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TERPENE ALCOHOL PHEROMONE PRODUCTION BY *Dendroctonus ponderosae* AND *Ips paraconfusus* (COLEOPTERA: SCOLYTIDAE) IN THE ABSENCE OF READILY CULTURABLE MICROORGANISMS[1]

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Abstract—*Dendroctonus ponderosae* Hopkins and *Ips paraconfusus* Lanier of both sexes produced most of their 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 also 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 α-pinene as vapors or through feeding, but was found in wild *D. ponderosae* exposed to α-pinene through feeding on bolts of lodgepole pine, *Pinus contorta* var. *latifolia* Engelmann.

Key Words—*Dendroctonus ponderosae, Ips paraconfusus,* Coleoptera, Scolytidae, pheromones, terpene alcohols, axenic-rearing, bark beetles, microorganisms.

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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. Certain species of microorganisms found in association with scolytids produced some of their hosts' terpene alcohol pheromones when exposed to monoterpene precursors of those pheromones (Brand et al., 1975; Chararas et al., 1980). This finding is not surprising since these microorganisms are exposed in nature to the same monoterpenes as the host beetles, and many species of microorganisms can oxidize monoterpene hydrocarbons (Bhattacharyya et al., 1960; Prema and Bhattacharyya, 1962; Shukla et al., 1968; Fonken and Johnson, 1972; Keislich, 1976). However, there is little evidence that microbe-produced terpene alcohols are being used in nature as pheromones by the host beetles or that these compounds are even being produced by the symbionts in quantities that are biologically significant to the host beetles.

In order to assess the importance of the microbial contribution to overall pheromone production, it is essential to ascertain the metabolic capabilities of beetles that are relatively free of microbial symbionts. Chararas (1980) claimed that after *Ips sexdentatus* Boerner, *Ips typographus* (L.), and *Ips acuminatus* Gyllenhal had been fed a wide-spectrum antibiotic, 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 α-pinene, β-pinene, and Δ^3-carene, and less of the conversion products of these compounds, than did frass from normal beetles. Byers and Wood (1981), found that *Ips paraconfusus* (Lanier) that were fed streptomycin were unable to convert myrcene to the pheromones ipsenol and ipsdienol, while the synthesis of *cis*-verbenol from α-pinene was not inhibited. 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 that are unaffected by streptomycin at the concentrations used. However, while these results suggest that microorganisms are involved in pheromone production by *Ips* species, treatment of insects with dietary antibiotics can produce adverse physiological effects in the insects (Greenberg, 1970). It is possible that reductions in pheromone production through streptomycin-feeding (Chararas, 1980; Byers and Wood, 1981) were the result of such effects.

Axenic rearing is a useful technique for the study of symbiosis in insects (Rodriguez, 1966; 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 mountain pine beetle, *Dendroctonus ponderosae* Hopkins, and the California five-spined ips, *I. paraconfusus*, were chosen for this study because their pheromone chemistry is fairly well understood and because much of the research that 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 infests living *Pinus* species throughout western North America (Wood, 1982). Females convert the host tree monoterpene α-pinene to the aggregation pheromone *trans*-verbenol (Hughes, 1973; Pitman, 1971) and a variety of other terpene alcohols such as myrtenol and *cis*-verbenol. Two bicyclic ketals, *exo*-brevicomin and frontalin, which are of uncertain biosynthetic origin (Vanderwel and Oehlschlager, 1987), are apparently concentration-dependent, multifunctional pheromones, acting to promote aggregation (Rudinsky et al., 1974; Conn et al., 1983; Borden et al., 1983, 1987) or antiaggregation (Rudinsky et al., 1974; Pitman et al., 1978; Ryker and Rudinsky, 1982; Ryker and Libbey, 1982; Borden et al., 1987). *endo*-Brevicomin (Ryker and Rudinsky, 1982), verbenone (Ryker and Yandell, 1983; Borden et al., 1987), pinocarvone (Libbey et al., 1985), and ipsdienol (Hunt and Borden, 1988) all have activity as antiaggregation pheromones, while the monoterpenes α-pinene (Pitman, 1971), 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 (S.L. 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 α-pinene to *cis*-verbenol (Renwick et al., 1976) and myrcene to ipsdienol (Byers et al., 1979; Hendry et al., 1980) and then ipsenol (Fish et al., 1979). It utilizes ipsenol, ipsdienol, and *cis*-verbenol as aggregation pheromones (Wood et al., 1968).

Conn et al. (1984) established that axenically reared *D. ponderosae* and *I. paraconfusus* are capable of producing certain terpene alcohol pheromones. However, their results were somewhat inconsistent, and they did not establish whether axenically reared insects are capable of producing these compounds at levels equal to normal insects with their full complement of microorganisms. Our objective was to assess in as comprehensive and definitive a manner as

possible the effects of axenic rearing and antibiotic feeding on the production of terpene alcohols and ketones from α-pinene and myrcene in both sexes of *D. ponderosae* and *I. paraconfusus*.

METHODS AND MATERIALS

Collection of Insects and Host Material. Lodgepole pines, *P. contorta* var. *latifolia*, infested with *D. ponderosae* and uninfested lodgepole and ponderosa pine, *Pinus ponderosa* Laws., were collected near Princeton, British Columbia. Adult *I. paraconfusus* were obtained from D.L. Wood (Department of Entomological Sciences, University of California-Berkeley, California 94720), and a colony was maintained on ponderosa pine bolts. Adult *D. ponderosae* and *I. paraconfusus* were collected daily after they emerged from caged logs 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 that had been stored a maximum of two weeks were rewarmed to room temperature, and normal and healthy-appearing individuals were selected.

Axenic Rearing. An axenic rearing technique (Whitney and Spanier, 1982) was used to obtain beetles that were devoid of readily culturable microorganisms. The outer bark was shaved off fresh pine bolts (lodgepole pine for *D. ponderosae* and ponderosa pine for *I. paraconfusus*), and the exposed phloem was removed and cut into 1-cm^2 pieces. The phloem was frozen at −25°C, ground in a prechilled Waring blender, and passed through No. 12 mesh screen. Grinding and sieving were conducted in a −25°C walk-in cold-room, and the ground phloem was stored in plastic bags at −20°C. 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 19 × 55-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 stainless-steel culture closures (18 mm), pasteurized in an Amsco isothermal sterilizer for 1.0 hr at 85°C, held at room temperature for four days, and then repasteurized. 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 bolts of lodgepole or ponderosa pine, respectively, using gelatin capsules (Lanier and Wood, 1968). After 24 hr, a mate was introduced to each capsule and the bolts were kept at 26–28°C. After 10 days for *D. ponderosae* or seven days for *I. paraconfusus*, the bark was removed. Using a blunt probe, the eggs were collected from the parent galleries, 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 three 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, New Jersey), rinsed six times in sterile distilled water, and placed individually in the vials of sterilized diet. Sterilized Pasteur pipets were used to transfer the eggs and the procedure was performed aseptically on a sterile air bench. The eggs were allowed to mature to adults in the vials, and emergent, axenically reared adults remained in the rearing vials until used in experiments.

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, potato dextrose agar, and plate count agar. 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 (alphacellulose, Sigma Chemical Co., St. Louis, Missouri), 8 g of sucrose and 22 g of ground pine phloem (lodgepole and ponderosa pine phloem for *D. ponderosae* and *I. paraconfusus,* respectively) was added to 62 ml of distilled water containing 10 mg of streptomycin sulfate (Sigma) per milliliter of water. The ingredients were thoroughly mixed and then dispensed into 19 × 55-mm shell vials at approximately 0.75 g/vial using a 10-ml plastic syringe with the bottom removed. An adult beetle was added to each vial and the insects were allowed to feed for 96 hr at 22–24°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.

Maturation Experiments. The term "wild" in this paper indicates that the beetles have not had their normal complement of microorganisms removed or reduced. When wild adults of a known age were required, the bark was removed from infested bolts and pupae were collected, placed in Petri dishes containing moistened filter paper, and examined daily until eclosion (the term "eclosion" in this paper refers specifically to molt from pupa to adult; posteclosion age refers to the number of days as an adult). Callow adults were removed from the Petri dishes and allowed to mature in individual vials until reaching the desired age (we use "mature" to designate beetles of the posteclosion age at which peak levels of terpene alcohol production were observed to occur). Since fungi found in the pupal chamber are thought to be important in the maturation feeding of callow scolytids (Whitney, 1971; Barras, 1973), moistened 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.

Pheromone Production and Extraction Techniques. Pheromone production was induced using one of two methods: (1) Beetles that had bored in the inner bark of pine logs for 24 hr were chipped out and held over Dry Ice until a sufficient number had accumulated for dissection. (2) Beetles were placed individually in upright, open-ended glass cylinders (1.4 cm ID) (Conn, 1981) inside a 500-ml glass jar. Open vials (1.8 ml screw-cap) containing 25 μl of the pheromone precursors α-pinene and/or myrcene were also placed in the jar, and the jar was sealed and held in the dark for 24 hr.

Beetle abdomens were removed using fine forceps, and immediately immersed in 100 μl of double-distilled pentane in individual 1.8-ml glass vials over Dry Ice. The excised abdomens included the hindgut, Malpighian tubules, and a large portion of the midgut. Each abdomen was macerated with the tip of a spatula in the pentane over Dry Ice, and the vial was then sealed and allowed to sit at room temperature for approximately 15 min. The pentane extract was then transferred with a syringe 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 Chromatographic Analyses. Single beetle extracts were analyzed on a Hewlett Packard 5880A gas chromatograph equipped with a capillary inlet system and a flame ionization detector. A glass capillary column (30 m $\times$ 0.66 mm ID) coated with SP-1000 (Supelco, Inc., Bellefonte, Pennsylvania) was used with the temperature set at 120°C for 2 min, and then increasing 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 analyzed by gas chromatography, a standard sample made up of 2- and 3-octanol, α-pinene, myrcene, *cis*- and *trans*-verbenol, myrtenol, verbenone, ipsenol, ipsdienol, and myrcenol was also analyzed for comparison of retention times. In addition, these compounds were periodically added to an abdominal extract, and that mixture was analyzed to ensure correct identification by cochromatography with unknown compounds in the extract. Selected extracts were analyzed using gas chromatography–mass spectroscopy to ensure proper identification of compounds. For most samples, the detection limit for compounds of interest was set at 5 ng/abdomen.

Experiments Conducted. Twenty-one experiments were conducted with *D. ponderosae* and *I. paraconfusus* adults of both sexes, with experiment number and objectives as outlined below. Details of experimental treatments and replicates are given in Tables 1–3, and Figures 1–3.

Female D. ponderosae.

I. To assess the effect of axenic rearing or streptomycin feeding on the conversion of α-pinene into terpene alcohols.

II. To assess the effect of maturation on the ability of axenically reared beetles to produce terpene alcohols.

III. To compare the effect of various degrees of maturation on the ability of axenically reared and wild beetles to produce terpene alcohols.

IV. To compare the levels of terpene alcohol production by mature, axenically reared beetles, mature, wild beetles, and emerged, wild beetles.

V and VI. To compare the effects of streptomycin-feeding on terpene alcohol production in beetles exposed to α-pinene either as vapors or through feeding.

VII. To determine the effects of axenic rearing on the conversion of ingested α-pinene into terpene alcohols.

VIII. To determine the ability of females to convert myrcene into terpene alcohols.

Male D. ponderosae.

IX and X. To determine the effects of axenic rearing and streptomycin feeding on the conversion of α-pinene (experiment IX) and myrcene (experiment X) vapors into terpene alcohols.

Male I. paraconfusus.

XI and XII. To compare the effect of various degrees of maturation on the ability of axenically reared and wild beetles to produce α-pinene-derived (experiment XI) and myrcene-derived (experiment XII) terpene alcohols.

XIII. To compare the levels of myrcene-derived terpene alcohol production by mature, axenically reared beetles; mature, wild beetles; and emerged, wild beetles.

XIV. To compare the levels of terpene alcohol production by mature, axenically reared beetles; mature, wild beetles; and emerged, wild beetles.

XV. To assess the effects of streptomycin-feeding on terpene alcohol production in wild beetles exposed to α-pinene vapors.

XVI and XVIII. To determine the effects of streptomycin feeding (experiment XVI) or axenic rearing (experiment XVIII) on terpene alcohol production in beetles exposed to α-pinene through feeding.

XVII. To compare the efficacy of two different batches of streptomycin in reducing the production of myrcene-derived terpene alcohols.

XIX. To compare the levels of terpene alcohol production in beetles exposed to myrcene vapors, myrcene and α-pinene vapors simultaneously, or the myrcene and α-pinene encountered while feeding on bolts of *P. ponderosa.*

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

Female I. paraconfusus.

XXI. To determine the effects of axenic rearing and streptomycin feeding on the conversion of α-pinene vapors into terpene alcohols.

Statistical Analysis. 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 (1983). The data were heteroscedastic; therefore, they were analyzed using the Kruskal-Wallis test (Sokal and Rohlf, 1981) followed by a nonparametric multiple comparisons test (Conover, 1980, p. 231), $P < 0.05$.

RESULTS AND DISCUSSION

Axenically Reared Beetles. During axenic rearing, the few vials with visible signs of contamination were discarded. In one batch of axenically reared *D. ponderosae,* there were many contaminated vials, and closer examination revealed numerous mites in the vials. 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 subsequently avoided 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,* revealed no microorganisms. In developing the technique, Whitney and Spanier (1982) had conducted even more extensive culturing on axenically reared *D. ponderosae,* which also revealed no microorganisms. In addition, no microorganisms other than brewer's yeast cells were observed on

the external cuticular surface of axenically reared adults examined by scanning electron microscopy.

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 possible that transovarially transmitted, obligate symbionts may still be associated with these beetles. Therefore, we refer to beetles obtained using this technique as "axenically reared" or "microbe reduced," and not "axenic."

Of the *D. ponderosae* eggs that were surface sterilized and placed in vials of sterilized diet, generally 60–80% survived to adulthood. Only 40–60% of axenically reared *I. paraconfusus* survived, probably because the eggs of this species are more fragile and were more easily damaged during handling.

Initial Experiments on Pheromone Production by Axenically Reared Beetles. Experiment 1 (Table 1) failed to verify the results of Conn et al. (1984) in which female *D. ponderosae* free of readily culturable microorganisms contained far more *trans*-verbenol than wild control beetles. Rather, axenically reared beetles in experiment 1 contained *trans*- and *cis*-verbenol and myrtenol at levels too low to be quantified (Table 1, experiment I). Streptomycin-fed beetles and wild controls also contained unusually low levels of these terpene alcohols (Table 1, experiment 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, produced significant levels of *trans*- and *cis*-verbenol, and myrtenol when exposed to α-pinene vapors (Table 1, experiment II). Normal levels of production were found in wild, control beetles. 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 different in the axenically-reared individuals (Table 1, experiment II).

Effect of Maturation on Terpene Alcohol Production in Female D. ponderosae. The axenically reared female *D. ponderosae* in experiment I were fully darkened adults, and it was initially assumed that they had reached physiological maturity. However, since individuals taken from the same batch 14 days later produced terpene alcohols at higher levels (Table 1, experiment II), and because no record was made of the age of the axenically reared females used by Conn et al. (1984), we hypothesized that maturation beyond the point of darkening of axenically reared *D. ponderosae* was required before peak terpene alcohol production occurred.

To test this hypothesis, we exposed axenically reared females of varying posteclosion ages to α-pinene vapors. 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 much higher than those in wild emerged beetles (Figures 1–3). Thus a three- to four-week maturation period is apparently required before adult *D. ponderosae*

TABLE 1. OXYGENATED MONOTERPENE PRODUCTION IN AXENICALLY REARED, STREPTOMYCIN-FED, AND WILD *Dendroctonus ponderosae* FEMALES (EXPERIMENTS I–VIII) AND MALES (EXPERIMENTS IX AND X)

Exp. No.	Sex	Treatment	No. beetles	Mean amount of volatiles in abdominal extract (ng/beetle)[a]			
				trans-verbenol	*cis*-verbenol	myrtenol	
I	Females	Wild/air	7	<5 a	<5 a	<5 a	
		Wild/α-pinene vapors	8	46 b	20 a	7 a	
		Wild/streptomycin/air	7	20 ab	8 a	<5 a	
		Wild/streptomycin/α-pinene vapors	8	44 b	17 a	7 a	
		Axenically reared/air	8	<5 a	<5 a	<5 a	
		Axenically reared/α-pinene vapors	7	<5 a	<5 a	<5 a	
		Axenically reared/streptomycin/air	8	8 a	<5 a	<5 a	
		Axenically reared/streptomycin/α-pinene vapors	7	<5 a	<5 a	<5 a	
				trans-verbenol	*cis*-verbenol	myrtenol	
II	Females	Wild/air	10	<5 a	<5 a	<5 a	
		Wild/α-pinene vapors	8	446 b	70 b	79 b	
		Axenically reared/air	9	7 a	<5 a	<5 a	
		Axenically reared/α-pinene vapors	8	177 b	26 b	28 c	
				trans-verbenol	*cis*-verbenol	myrtenol	verbenone
IV	Females	Wild/emerged/air	10	<5 a	<5 a	<5 a	<5 a
		Wild/emerged/α-pinene vapors	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 vapors	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 vapors	10	1216 c	87 c	112 c	<5 a
				trans-verbenol	*cis*-verbenol	myrtenol	
V	Females	Wild/air	7	<5 a	<5 a	<5 a	
		Wild/α-pinene vapors	7	730 b	133 b	252 b	
		Wild/streptomycin/air	7	<5 a	<5 a	<5 a	
		Wild/streptomycin/α-pinene vapors	7	2056 b	104 b	172 b	

				trans-verbenol	*cis*-verbenol	myrtenol	
VI	Females	Wild/air	6	<5 a	<5 a	<5 a	
		Wild/α-pinene vapors	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 vapors	10	3375 c	155 c	264 c	
		Wild/streptomycin/*P. contorta*	5	17 a	<5 a	<5 a	
				trans-verbenol	verbenone		
VII	Females	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*)-1-myrcenol	(*E*)-1-myrcenol	unknown (*R*, 14.74)
VIII	Females	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 vapors	8	<10 a	10 a	641 b	103 b
				trans-verbenol	*cis*-verbenol	myrtenol	
IX	Males	Wild/air	7	<5 a	<5 a	<5 a	
		Wild/α-pinene vapors	7	169 b	98 b	91 b	
		Wild/streptomycin/air	7	<5 a	<5 a	<5 a	
		Wild/streptomycin/α-pinene vapors	11	1534 c	482 c	180 c	
		Axenically reared/air	9	<5 a	<5 a	<5 a	
		Axenically reared/α-pinene vapors	10	1735 c	571 c	207 c	
				ipsdienol	(*Z*)-1-myrcenol	(*E*)-1-myrcenol	unknown (*R*, 14.74)
X	Males	Wild/air	10	<10 a	<5 a	<5 a	<5 a
		Wild/myrcene vapors	11	1906 b	156 b	1341 b	167 b
		Wild/streptomycin/air	10	<10 a	<5 a	<5 a	<5 a
		Wild/streptomycin/myrcene vapors	10	1867 b	57 c	277 c	36 c
		Axenically reared/air	10	<10 a	<5 a	<5 a	<5 a
		Axenically reared/myrcene vapors	12	3122 b	110 bc	222 c	62 c

[a] Data analyzed using the Kruskal-Wallis test followed by a nonparametric 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.

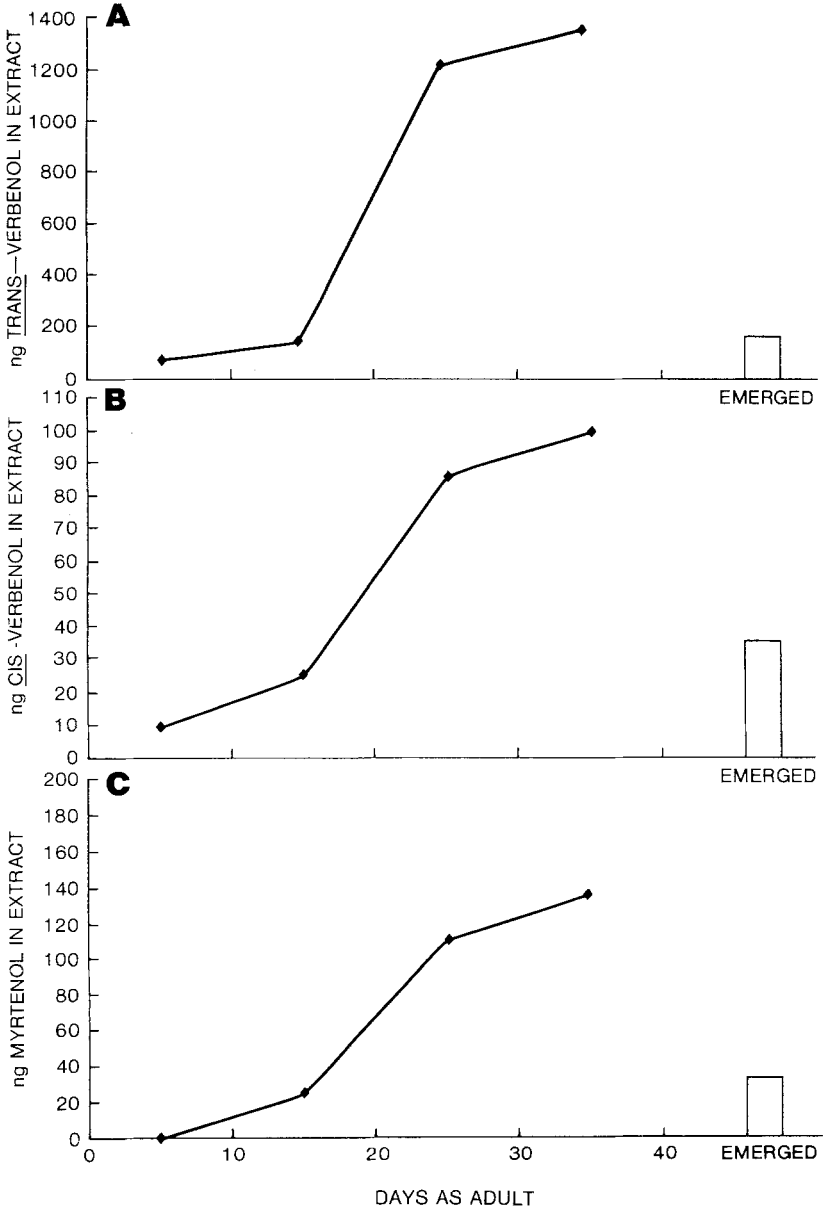

FIG. 1. Quantities (nanograms) of *trans*-verbenol (A, D), *cis*-verbenol (B, E), and myrtenol (C, F), produced in experiment III by individual axenically reared (A–C) and wild (D–F) female *D. ponderosae* of various posteclosion ages that were exposed to α-pinene vapors. Numbers of beetles used for 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; 16 days, 6; 27 days, 6; 40 days, 9. Wild, group 2: 5 days, 11; 15 days, 8; 25 days, 10; 35 days, 10.

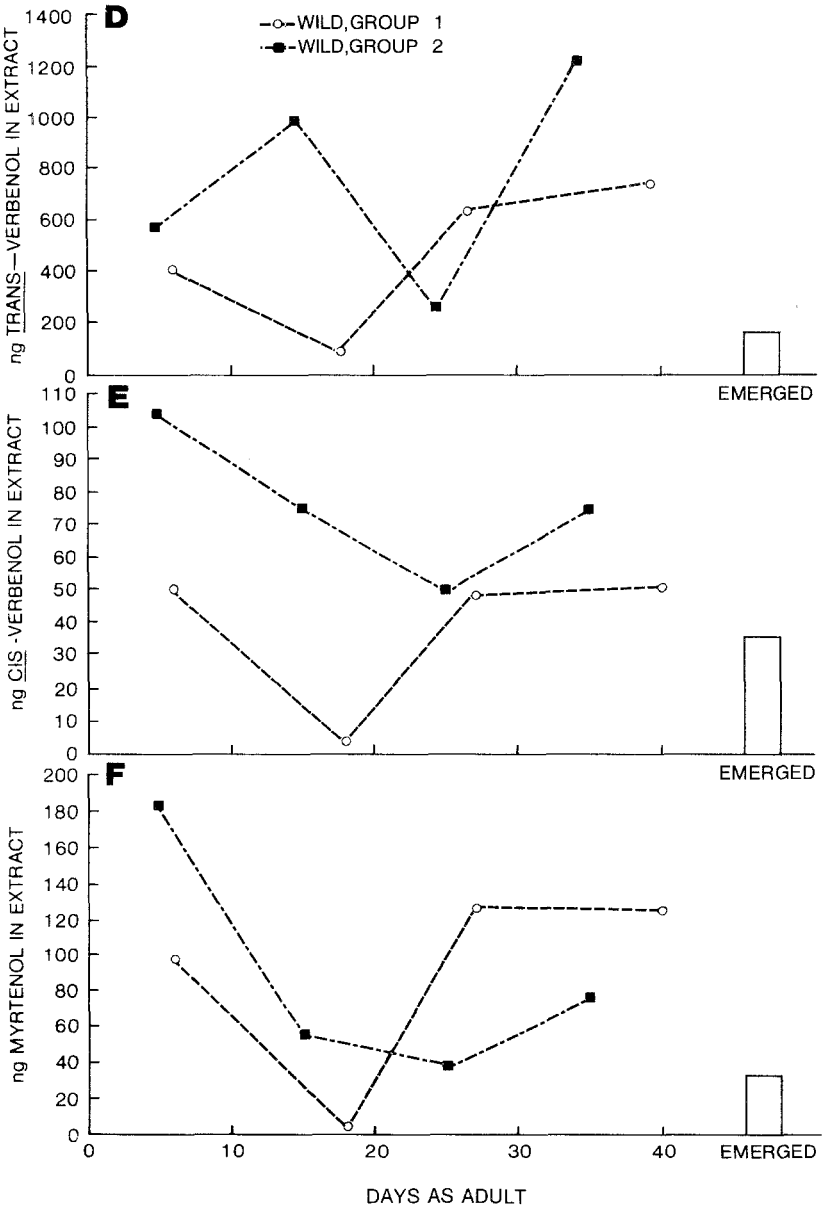

FIG. 1. Continued.

develop maximal enzymatic capacity for the oxidation of α-pinene into terpene alcohols.

Since the posteclosion age of *D. ponderosae* is evidently important in determining the physiological maturity of axenically reared beetles, much of the data reported by Conn et al. (1984) must be questioned. Although Conn et al. (1984) did establish that axenically reared *D. ponderosae* can produce 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 immature beetles. 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.

The pattern of terpene alcohol production in relation to age for wild female *D. ponderosae* was different than that for axenically reared individuals (Figure 1). During the first 15 days posteclosion, the production of *trans*- and *cis*-verbenol and myrtenol in two groups of beetles was moderate to high, then declined sharply, and returned to higher levels in older beetles (Figure 1). The terpene alcohol production in wild callow adults of 5–15 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 much lower levels (Figure 1). Possibly the *cis*- and *trans*-verbenol and myrtenol produced by wild beetles of 5–15 days posteclosion is metabolized from derivatized α-pinene sequestered by the insects as larvae or pupae (Hughes, 1975), rather than by conversion of inhaled α-pinene vapors. 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 (Table 2). The increase in terpene alcohol production in wild beetles 25 days posteclosion would, as in axenically reared beetles, be due to the maturation of the enzyme systems responsible for oxidizing the monoterpenes encountered as the mature adult attacks a new host.

The peak levels of terpene alcohol production were generally much higher

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

in wild beetles of 27–40 days posteclosion than in wild emerged beetles (Figure 1). 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 that are not yet responsive to attractive semiochemicals contain higher levels of lipids and that these individuals become responsive through a metabolic feedback process activated through flight exercise (Atkins, 1966, 1969). We hypothesize that a period of lipid metabolism through flight exercise may also be 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, particularly under epidemic conditions, as these individuals would be more likely to encounter suitable hosts, thus assuring 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 (reared as pupae and held 35–40 days posteclosion), which show much higher levels of pheromone production than wild emerged beetles (Figure 1), may be metabolically similar to emerged beetles that have completed a dispersal flight. They have probably lost lipid reserves through walking exercise in the Petri dishes and also may have faced a low-quality diet due to drying out of the phloem on which they were fed. Sanders (1983) showed that starvation or walking activity released host-positive responses in host-negative *Pityogenes chalcographus* (L.). Similarly, Gries (1984) showed that *I. typographus* that were denied a chance to fly became host-responsive only after incurring a mean weight loss equivalent to that lost during a 7-km flight. Therefore, we hypothesize that the older wild beetles shown in Figure 1 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.

When pupae were collected from infested bolts for use in the wild beetle maturation experiments, the beetles from two additional infested bolts from the same tree were allowed to emerge naturally. The average period of time between eclosion to adults in the Petri dishes and emergence of wild beetles from infested bolts was approximately 30 days. This is a very rough estimate of the time that adults wait before emerging in nature, 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, probably resulted in accelerated emergence. Nonetheless, this result supports the hypothesis that the 35-to-40-day posteclosion adults in Figure 1 were metabolically equivalent to emerged and dispersed adults, which would be expected to produce terpene alcohols at higher levels than emerged beetles.

An alternative explanation for 27-to-40-day posteclosion beetles producing higher levels of terpene alcohols than emerged beetles (Figure 1) is that the adults that were collected as pupae and allowed to mature on phloem strips may be microbe-deficient. Since these beetles did not feed *in situ* as callow adults, they may possess a microbial fauna that is substantially reduced or different from wild emerged individuals. Some of the microorganisms that would be present at reduced levels may utilize the terpene alcohols as substrates, metabolizing them into other products.

Although Figure 1 indicates that wild emerged beetles are apparently not capable of maximal terpene alcohol production, wild emerged beetles were used as a standard control for comparison in all subsequent experiments with axenically reared and streptomycin-fed 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 α-pinene and myrcene vapors. Axenically reared individuals of five 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 (Figure 2). Adults that were allowed to mature to 69 days posteclosion showed declining levels of terpene alcohol production. Wild beetles also matured in their ability to oxidize α-pinene (Figure 2), but, with the exception of myrtenol, did not exhibit the early peak in terpene alcohol production noted in young *D. ponderosae* adults (Figure 1). High levels of mortality prevented the testing of wild *I. paraconfusus* beyond 28 days posteclosion.

The peak levels of terpene alcohols produced by wild *I. paraconfusus* were only half of those produced by axenically reared beetles (Figure 2). 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 (Figure 2). Oxidation products of myrcene, such as ipsdienol and ipsenol, were not generally detectable at quantifiable levels in either axenically reared beetles or wild controls exposed to myrcene vapors.

Axenically reared male *I. paraconfusus* of eight days posteclosion that 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 similar to those in wild

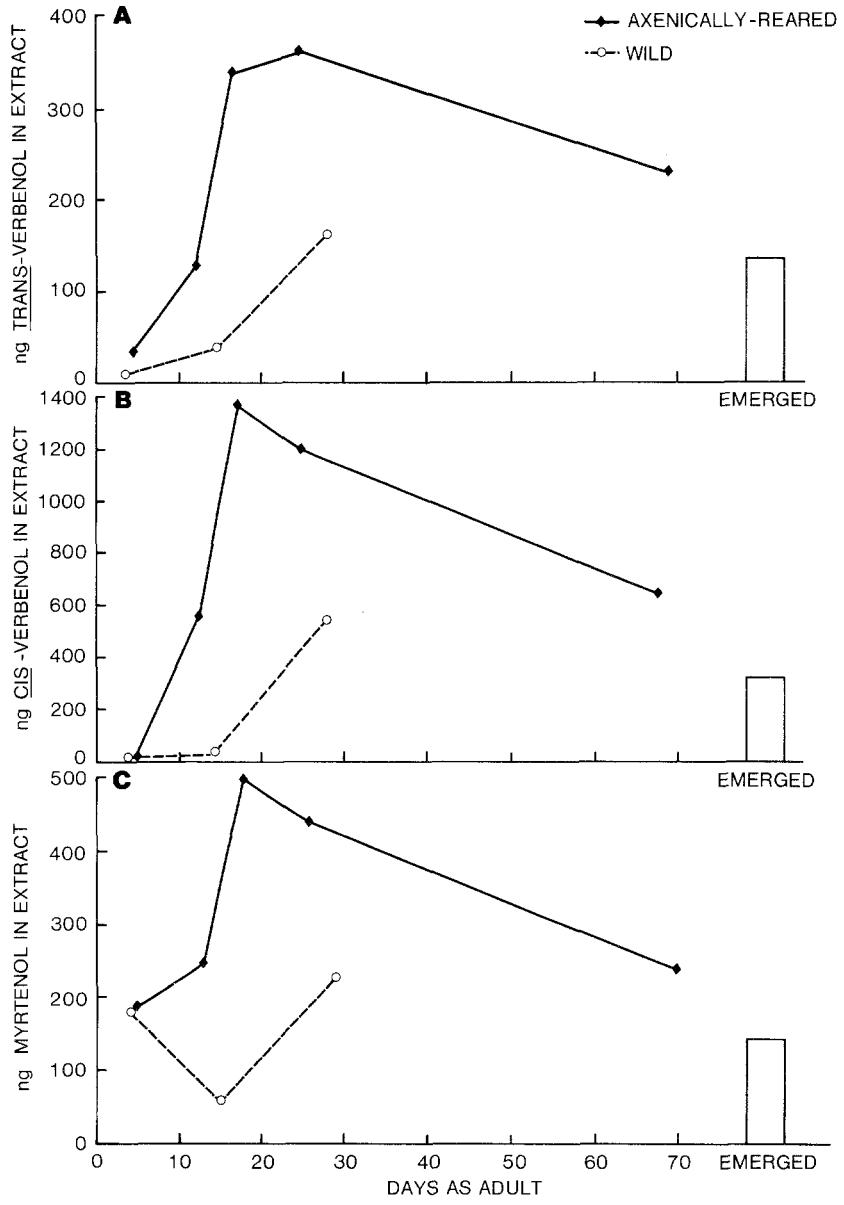

FIG. 2. Quantities (nanograms) of *trans*-verbenol (A), *cis*-verbenol (B), and myrtenol (C), produced in experiment XI by individual male *I. paraconfusus* of various posteclosion ages that were exposed to α-pinene vapors. Numbers of beetles used for each treatment were as follows. Axenically reared: 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.

emerged beetles (Figure 3). When mature (24 days posteclosion), axenically reared individuals were fed on ponderosa pine bolts, they contained ipsenol and ipsdienol at levels that were not significantly different from those in mature wild beetles or wild emerged beetles (Table 3, experiment XIII).

As with *D. ponderosae,* Conn et al. (1984) did not report the posteclosion ages of *I. paraconfusus* used in their experiments. Although they did establish that axenically reared *I. paraconfusus* are capable of producing terpene alcohol pheromones, the generally low levels of production that they report probably

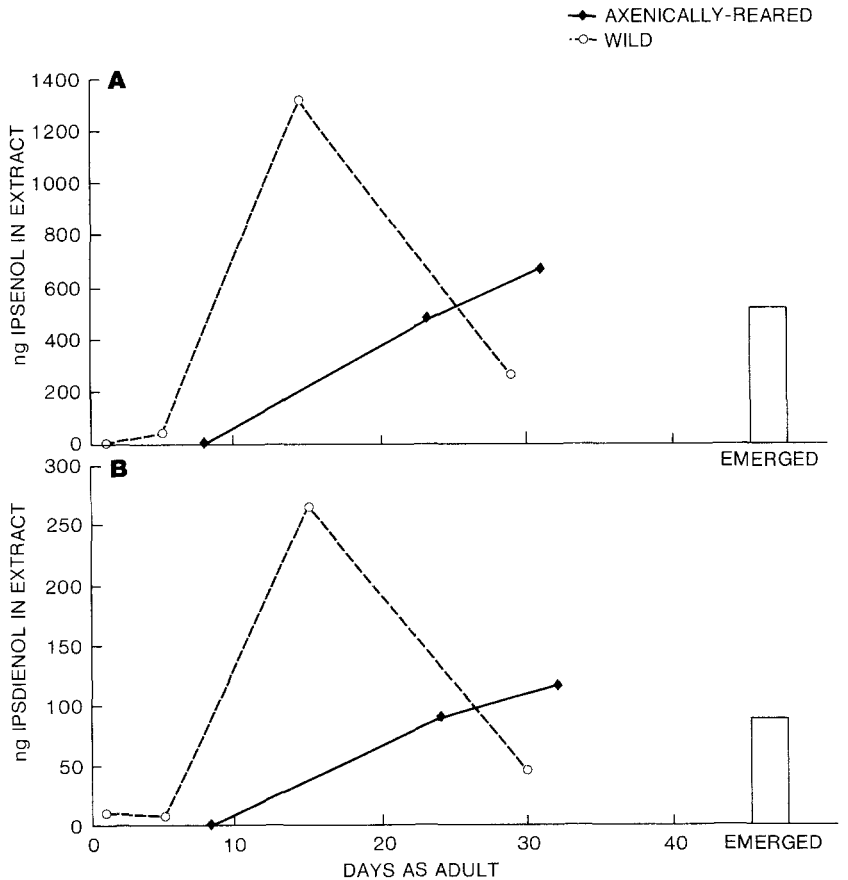

FIG. 3. Quantities (nanograms) of ipsenol (A), and ipsdienol (B), produced in experiment XII by individual male *I. paraconfusus* of various posteclosion ages that were fed on bolts of ponderosa pine. Numbers of beetles used for each treatment were as follows. Axenically reared: 8 days, 7; 24 days, 10; 32 days, 12. Wild: 1 day, 9; 5 days, 9; 15 days, 13; 30 days, 8.

reflect the use of axenically reared beetles that were not yet mature. The maturation period required before adult *I. paraconfusus* can convert myrcene into ipsenol and ipsdienol (Figure 3) and before adult *I. paraconfusus* and *D. ponderosae* can convert α-pinene into *cis*- and *trans*-verbenol and myrtenol (Figures 1 and 2) may explain why immature *I. paraconfusus* were unattractive to conspecifics 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 α-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 α-pinene.

Microbial Involvement in α-Pinene Vapor Oxidation. Mature, axenically reared, female (Table 1, experiment IV) and male (Table 1, experiment IX) *D. ponderosae* produced more *trans*- and *cis*-verbenol and myrtenol upon exposure to α-pinene vapors than wild, emerged beetles. In addition, streptomycin-fed *D. ponderosae* females produced more of these terpene alcohols than wild, emerged beetles in one of two experiments (Table 1, experiments V and VI). Streptomycin-fed male *D. ponderosae* also produced more of these terpene alcohols than wild, emerged males (Table 1, experiment IX). Similarly, mature, axenically reared, male *I. paraconfusus* also produced more *trans*- and *cis*-verbenol and myrtenol upon exposure to α-pinene vapors than wild, emerged beetles (Table 3, experiment XIV). Mature, axenically reared, female *I. paraconfusus* (Table 3, experiment XXI), and streptomycin-fed male (Table 3, experiment XV) and female (Table 3, experiment XXI) *I. paraconfusus* also produced these terpene alcohols at levels not significantly different from wild, emerged beetles.

Evidently both male and female *D. ponderosae* and *I. paraconfusus* that have had their natural levels of symbiotic microorganisms reduced through axenic rearing or streptomycin feeding are capable of converting α-pinene vapors into the terpene alcohols *trans*- and *cis*-verbenol and 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 terpene alcohols in these beetles. The fact that terpene alcohol levels were significantly higher in microbe-reduced than in wild beetles in several experiments indicates that certain microorganisms may metabolize the available α-pinene precursor into other products, so that the α-pinene is unavailable to the 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 aggregation

TABLE 3. OXYGENATED MONOTERPENE PRODUCTION IN AXENICALLY REARED, STREPTOMYCIN-FED, AND WILD *Ips paraconfusus* MALES (EXPERIMENTS XI–XVIII) AND FEMALES (EXPERIMENT XXI)

Exp. No.	Sex	Treatment	No. beetles	Mean amount of volatiles in abdominal extract (ng/beetle)[a]				
				ipsdienol	ipsenol			
XIII	Males	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		
XIV	Males	Wild/emerged/air	10	<5 a	<5 a	<5 a		
		Wild/emerged/α-pinene and myrcene vapors	10	123 b	298 b	137 b		
		Wild/mature/air	5	<5 a	<5 a	<5 a		
		Wild/mature/α-pinene and myrcene vapors	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 vapors	12	336 c	1359 c	498 c		
				trans-verbenol	*cis*-verbenol	myrtenol		
XV	Males	Wild/air	10	<5 a	<5 a	<5 a		
		Wild/α-pinene vapors	11	160 b	320 b	150 b		
		Wild/streptomycin/air	9	<5 a	<5 a	<5 a		
		Wild/streptomycin/α-pinene vapors	10	209 b	413 b	195 b		
				trans-verbenol	*cis*-verbenol	myrtenol	ipsdienol	ipsenol
XVI	Males	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			
XVII	Males	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
XVIII	Males	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
				trans-verbenol	*cis*-verbenol	myrtenol	ipsdienol	ipsenol
XIX	Males	Wild/air	10	<5 a	<5 a	<5 a	<5 a	<5 a
		Wild/myrcene vapors	10	22 b	7 a	17 a	10 a	29 a
		Wild/α-pinene and myrcene vapors	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
				ipsdienol	ipsenol			
XX	Males	Wild/myrcene vapors	10	<5 a	11 a			
		Wild/cellulose fed/myrcene vapors	11	6 a	37 a			
				trans-verbenol	*cis*-verbenol	myrtenol		
XXI	Females	Wild/air	10	<5 a	<5 a	<5 a		
		Wild/α-pinene vapors	10	602 b	152 b	316 b		
		Axenically reared/air	10	<5 a	<5 a	<5 a		
		Axenically reared/α-pinene vapors	11	211 b	79 b	322 b		
		Wild/air/streptomycin	10	<5 a	<5 a	<5 a		
		Wild/α-pinene vapors/streptomycin	10	307 b	129 b	301 b		

[a] Data analyzed using the Kruskal-Wallis test followed by a nonparametric 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.

is complete. Alternatively, it is possible that reducing the levels of microorganisms in the beetles through axenic rearing or streptomycin feeding may reduce the levels of microorganisms that are mildly pathogenic to the beetles and that the resultant healthier beetles simply produce higher levels of terpene alcohols. In support of this hypothesis, Gueldner et al. (1977) found reduced levels of pheromone production in boll weevils, *Anthonomus grandis* Boheman that were artificially contaminated with *Streptococcus* sp., *Micrococcus varians* Migula, and *Enterobacter aerogenes* Hormaeche and Edwards, all isolated from apparently normal, insectary-reared weevils.

Two Sites and Two Methods of α-Pinene Oxidation. While axenically reared or streptomycin-fed *D. ponderosae* readily produced *trans*- and *cis*-verbenol and myrtenol from α-pinene vapors, axenically reared beetles were unable to produce *trans*-verbenol from ingested α-pinene (Table 1, experiment VII). This result suggests that there are two production systems for this pheromone, one by the beetle's own enzymes and one by symbiotic microorganisms. The low levels of *trans*-verbenol in extracts of wild beetles fed on lodgepole pine probably result from the conversion of α-pinene by microorganisms in the gut. The high levels of *trans*-verbenol, *cis*-verbenol, and myrtenol in extracts of axenically reared and streptomycin-fed *D. ponderosae* exposed to α-pinene vapors would then be due to oxidation of inhaled vapors by the beetle's own enzymes.

Since axenically reared *D. ponderosae* produce *trans*-verbenol from inhaled, rather than ingested α-pinene (Table 1, experiments IV and VII), it is likely that the conversion does not occur in the gut, as suggested for other scolytids (Pitman et al., 1965; Zethner-Møller and Rudinsky, 1967). *trans*-Verbenol is probably produced in the hemolymph, transported through the Malpighian tubules into the hindgut, and released through the anus. This explanation is consistent with Hughes' (1973) finding of *trans*-verbenol and other monoterpene oxidation products in the hemolymph of *D. ponderosae* and his hypothesis that terpene metabolism occurs outside the gut.

Wild female *D. ponderosae* that had fed in lodgepole pine bolts for 24 hr contained significant amounts of verbenone, while axenically reared individuals contained only trace quantities (Table 1, experiment VII). However, after exposure to α-pinene vapors, neither wild nor axenically reared female *D. ponderosae* contained quantifiable levels of verbenone (Table 1, experiment IV), even when the extracts were concentrated to approximately 5 μl over a stream of nitrogen before gas chromatographic analysis. Similarly, exposure to α-pinene vapors does not result in verbenone production by *D. frontalis* (Renwick et al., 1973), or *D. brevicomis* (Byers, 1983). Therefore, the production of verbenone by female *D. ponderosae* is probably due solely to the conversion of beetle-ingested precursor by gut microorganisms. This suggests that *D. ponderosae* is dependent on its symbiotic microorganisms for the production of verbenone, its

principal antiaggregation pheromone (Ryker and Yandell, 1983; Libbey et al., 1985; Borden et al., 1987).

Microbial Involvement in Myrcene Oxidation. Both axenic rearing and streptomycin feeding reduced the levels of (*E*)-1-myrcenol (1-myrcenol = 2-methyl-6-methylene-octa-2,7-dien-1-ol) and an unknown product in male *D. ponderosae* exposed to myrcene vapors (Table 1, experiment X). The levels of (*Z*)-1-myrcenol were reduced by streptomycin feeding, while the reduction caused by axenic rearing was not significant. This is further evidence that microorganisms are partially responsible for the production of certain conversion products of monoterpenes in this species. However, streptomycin-fed and wild control females exposed to myrcene vapors for 24 hr produced (*Z*) and (*E*)-1-myrcenol and an unknown product at similar levels (Table 1, experiment VIII).

Axenically reared or streptomycin-fed male *D. ponderosae* that were subsequently exposed to myrcene vapors contained ipsdienol at levels that were not significantly different from those in wild, emerged beetles (Table 1, experiment X). None of the females tested produced detectable levels of ipsdienol (Table 1, experiment VIII).

Two Pheromone Production Systems. The hypothesis that both the beetle and its symbiotic microorganisms can produce *cis*- and *trans*-verbenol, as well as other terpene alcohols, is consistent with the report by Chararas et al. (1980) that the frass of *I. sexdentatus*, *I. typographus*, or *I. acuminatus* that had been fed a wide-spectrum antibiotic showed reduced attractiveness to conspecifics. Chararas et al. (1980) also reported that the frass of antibiotic-fed beetles had more α-pinene, β-pinene, and Δ_3-carene and less of their conversion products than normal beetles. They concluded that microbial conversion was responsible for a portion of total pheromone production, but provided minimal methodological details and presented no data to support this conclusion.

The existence of two pheromone production systems, beetle and microbial, is also consistent with what is known about polysubstrate monooxygenases (PSMOs). PSMOs are enzymes that catalyze numerous oxidation reactions that render lipophilic compounds, such as monoterpenes, hydrophilic, so that they can be more easily excreted. They are likely the most important enzymes involved in the metabolism of foreign substances, such as monoterpenes (Sturgeon and Robertson, 1985), and compounds that inhibit PSMO activity have been shown to inhibit partially terpene alcohol pheromone production in *D. ponderosae* (Hunt and Smirle, 1988). Since PSMOs are believed to be present in all aerobic organisms (Brattsten, 1979), and since bark beetles and their microorganisms are frequently exposed to monoterpenes that are toxic to both the insects (Smith, 1965; Reid and Gates, 1970; Coyne and Lott, 1976; Raffa and Berryman, 1983) 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 PSMOs to oxidize monoterpenes.

Effect of Streptomycin on I. paraconfusus. In experiment XVI (Table 3) streptomycin feeding resulted in virtual elimination of the production of ipsdienol and ipsenol, a result in agreement with those of Byers and Wood (1981). However, in experiment XVII (Table 3), streptomycin-feeding caused a significant, but not total, reduction in ipsdienol and ipsenol production. Although the streptomycin used in these two experiments was from the same batch, there were several months between the experiments. Therefore, newly purchased streptomycin was used to determine whether the streptomycin in experiment XVII had lost some of its activity. However, the levels of ipsenol and ipsdienol in beetles fed the old or the new streptomycin were not significantly different (Table 3, experiment XVII).

Our *I. paraconfusus* colony was initiated with beetles from the Sierra Nevada, and should have been very similar to those used by Byers and Wood (1981). We had reared these beetles through several generations by the time experiment XVII (Table 3) was done, and it appears that this resulted in a shift of the population of insects or their symbiotic microorganisms toward some characteristic that promoted resistance to the effects of streptomycin.

Streptomycin Feeding vs. Axenic Rearing. There was only one discrepancy between the results obtained using axenically reared or streptomycin-fed beetles. The levels of ipsenol and ipsdienol in axenically reared and wild male *I. paraconfusus* were not significantly different (Table 3, experiments XIII and XVIII). However, streptomycin-feeding of male *I. paraconfusus* significantly reduced the production of these terpene alcohols (Table 3, experiments XVI and XVII), as previously shown by Byers and Wood (1981). This anomaly could indicate that the reduction in ipsenol and ipsdienol production due to streptomycin feeding may be caused by toxic effects on insect cells rather than the elimination of certain microorganisms. This hypothesis appears to be somewhat unlikely since the production of *cis*- and *trans*-verbenol and myrtenol is not affected by streptomycin-feeding. Alternatively, the conversion of myrcene to ipsenol and ipsdienol could be performed by symbiotic microorganisms that are eliminated by streptomycin feeding but not by axenic rearing. These microorganisms would have to be transovarially transmitted, obligate symbionts to avoid elimination through axenic rearing and detection during the culturing of axenically reared beetles. Some evidence for transovarial transmission of bacteria in bark beetles has previously been reported (Buchner, 1965).

Vapors 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 α-pinene is converted to *cis*- and *trans*-verbenol and myrtenol more efficiently following exposure to vapors (Vité et al., 1972). Experiment XIX (Table 3) confirms that this trend is also true for *I.*

paraconfusus. When males were exposed to α-pinene and myrcene vapors simultaneously, conversion of α-pinene to *trans*- and *cis*-verbenol and myrtenol was much more efficient than conversion of myrcene to ipsenol and ipsdienol. When beetles were exposed only to myrcene vapors, slightly more ipsenol and ipsdienol were produced, but the effect was not significant. However, when beetles were exposed by feeding to the myrcene and α-pinene in ponderosa pine bolts, the conversion of myrcene to ipsenol and ipsdienol was much more efficient than with vapor exposure, while the conversion of α-pinene was significantly less efficient (Table 3, experiment XIX). As a result, experiments with *I. paraconfusus* generally used vapor exposure to study the conversion of α-pinene, and feeding to study the conversion of myrcene.

Hughes and Renwick (1977) 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 (1977), 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 vapors. This approach was criticized by Byers (1981), who suggested that the distension of the gut with air would enhance the diffusion of myrcene vapors in the gut, thus promoting pheromone synthesis artificially by increasing precursor diffusion to the site of synthesis. Our 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 vapors. This treatment resulted in a 3.5-fold increase in ipsenol content and a slight increase in ipsdienol content compared to controls (Table 3, experiment XX). However, these differences were not significant due to the large numbers of zeros in the data.

D. ponderosae also convert α-pinene more efficiently through vapor exposure than through feeding (Table 1). Female beetles fed on lodgepole pine phloem produced very little *cis*- and *trans*-verbenol and myrtenol (Table 1, experiment VI), while beetles exposed to α-pinene vapors produced much larger quantities of these terpene alcohols (Table 1, experiment VI). In contrast to *I. paraconfusus,* male *D. ponderosae* exposed to myrcene vapors produced large quantities of ipsdienol, as well as (*Z*)- and (*E*)-1-myrcenol (Table 1, experiment X), while males fed on bolts of lodgepole pine did not produce these products in quantifiable amounts.

Since myrcene is generally converted more efficiently through feeding and α-pinene is converted more efficiently through vapor exposure, ipsdienol has been termed a "frass" pheromone, while *cis*- and *trans*-verbenol are termed "contact" pheromones (Vité et al., 1972). Our data, as well as those of Byers (1982) and Hughes (1974), indicate that in *Dendroctonus* species myrcene is actually oxidized much more efficiently with vapor exposure than with feeding.

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. It is therefore logical that they would depend on rapidly produced contact pheromones. Less aggressive species, which generally attack trees with weakened or nonexistent defenses, can postpone mass attack until pioneer individuals have established, through feeding and defecation, that the host phloem is of suitable quality.

CONCLUSIONS

We have found that physiological maturation is required before axenically reared bark beetles can produce terpene alcohol pheromones maximally from monoterpenes. Our data provide a rationale for the extended maturation feeding by bark beetles prior to emergence from their brood host. Premature emergence would result in an unfit beetle incapable of producing optimal amounts of pheromone.

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* is more dependent on its symbiotic microorganisms than is *D. ponderosae.* Because it is a very aggressive bark beetle, *D. ponderosae* 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 enough individuals to overcome host resistance. However, *I. paraconfusus* generally breeds in fallen or cut trees (S.L. 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 less efficient production of aggregation pheromones is adequate in overcoming host resistance. Thus *I. paraconfusus* can 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 may be 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 lit-

erature that 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, our 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 here and by Byers and Wood (1981). Our data do add support to the assertion that certain microorganisms are capable of oxidizing monoterpenes, but establish 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.

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 by *D. ponderosae*. This ketone was not produced in quantifiable levels by axenically reared or streptomycin fed beetles that were exposed to α-pinene as vapors or through feeding. However, significant quantities of verbenone were found in wild *D. ponderosae* that were exposed to α-pinene through feeding on bolts of lodgepole pine. This result suggests that verbenone is not formed by the beetle, but is produced from *trans*-verbenol by microorganisms in its gut. This hypothesis is supported by reports that yeasts capable of converting verbenols to verbenone have been isolated from *D. ponderosae* (Hunt and Borden, 1989) and *I. typographus* (Leufvén et al., 1984). We hypothesize that similar oxidative metabolism of terpene alcohols by microorganisms may be responsible for the significantly lower levels of these compounds in wild beetles than in microbe-reduced beetles. In this way microorganisms would regulate pheromone levels in the host insects.

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