs. 23-25) generally supported the results obtained in 1984. In all 3 experiments traps baited with (±), (+), or (-)-ipsdienol were not significantly more attractive than unbaited control traps. In 2 of 3 experiments, (±)-ipsdienol significantly inhibited response to myrcene + *trans*-verbenol + *exo*-brevicomin. (+)-Ipsdienol significantly inhibited the attractiveness of myrcene + *trans*-verbenol + *exo*-brevicomin in 1 of 3 experiments. (-)-Ipsdienol did not induce a significant inhibition in any of the field experiments.

Due to the large numbers of beetles caught in traps in the 1985 experiments sex was only determined for the beetles caught in the July 26-29 experiment. The ratios of males to females caught were not significantly different ($P > 0.05$, Newman-Keuls test) for all baits containing myrcene + *trans*-verbenol + *exo*-brevicomin. However, these ratios (range 59-70% male) were all significantly higher ($P < 0.05$, Newman-Keuls test) than those for baits which contained only ipsdienol, or for unbaited traps (range 22-34% male). This result may indicate that traps baited with the standard semiochemical bait composed of myrcene + *trans*-verbenol + *exo*-brevicomin were more attractive to males than females. Ipsdienol had no effect on the sex ratios of the beetles caught in traps.

In 1984 and 1985 the mean attack densities on trees baited with myrcene + *trans*-verbenol + *exo*-brevicomin were higher than on trees which were also baited with (±), (+) or (-)-ipsdienol (Table 15), although the effect was never statistically significant. Neither (±), (+) nor (-)-ipsdienol alone induced attack on the baited trees at levels above unbaited controls.

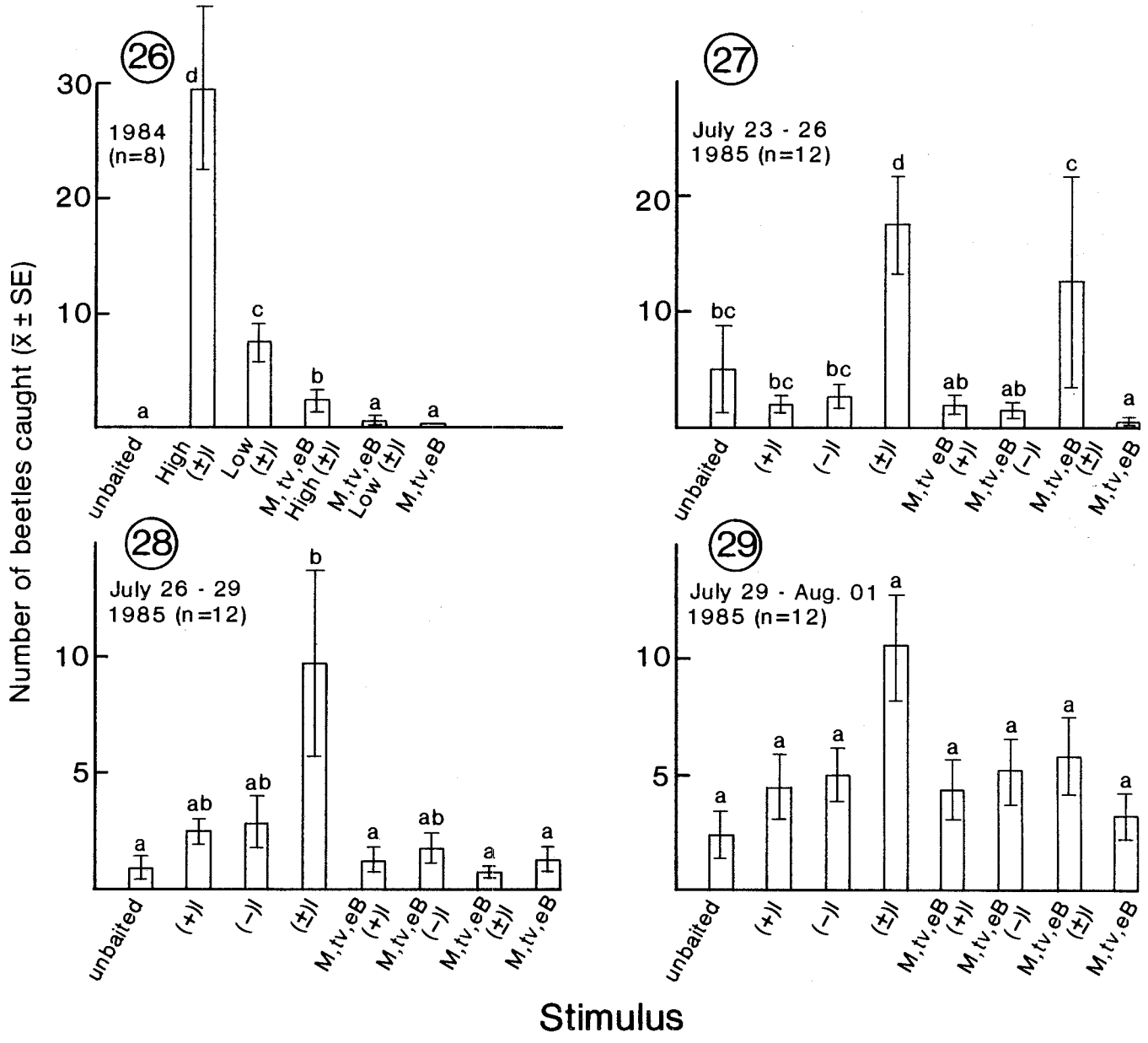
Although the field trapping studies were designed to test the responses of *D. ponderosae*, the traps also caught numerous *I. pini* (Figs. 26-29). The 1984 data indicated that *I. pini* were strongly attracted to (±)-ipsdienol, with the high release rate being significantly more attractive than the low release rate (Fig. 26). Traps baited with myrcene + *trans*-verbenol + *exo*-brevicomin were not significantly more attractive than the unbaited control traps. When this 3-component mountain pine beetle bait was added to (±)-ipsdienol, the attractiveness of (±)-ipsdienol to *I. pini* was significantly reduced. Thus, either myrcene, *trans*-verbenol, *exo*-brevicomin or some combination of these

Table 15. Ranked attack densities of *D. ponderosae* on lodgepole pines (*Pinus contorta*) baited with host monoterpenes and beetle-produced volatiles. Shinish Cr. Road, 30 km northeast of Princeton, B.C.

Year and no. of replicates	Treatment	dbh ($\bar{x}$±SE[a])	Trees attacked No.	Trees attacked %	Attack density/m^2 ($\bar{x}$±SE[a])
1984 (n=10)	M,tV,eB,High(±)I	24.6±1.5 a	5	50	17±12 a
	Blank	25.0±1.7 a	2	20	18± 9 a
	High(±)I	24.3±1.1 a	5	50	23± 7 a
	M,tV,eB	23.0±0.6 a	6	60	24±18 a
1985 (n=11)	Blank	25.7±0.6 a	1	9	2± 2 a
	(+)I	25.3±0.9 a	1	9	3± 3 a
	(-)I	25.6±0.8 a	1	9	6± 6 a
	(±)I	26.0±0.8 a	2	18	7± 6 a
	M,tV,eB,(+)I	25.8±0.6 a	9	82	40± 9 b
	M,tV,eB,(±)I	25.7±1.0 a	10	91	43±13 b
	M,tV,eB,(-)I	26.9±1.0 a	9	82	44± 8 b
	M,tV,eB	28.1±1.0 a	10	91	55±10 b

[a]Values for treatments in each experiment followed by the same letter are not significantly different ($P > 0.05$), Newman-Keuls test.

Figures 26-29. Average numbers of *I. pini* caught (±SE) in multiple funnel traps baited with beetle-produced volatiles and host monoterpenes. Experiments conducted in 1984 and 1985 at Shinish Cr. Road, 30 km northeast of Princeton, B. C. In each experiment values with the same letter are not significantly different ($P > 0.05$), non-parametric multiple comparisons test (Conover, 1980, p. 231).


26
1984
(n=8)
27
July 23 - 26
1985 (n=12)
28
July 26 - 29
1985 (n=12)
29
July 29 - Aug. 01
1985 (n=12)
Number of beetles caught (X̄ ± SE)
Stimulus
unbaited
High (±)
Low (±)
M,tv,eB High (±)
M,tv,eB Low (±)
M,tv,eB
(+)
(−)
(±)
M,tv eB (+)
M,tv,eB (+)
M,tv,eB (−)
M,tv,eB (±)

compounds inhibited the response of *I. pini* to its aggregation pheromone.

The 1985 data for *I. pini* generally supported the 1984 data, although in the July 29 - Aug. 01 experiment there were no significant differences in responses (Figs. 27-29). In addition in all 1985 tests neither (+) nor (-)-ipsdienol were more attractive than an unbaited control, indicating that the 2 enantiomers were synergistic in attracting *I. pini* in this region.

Discussion

Bioassays show that (+)-ipsdienol may weakly inhibit the attraction of both sexes of *D. ponderosae* to the attractive semiochemicals present in extracts of female frass in the laboratory (Table 14), as well as inhibiting attraction in the field to a blend of myrcene + *trans*-verbenol + *exo*-brevicomin (Figs. 22-25; Table 15). Thus, the 94.5-97.0% (+)-ipsdienol produced in large quantities by male *D. ponderosae* from 2 regions of southern B. C. (Table 12) may function as an antiaggregation pheromone in this species. In this way male beetles could regulate the attack density and/or terminate the attack on host trees, which would help to prevent overly high attack densities and increased competition within hosts and reduced brood survival (Reid 1963). When the (+)-ipsdienol concentration reached a high level, in-coming beetles would avoid that host and attack adjacent trees. It is also possible that the weakly inhibitory (+)-ipsdienol could act as an epideictic or spacing pheromone on individual host trees, facilitating a more efficient partitioning of the phloem in that host.

Several other compounds which are produced by *D. ponderosae* have proven to be inhibitory to these beetles in field tests, leading to speculation that they may act as antiaggregation pheromones. These compounds include frontalin (Ryker and Libbey 1982), *exo*- and *endo*-brevicomin (Ryker and Rudinsky 1982), verbenone (Ryker and Yandell 1983) and pinocarvone (Libbey *et al.* 1985). The reduction in trap catch due to ipsdienol (Figs. 22-25) is similar in magnitude to the reductions caused by these other compounds. Further study is required to determine how any or all of these compounds act to modify the behaviour of *D. ponderosae* in the natural system, as well as whether these compounds have any potential as pest management tools to protect stands of pine from attack by *D. ponderosae*.

Although male *D. ponderosae* produce large quantities of (+)-ipsdienol (Table 12), (±)-ipsdienol has a much stronger inhibitory effect than (+)-ipsdienol on the attraction of *D. ponderosae* to its aggregative semiochemicals (Figs. 22-25; Tables 14, 15). This strongly inhibitory activity of (±)-ipsdienol suggests that *D. ponderosae* in southwestern B. C. may be exposed to an approximately racemic mixture of ipsdienol in nature. Although *I. pini* from California, Idaho and southeastern B. C. produce predominantly (-)-ipsdienol (Birch *et al.* 1980; Plummer *et al.* 1976; Slessor *et al.* 1985), *I. pini*

from Manning Park, about 65 km southwest of my study site, produce an average of 34% (-)-ipsdienol with tremendous variation among individuals (Fig. 19). Possibly the strong inhibitory effect of (±)-ipsdienol on *D. ponderosae* in my experiments represents an adaptation to avoid competition from a sympatric population of *I. pini* that produces both enantiomers of ipsdienol in substantial amounts. In this way *D. ponderosae* in the Princeton-Manning Park area could identify and avoid hosts which have been attacked by *I. pini* based on the presence of the 34% (-)-ipsdienol produced by *I. pini* in this region. This avoidance response would be advantageous to *D. ponderosae*, as *I. pini* develop more rapidly and would likely outcompete *D. ponderosae* within host trees.

It would be interesting to test for inhibitory effects of (+), (-) and (±)-ipsdienol on *D. ponderosae* in California, Idaho and southeastern B. C. where *I. pini* produce predominantly (-)-ipsdienol (Birch *et al.* 1980; Plummer *et al.* 1976; Slessor *et al.* 1985). It is possible that selection pressure due to competition with *I. pini* in these areas would favour *D. ponderosae* which were more strongly inhibited by (-)-ipsdienol than by (±)-ipsdienol. However, my data indicate that the chirality of ipsdienol produced by *D. ponderosae* in southeastern B. C. (Table 12, Fig. 18) and southwestern B. C. (Table 12, Fig. 17) does not reflect the differences in chirality found in *I. pini* from these regions (Fig. 19, 20).

It is not known why the inhibitory effect of ipsdienol on the attraction of *D. ponderosae* to extracts of female frass in the laboratory was more pronounced at low than at high concentrations (Table 14). The decreased inhibition at higher concentrations may be due to the slight attractive effect of ipsdienol which was noted at higher concentrations (Table 13).

The pronounced attractiveness of ipsdienol in the laboratory at the highest concentrations tested (400,000 ng/20 µl pentane and 40,000 ng/20 µl pentane) (Table 13) is somewhat surprising because of the inhibitory effects of (+), (-) and (±)-ipsdienol on the response of *D. ponderosae* to frass extracts (Table 14). However, the concentrations of ipsdienol which are significantly more attractive than the pentane controls are probably higher than any concentrations likely to occur in nature. Although it is possible that ipsdienol could be a multi-functional pheromone in *D. ponderosae* and that it has different effects depending on the concentration, it is more likely that the attraction was an artifact of using artificially high concentrations, or that a minor contaminant in the ipsdienol was causing the

attraction.

Although Pitman *et al.* (1966) cautioned that variation in olfactory behavior between laboratory and field bioassays greatly limits the usefulness of laboratory bioassays, their reservations have proven to be without foundation for many species. However, *D. ponderosae* bioassay results are often very different depending on whether the study is conducted in the field or on walking beetles in a laboratory olfactometer. While myrcenol was attractive to *D. ponderosae* of both sexes in laboratory bioassays (Conn 1981), it slightly inhibited the response to other attractants in the field (Conn *et al.* 1983). Similarly, 3-caren-10-ol was attractive in the laboratory (Conn 1981), but did not affect total trap catch in the field (Conn *et al.* 1983). My laboratory and field results for ipsdienol are also contradictory. The divergence of laboratory and field results may be due to differences in the responses to natural frass in the laboratory and the 3-component semiochemical bait in the field. Alternatively, these differences may be due to concentration effects, or to the test compounds having different effects on flying and walking insects, as well as different effects when tested alone or in combination with other compounds. Until proven reliable, bioassays conducted with walking beetles in the laboratory should not be used as anything more than a preliminary test of compounds which should subsequently be field tested.

The response of *I. pini* to multiple funnel traps baited with (±)-ipsdienol, as well as the lack of a response to (+) or (-)-ipsdienol (Figs. 26-29), indicates that *I. pini* in this region requires both enantiomers to induce a response. This result is in agreement with the findings of Miller *et al.* (1987) that *I. pini* from nearby Manning Park produces an approximately 34:66 ratio of (-) to (+)-ipsdienol on the average (Fig. 19). This ratio of ipsdienol enantiomers suggests that *I. pini* from southwestern B. C. are genetically close to New York populations, which also produce both enantiomers in a similar ratio, and for which both enantiomers act synergistically to produce a response (Lanier *et al.* 1980). These 2 populations are clearly distinct from populations from California (Birch *et al.* 1980), Idaho (Plummer *et al.* 1976) and the Kootenay region of southeastern B.C. (Fig. 20).

The inhibitory effect of the 3-component mountain pine beetle bait on the response of *I. pini* to (±)-ipsdienol indicates that either myrcene, *trans*-verbenol, *exo*-brevicomin or some combination of these compounds inhibits the response of *I. pini* to its aggregation pheromone. Birch *et al.* (1980) have

shown that *I. pini* from California are attracted to (-)-ipsdienol, the naturally predominating enantiomer, while (+)-ipsdienol interrupted the response of *I. pini* to an attractive source in field tests. Noteably (+)-ipsdienol is produced by *I. paraconfusus* (Silverstein *et al.* 1966) and *D. brevicomis* (Byers 1982), both of which are sympatric competitors of *I. pini* in California. Thus, in regions where *I. pini* is repelled by (+)-ipsdienol it could act as a repellent allomone to keep *I. pini* out of trees attacked by *I. paraconfusus* (Birch *et al.* 1980), *D. brevicomis* (Byers 1982) or *D. ponderosae*. It should be noted that Byers (1982) suggested that ipsdienol may be involved in regulating the colony density of *D. brevicomis* without testing the effect of (+)-ipsdienol. My data on the inhibitory effects of (+) and (±)-ipsdienol for *D. ponderosae* suggests that Byers (1982) may not have been justified in assuming that the inhibitory activity which he found in (±)-ipsdienol would necessarily be present with (+)-ipsdienol, the enantiomer produced by *D. brevicomis*.

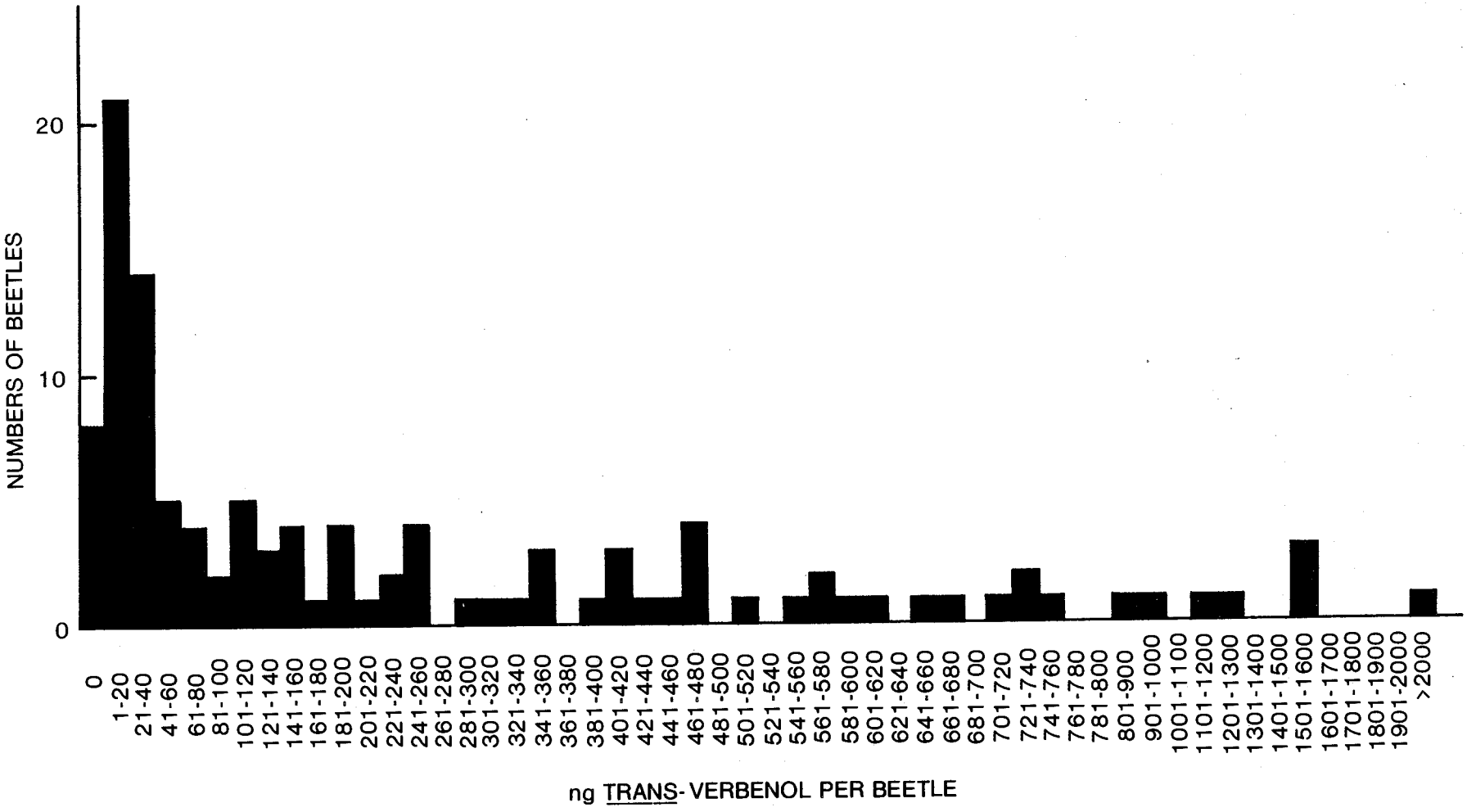
If *trans*-verbenol or *exo*-brevicomin, which are both produced by *D. ponderosae*, are involved in inhibiting the attraction of *I. pini* to (±)-ipsdienol then *D. ponderosae* would be using them as repellent allomones to reduce interspecific competition for the same hosts. In this way the activity of (+)-ipsdienol as a repellent allomone against *I. pini* may be replaced in southwestern B. C. by the inhibitory activity of one of these compounds. Alternatively, *I. pini* may simply be avoiding high concentrations of myrcene due to their preference for dead and dying trees which probably have more tolerable levels of monoterpenes.

VIII. INDIVIDUAL VARIATION IN PHEROMONE CONTENT AND ITS POSSIBLE SIGNIFICANCE IN NATURE

The production by *D. ponderosae* and *I. paraconfusus* of all of the terpene alcohols which were quantified during this study generally followed the distributions shown for the production of *trans*-verbenol by individual female *D. ponderosae* (Fig. 30), and ipsdienol by male *D. ponderosae* (Fig. 21). In all experiments and with all treatments there were large numbers of beetles which did not produce quantifiable levels of any terpene alcohol. This large number of zeros in the data, combined with the heterogeneity of variances and lack of normality, resulted in some difficulty in finding an appropriate statistical test for use with these data. As a result some treatments which resulted in a 2 to 3 fold change in levels of production of terpene alcohols were not statistically significant with the nonparametric multiple comparisons test used (Conover 1980, p. 231). It may be justifiable to drop the zeros from the data before statistical analysis on the grounds that comparisons of terpene alcohol production levels should only be made for those individuals which are capable of producing those compounds. Alternatively, if these values are not truely zeros, but are simply quantities which are below the detection threshold for the analytical techniques used, then it may be possible to assign values to these samples. Either of these procedures would result in a number of additional significant differences in comparisons between treatments.

The pattern of variation in total *trans*-verbenol content in individual female *D. ponderosae* (Fig. 30) is in agreement with data for ipsdienol in *I. pini* (Borden *et al.* 1986) and *cis*-verbenol in *I. typographus* (Birgersson *et al.* 1984). Borden *et al.* (1986) speculated that the few beetles which produce very large quantities of aggregation pheromones may be those that are successful pioneer beetles. It is also possible that there are basically 2 different strategies being employed by individual bark beetles. The small proportion of the population which produces large quantities of aggregation pheromones, the "producers", could be composed of pioneer individuals which initiate attacks on new host trees by starting very attractive sources of aggregation. This segment of the population risks being killed by the defences of vigorous hosts, but benefits by increasing the likelihood of obtaining an

Figure 30. Content of *trans*-verbenol in individual female *D. ponderosae* extracted after 24 h exposure to *alpha*-pinene vapour. Data combined from several experiments (n=114), all beetles from southwestern B. C.


NUMBERS OF BEETLES
20
10
0
0
1-20
21-40
41-60
61-80
81-100
101-120
121-140
141-160
161-180
181-200
201-220
221-240
241-260
261-280
281-300
301-320
321-340
341-360
361-380
381-400
401-420
421-440
441-460
461-480
481-500
501-520
521-540
541-560
561-580
581-600
601-620
621-640
641-660
661-680
681-700
701-720
721-740
741-760
761-780
781-800
801-900
901-1000
1001-1100
1101-1200
1201-1300
1301-1400
1401-1500
1501-1600
1601-1700
1701-1800
1801-1900
1901-2000
>2000
ng TRANS-VERBENOL PER BEETLE

adequate supply of high quality phloem in which to feed and breed. The remainder of the population would then be composed

of "responder" individuals which do not produce large quantities of aggregation pheromones, but instead rely on the pheromones produced by successful pioneers. These beetles would not face the same risks from the host tree defences since they do not attack until mass-aggregation has begun to weaken the tree. However, they would risk the possibility of attacking a host too late to obtain a portion of the phloem suitable for gallery construction and brood survival, as well as the risk of being unable to attract a mate. It would be interesting to test for a relationship between the relative time of emergence from an individual host tree and the quantities of aggregation pheromones subsequently produced, as "producers" (or pioneers) may emerge earlier than "responders".

In *D. pseudotsugae* a portion of the emergent adults contain <20% lipid and are responsive to attractant semiochemicals without a dispersal flight, while the remainder of the beetles, which contain greater lipid reserves, are not responsive until flight exercise has metabolized a portion of these reserves (Atkins 1966, 1969). The latter individuals, which are initially photopositive and host-negative, sacrifice the opportunity to breed in nearby hosts for the chance to interbreed in more distant trees which may be more suitable hosts. These 2 strategies also appear to be found in *S.multistriatus* for which beetles which emerged from host logs surrounded by traps in 5 concentric circles responded in greatest numbers to the traps in the nearest ring (20 m) and the most distant ring (600 m) (Lanier *et al.* 1976). It is possible that there is a relationship in bark beetles between the quantities of aggregation pheromones produced, the time of emergence in relation to other individuals, the lipid content, and the need for dispersal flight exercise before becoming responsive to attractive semiochemicals. Possibly early-emergers are "producers" (pioneers) which contain large lipid reserves and disperse long distances before producing or becoming responsive to attractive semiochemicals.

As discussed in Section III, adult female *D. ponderosae* which are held on bark in petri dishes for a longer time than adult beetles naturally spend beneath the bark are capable of producing *trans*- and *cis*-verbenol and myrtenol at levels which are higher than those found in wild beetles (Figs. 1-3). This capability may be due to the beetles metabolizing nutrient reserves during the prolonged period of

walking exercise in the petri dishes, which may mimic the effect of lipid metabolism during flight exercise, as Gries (1984) demonstrated for *I. typographus*. Thus an alternative hypothesis to explain the distribution of terpene alcohol production in Fig. 30 is that the individuals which can produce large amounts of pheromone immediately following emergence (Fig. 30; Borden *et al.* 1986; Birgersson *et al.* 1984) are simply those beetles which have low lipid reserves and which produce and respond to pheromones without a dispersal flight. Those individuals which can not produce large quantities of terpene alcohols upon emergence (Fig. 30; Borden *et al.* 1986; Birgersson *et al.* 1984) might become capable of greatly increased pheromone production following the depletion of their nutrient reserves through flight exercise or starvation.

IX. CONCLUSION

The oxidation of host tree monoterpenes by *D. ponderosae* and *I. paraconfusus* has been fairly well studied by a variety of workers, as has the behavioural activity of the resulting compounds. However, there is still relatively little known about the mechanisms of production of these compounds.

My data indicate that both sexes of *D. ponderosae* and *I. paraconfusus* are capable of producing their full complement of terpene alcohols at normal or elevated levels in the absence of readily-culturable microorganisms. The only exception to this trend is the production of ipsenol and ipsdienol in male *I. paraconfusus*, for which there is some evidence that microbial involvement may be required to produce normal levels of these compounds. The increased levels of certain terpene alcohols in axenically-reared or streptomycin-fed beetles compared to wild beetles suggests that certain of their symbiotic microorganisms may actually be responsible for breaking down these compounds rather than producing them. The wide-spread ability of certain microorganisms, such as the yeasts examined in this and other studies, to metabolize verbenols to verbenone is supportive of this hypothesis. It is also possible that certain microorganisms found in association with *D. ponderosae* and *I. paraconfusus* may metabolize monoterpene precursors of pheromones into products other than terpene alcohols, so that some of the precursor is unavailable to the beetles. Alternatively, it is possible that reducing the levels of microorganisms in the beetles through axenic-rearing or antibiotic-feeding may remove microorganisms which are mildly pathogenic to the beetles, and that the resultant healthier beetles simply produce terpene alcohols at higher than normal levels.

The production of normal or elevated quantities of most terpene alcohols by both sexes of *D. ponderosae* and *I. paraconfusus* in the absence of readily-culturable microorganisms does not necessarily indicate that microorganisms associated with these beetles are unable to produce these same compounds. Wild female *D. ponderosae* which were fed on lodgepole pine were found to contain low levels of *trans*-verbenol, while axenically-reared insects contained high levels of *trans*-verbenol following exposure to *alpha*-pinene vapours but not after feeding. This apparent anomaly suggests that following inhalation of *alpha*-pinene vapours, adult *D. ponderosae* produce

most of their *trans*-verbenol somewhere other than the gut. This *trans*-verbenol is then transported to the hindgut through the Malpighian tubules, where it is probably augmented by small amounts of *trans*-verbenol produced by gut microorganisms.

In contrast to the aggregation pheromones and other terpene alcohols examined during this study, there is considerable evidence for significant microbial involvement in the production of the antiaggregation pheromone verbenone in *D. ponderosae*. This ketone was not produced in quantifiable levels by axenically-reared or streptomycin-fed beetles which were exposed to *alpha*-pinene as vapours or through feeding. However, significant quantities of verbenone were found in wild *D. ponderosae* which were exposed to *alpha*-pinene through feeding on bolts of lodgepole pine. This result suggests that verbenone is not formed by the beetle, but is produced by microorganisms in its gut. The ability of *H. capsulata*, *P. pinus* and other yeasts which are closely associated with *D. ponderosae* and other bark beetles to convert verbenols to verbenone *in vitro* is supportive of the hypothesis that microorganisms, rather than beetles, produce verbenone. The substantial growth of these yeasts in bark beetle galleries, particularly during phases of attack at which verbenone is known to be produced in large quantities (Leufvén and Nehls 1986), is also supportive of this hypothesis, as is the consistent presence of *H. capsulata* and *P. pinus* in *D. ponderosae* galleries (Whitney 1971) and mycangia (Whitney and Farris 1970).

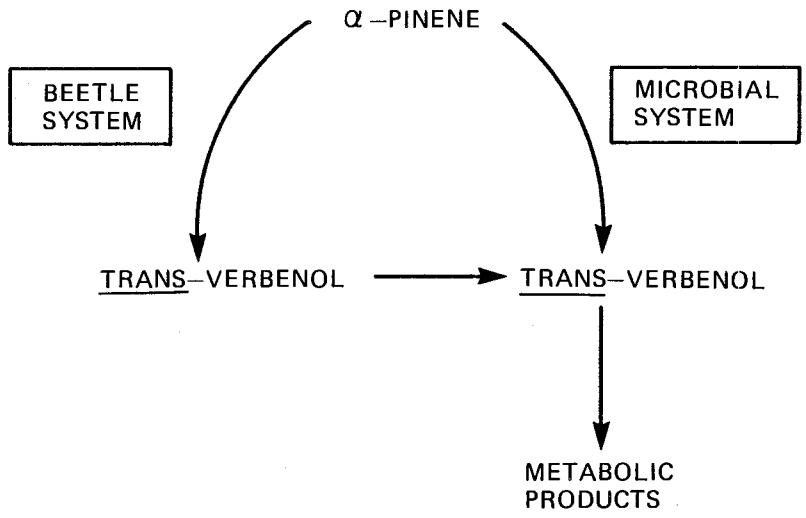
I have presented evidence that polysubstrate monooxygenase enzymes are involved in the production of pheromones and other terpene alcohols from host monoterpenes. However, this finding is not inconsistent with the hypothesis that microorganisms are involved in producing their hosts' pheromones, since these reactions could be catalyzed by monooxygenase enzymes present in the beetles, in their symbiotic microorganisms, or both. The question of which organism's enzymes are involved could be addressed by applying monooxygenase inhibitors to microorganisms which are capable of the *in vitro* production of their hosts' pheromones, as well as by treating axenically-reared beetles with monooxygenase inhibitors. Further research on the role of polysubstrate monooxygenase enzymes in the production of bark beetle pheromones should be conducted by exposing the microsomal fraction of these insects to pheromone precursors *in vitro*.

I present evidence that *alpha*-pinene can autoxidize to *trans*- and *cis*-verbenol and verbenone. This reaction may occur at behaviourally significant levels under certain situations. Many species of bark beetles which use *trans*- or *cis*-verbenol as a pheromone have also been reported to use *alpha*-pinene as a kairomone. It is possible that for some species this conclusion is erroneous, and that the activity of *alpha*-pinene is partly or entirely due to verbenol or verbenone "contaminants" produced through autoxidation. In addition, certain phenomena, such as the high incidence of attack by bark beetles on lightning-struck and wounded trees, could be explained by an induction of attack by the products of *alpha*-pinene autoxidation. If further research which quantifies the autoxidation of *alpha*-pinene from wounds in living trees and from *alpha*-pinene used in semiochemical baits, confirms that this process is occurring at a biologically significant rate, then experiments involving *alpha*-pinene would need to be re-evaluated, as would the use of *alpha*-pinene in semiochemical baits used in beetle management.

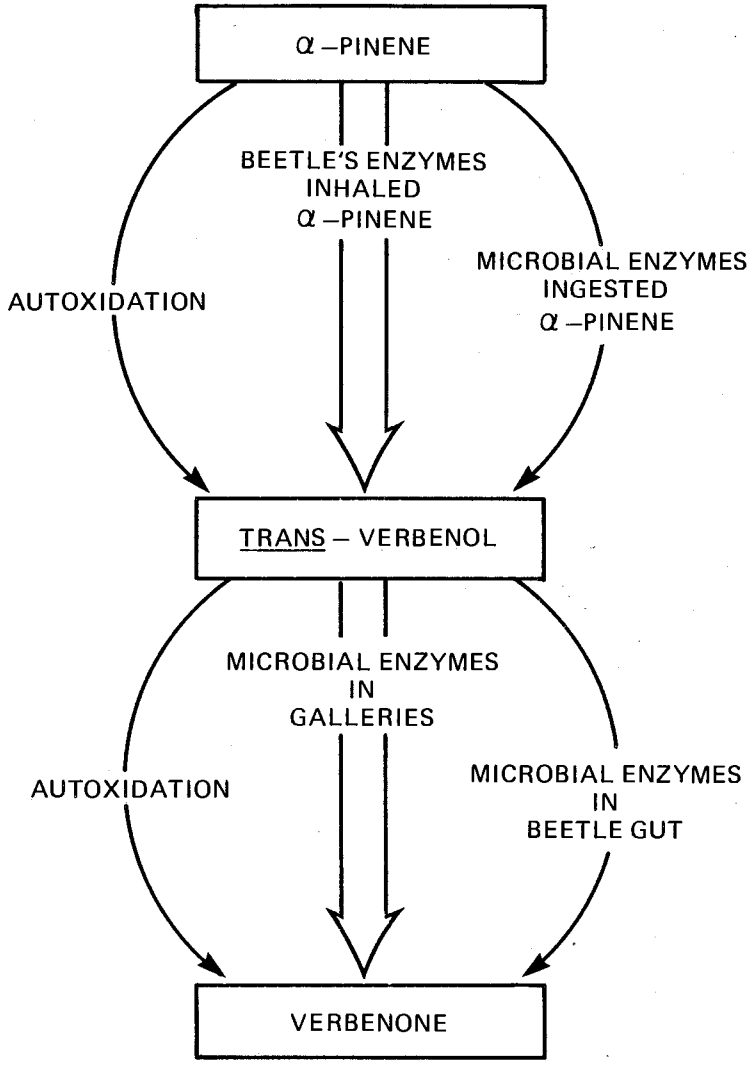
Male *D. ponderosae* which were exposed to myrcene vapours were found to produce large quantities of ipsdienol, which was predominantly the (+) enantiomer. This terpene alcohol reduced the attraction of both sexes of this beetle to mixtures of aggregation pheromones and host tree monoterpenes, but attracted the sympatric competitor, *I. pini*. Thus ipsdienol apparently has a duel function of an antiaggregation pheromone for *D. ponderosae* and a host-finding kairomone for *I. pini*.

For the production of *alpha*-pinene-derived pheromones in *D. ponderosae* it is now possible to refine and expand the model proposed by Borden (1984). The new model (Fig. 31) proposes that most of the aggregation pheromone *trans*-verbenol which is formed in trees attacked by *D. ponderosae* is produced from inhaled *alpha*-pinene by the beetles' own enzymes. The conversion of ingested *alpha*-pinene by microorganisms in the beetles' guts, as well as autoxidation of *alpha*-pinene, also contribute towards *trans*-verbenol production. *trans*-Verbenol may also be formed by microorganisms present in the beetles' galleries, although I have not presented any data to support this possibility, and, therefore, this route is excluded from the model. The antiaggregation pheromone verbenone appears to be produced almost entirely by microorganisms, with the beetles' enzymes not involved. This conversion is probably performed by microorganisms in the beetles' galleries, as well as, to a lesser extent, by gut symbionts. Autoxidation of *trans*-verbenol to verbenone supplements the

Figure 31. Pathways for the production of *alpha*-pinene-derived pheromones in *D. ponderosae* as proposed by Borden (1984) and as modified and expanded in this thesis.Wide arrows down the middle of the modified model denote major pathways. Narrower arrows on either side denote minor pathways.



BORDEN (1984)



HUNT (1987)

other routes to an unknown extent. There is no evidence as to whether or not verbenone is metabolized further by any system.

The principal conclusion to be drawn from the above studies is that oxygenated terpene alcohol pheromones of *D. ponderosae* and *I. paraconfusus* are produced in 3 major ways: 1) synthesis by the beetles, probably as a result of detoxication of host tree monoterpenes by polysubstrate monooxygenase enzymes; 2) synthesis by microorganisms associated with the beetles, which probably occurs in the guts and the galleries; and 3) autoxidation of the host tree monoterpene *alpha*-pinene.

X. FOOTNOTES

1. Chemical names and structures of compounds which are listed in the text by common name are contained in Appendix I.
2. Entomological Sciences Dept., Wellman Hall, University of California, Berkeley, California 94720, U. S. A.
3. A publication by Conn *et al.* (1984) includes a portion of the research reported in this section.
4. Pacific Forestry Centre, 506 West Burnside Road, Victoria, B. C. V8Z 1M5.
5. The term "wild" in this thesis only indicates that the beetles have not had their normal complement of microorganisms removed or reduced; beetles which have been reared in the laboratory are still referred to as "wild".
6. The term "eclosion" in this thesis refers specifically to moult from pupa to adult; posteclosion age refers to the number of days as an adult.
7. The term "mature" in this thesis refers to beetles of the posteclosion age at which peak levels of terpene alcohol production were observed to occur.
8. Southern Forest Experiment Station, Forest Service - U. S. Department of Agriculture, Pineville, Louisiana 71360, U. S. A.
9. Northern Utilization Research and Development Division, U. S. Department of Agriculture, Peoria, Illinois, U. S. A. (now retired).
10. Phero Tech, Inc., Vancouver, B. C., V5L 3K3.
11. Department of Biological Sciences, Simon Fraser University, Burnaby, B. C., V5A 1S6.
12. The research reported in this section is part of a study which was completed with the collaboration of Mr. M. J. Smirle, Department of Biological Sciences, Simon Fraser University, Burnaby, B. C., V5A 1S6.

XI. APPENDIX I

Common and Chemical Names for Compounds Mentioned in the Text

exo-brevicomin: *exo*-7-ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]octane

endo-brevicomin: *endo*-7-ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]octane

frontalin: 1,5-dimethyl-6,8-dioxabicyclo[3.2.1]octane

ipsenol: 2-methyl-6-methylene-7-octen-4-ol

ipsdienol: 2-methyl-6-methylene-2,7-octadien-4-ol

myrcene: 2-methyl-6-methylene-2,7-octadien

myrcenol: 2-methyl-6-methylene-2,7,octadien-1-ol

myrtenol: 4,6,6-trimethylbicyclo[3.1.1]hept-3-en-10-ol

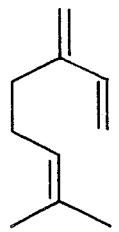
alpha-pinene: 2,6,6-trimethylbicyclo[3.1.1]hept-2-ene

cis-verbenol: *cis*-4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-ol

trans-verbenol: *trans*-4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-ol

verbenone: 4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-one

STRUCTURES



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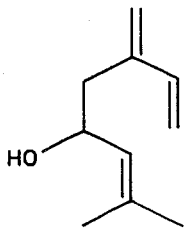
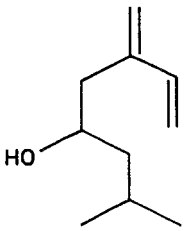


ipsdienol



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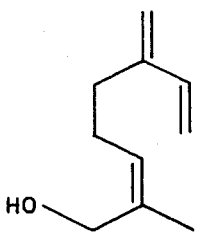
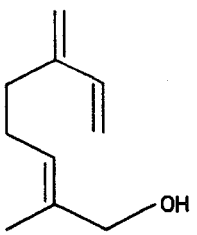


Z–myrcenol



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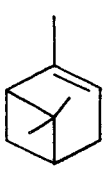
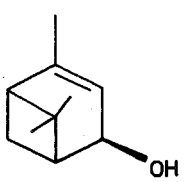
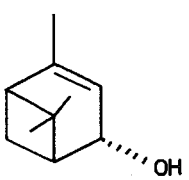


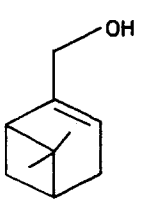
alpha–pinene



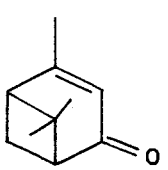
cis–verbenol



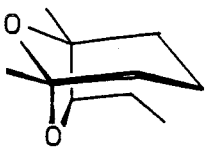
trans–verbenol



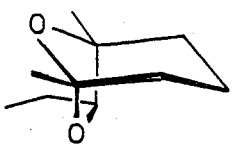
myrtenol



verbenone

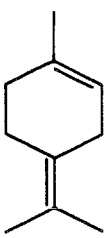


endo–brevicomin

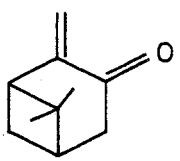


exo–brevicomin

frontalin



terpinolene



pinocarvone

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