Mutual Inhibition of the Attractive Response to Pheromones of *Dendroctonus brevicomis* and *Ips paraconfusus*, and the Physiology of Pheromone Production in *Ips paraconfusus*

By

John Allen Byers

ABSTRACT

Dendroctonus brevicomis (Coleoptera: Scolytidae) is attracted to low levels of the synthetic pheromones ipsenol, *cis*-verbenol, and ipsdienol (I,II,III) of *Ips paraconfusus* (Coleoptera: Scolytidae) in the laboratory assay, but *I. paraconfusus* is not attracted to the synthetic pheromones *exo*-brevicomin, frontalin, and myrcene (E,F,M) of *D. brevicomis*. Low level interspecific attraction in the field was not observed. Attractive pheromones of *I. paraconfusus* (I,II,III) inhibit the attractive response of *D. brevicomis* to E,F,M in the laboratory. Similarly, a ponderosa pine log infested with *I. paraconfusus* males inhibits *D. brevicomis* attraction in the field to either E,F,M or to attractive logs cut from ponderosa pine trees under attack. E,F,M does not inhibit the attractive response of *I. paraconfusus* in the laboratory or field. However, logs cut from trees under attack by *D. brevicomis* inhibit the attractive response of *I. paraconfusus* to logs infested with male *I. paraconfusus*. Verbenone inhibits the attractive response of *I. paraconfusus* to I,II,III in the laboratory and to male infested logs in the field. Verbenone was found predominately in male *D. brevicomis* dissected from attractive logs under attack during the same time that the attractive response of *I. paraconfusus* was

inhibited by these D. brevicomis infested logs in the field. Trans-verbenol and verbenone are present in relatively large amounts near the beginning of the aggregation phase of host colonization and decrease at rates similar to the attractive pheromones (E and F). Therefore, it is hypothesized that verbenone does not function in the termination of the aggregation phase but that a gradual decrease in attractive pheromones causes the observed reduction in catch. The mutual inhibition of the attractive response to pheromones appears to help segregate these bark beetle species in distinct areas of the tree and as a consequence of this behavior interspecific competition for food and space is reduced.

The first quantitative study of a sex-specific conversion of a host plant precursor to components of an attractive pheromone was conducted with I. paraconfusus. Only the males produce I and III when exposed to a series of concentrations of myrcene vapor, a hydrocarbon found in xylem oleoresin of ponderosa pine. Females do not produce I or III under similar conditions although myrcene is absorbed into the hindguts in quantities comparable to males.

I. paraconfusus males when exposed to vapors of the host precursors myrcene and (-) α-pinene accumulate the pheromone compounds I, II, and III in the posterior portion of the hindgut. Other portions of the digestive tract and head contained low or undetectable amounts of the pheromones. The antibiotics streptomycin sulfate and penicillin-G when added to a semi-defined diet which was eaten by the beetle causes the complete inhibition of the production of I and III when exposed to myrcene vapors. Another pheromone component, II, which is produced by both sexes and derived from the host precursor (-) α-pin-

ene, was not affected by the dosages of antibiotic used in the experiments.

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ACKNOWLEDGMENTS

I would like to express my gratitude to David Wood and Lloyd Browne (Department of Entomological Sciences, UCB) who taught me many of the techniques and theories necessary to conduct the research described in this dissertation. David Wood was especially helpful in focusing my research and in developing my understanding of the scientific method. I would like to thank Barbara Barr (Department of Entomological Sciences, UCB) for relating to me her understanding and experience with bark beetles. Joe Elkinton (Department of Entomological Sciences, UCB) contributed to my knowledge of statistics and encouraged me throughout my studies. Kenneth Lindahl (Group in Biostatistics, UCB) also gave me valuable advice and encouragement on the statistical methodology used in these studies. Eugene Zavarin (Forest Products Laboratory, UCB) and Werner Loher (Department of Entomological Sciences, UCB) were very helpful in reviewing my thesis. Eugene Zavarin gave me advice on analytical chemical isolation and identification methods and Werner Loher gave me perspective and breadth in the study of insect behavior.

I am deeply grateful to the late Donald Denna (Department of Horticulture, Colorado State University, Fort Collins) who guided me through my M.S. research and contributed to my scientific development. James Bamburg (Department of Biochemistry, CSU), Richard Johnsen, and Wayne Brewer (Department of Zoology and Entomology, CSU), all M.S. thesis advisors, as well as Ken Collyard also were very instrumental in the development of my research capabilities necessary to undertake these studies.

I would also like to thank William Bedard and Paul Tilden (USDA Forest Service, Berkeley) for their advice concerning the field behavior of bark beetles and for the use of their research facilities and materials at Miami Field Base, Oakhurst, California.

I am especially grateful to my family for their encouragement and support.

Larry Hendry and Bogan Piatek (Department of Chemistry, Pennsylvania State University, University Park) were responsible for the GC CIMS confirmation of compounds I, III, and myrcene in part II.

These studies were supported in part by the Rockefeller Foundation; USDA Forest Service; and Cooperative State Research Services (2598-RRF, W-110); and the National Science Foundation and Environmental Protection Agency through a grant (NSF GB-34719/BMS 75-04223) to the University of California.

PREFACE

The bark beetles Ips paraconfusus Lanier and Dendroctonus brevicomis LeC. (Coleoptera: Scolytidae) cohabit ponderosa pine in California. These species must aggregate on the tree in sufficient numbers to overcome host resistance mechanisms. This aggregation is a result of attractive behavior released by pheromones that are chemicals produced by beetles initiating entrance tunnels on the host. It has been theorized that pheromones may also play a role in the termination of the aggregation phase of host colonization, pairing of the sexes, and regulation of the attack density. Also, these pheromones influence the behavior of other species of insects arriving on the tree either through attraction or inhibition of attraction. Precursor compounds found in the host may be eaten or absorbed and then converted into the pheromones within the body of the beetle and released through defecation. Investigations of the ecological role, behavioral responses, sites of production and mechanisms of synthesis of the pheromones used by these beetles promises a better understanding of chemical ecology, behavior, and physiology and may contribute to the development of treatment methods for manipulation of these destructive insects.

PART I

MUTUAL INHIBITION OF THE ATTRACTIVE RESPONSE TO PHEROMONES OF *DENDROCTONUS BREVICOMIS* AND *IPS PARACONFUSUS*

INTRODUCTION

The western pine beetle, Dendroctonus brevicomis LeC. (Coleoptera: Scolytidae), and the California 5-spined engraver, Ips paraconfusus Lanier (Coleoptera: Scolytidae), colonize the phloem-cambium/xylem tissues of ponderosa pine, Pinus ponderosa Laws., during the same periods in California. These bark beetles may select their host through an olfactory response to primary attractants emanating from the host (Person, 1931; Vité and Gara, 1962; Werner, 1972), gustatory response to phagostimulants (Elkinton, 1978), and visual mechanisms (Tilden, 1976). Once the beetles begin feeding, attractive pheromones and other behavioral compounds accumulate in their hindguts and are excreted with the fecal pellets (Wood and Bushing, 1963; Pitman and Vité, 1963; Pitman et al., 1965; Wood et al., 1966; Renwick, 1967; Silverstein et al., 1968). The attractive pheromone components of D. brevicomis are exo-brevicomin (female), frontalin (male), and myrcene (host) (Silverstein et al., 1968; Kinzer et al., 1969, Pitman et al., 1969). The attractive pheromone components of I. paraconfusus are ipsenol (I) (male), cis-verbenol (II) (both sexes), and ipsdienol (III) (male) (Silverstein and Rodin, 1965; Silverstein et al., 1966a, b; Wood et al.,

1966). The 3 pheromones of each species must be released simultaneously for maximum activity in the laboratory and field bioassays, and there are no known interspecific effects of these compounds on D. brevicomis and I. paraconfusus (Wood et al., 1967, 1968; Bedard et al., 1969, 1970; Silverstein et al., 1968; Vité and Pitman, 1969a, b; Wood, 1970).

The female D. brevicomis initiates the entrance tunnel and is joined by a male usually within a day or two (Miller and Keen, 1960). The males of emerged D. brevicomis are reported to contain verbenone in their hindguts (Renwick, 1967; Vité and Renwick, 1970; Renwick and Vité, 1970) which is released (Libbey et al., 1974; Browne et al., 1978) and inhibits the attractive response of beetles (Renwick and Vité, 1970; Hughes and Pitman, 1970; Wood, 1972; Bedard et al., 1978a). Verbenone has been proposed to terminate a mass-attack when the tree is fully colonized (Renwick and Vité, 1970; McNew, 1970). By contrast, in I. paraconfusus the male initiates the attack and is then joined usually by 3 females. Intraspecific inhibitory compounds are not known for I. paraconfusus and the cessation of the aggregation phase may simply result from a reduction in pheromone production (Pitman et al., 1966).

D. brevicomis is known to be the most aggressive tree-killing bark beetle in California on ponderosa pine.

The beetle can attack and kill any presumably healthy tree, but the beetles sustain less mortality when killing weakened or suppressed trees (Miller and Keen, 1960; Vité and Wood, 1961). I. paraconfusus is considered somewhat less aggressive since it usually kills the tops of trees, young trees, or infests recently cut logs and limbs (Struble and Hall, 1955; Wood, 1962). It is generally believed that a D. brevicomis infestation would predispose a tree to colonization by I. paraconfusus in the top section unoccupied by D. brevicomis (Miller and Keen, 1960). However, reports by Miller and Keen (1960) and Struble and Hall (1955) have indicated that I. paraconfusus may sometimes precede D. brevicomis in the successful attack of ponderosa pine even though I. paraconfusus is apparently the less aggressive species. Therefore, another mechanism of host selection could be that one species is attracted to the pheromone of the other species.

D. brevicomis and I. paraconfusus appear to be segregated into two distinct areas of the tree, i.e., D. brevicomis in the lower trunk and mid bole and I. paraconfusus extending above in the top section of the tree. The mechanism of segregation may include the production and release of inhibitory compounds (as shown by Birch and Wood, 1975, for I. paraconfusus and I. pini Say) that maintain the integrity of the infestation

depending on which species arrives first on the host substrate. The objective of this study was to determine if the attractive pheromones or other compounds produced by D. brevicomis and I. paraconfusus have a behavioral effect on the other species. Interspecific attraction and inhibition, i.e., the lowering of attractive response to pheromones when inhibitory compounds are released was tested for each species in the field and in laboratory assays using natural substrates and synthetic pheromones.

METHODS AND MATERIALS

Collection, Rearing, and Handling of Beetles

I. paraconfusus were obtained by collecting infested ponderosa pine slash from the Sierra National Forest near Bass Lake, California, at an approximate elevation of 1000 m. The logging slash containing late larval stages and callow adults was placed in emergence cages for the rearing and collection of adult beetles (Browne, 1972). The beetles were sexed by the method of Wood (1961). D. brevicomis were obtained by peeling bark off naturally-attacked ponderosa pines from the same areas. The beetles were reared, collected, and stored in an identical way but sexed according to Tate and Bedard (1967). The emerged adults were stored from 1 to 15 days at 4° C on moist paper toweling until used in the laboratory or for field assays.

It was often necessary to group beetles from several days emergence to obtain a sufficient number for a particular experiment. Therefore, the beetles were randomized so that certain treatments or assays would not contain a disproportionate number of beetles from any one date. The beetles of one sex were randomized by taking individuals that could walk from each day's collection and allowing all of them to disperse on a crumpled paper towel in one container.

Purification of Verbenone

Verbenone (Chem. Samples Co., Columbus, Ohio) was purified with a Varian model 2700 f.i.d. gas-liquid chromatograph (GLC) fitted with a 1.83 m x 8 mm i.d. preparative glass column of 3% Apiezon L on 100/120 Gas Chrom Q. The operating conditions were 130° C and N_2 carrier gas at 300 ml per min. through the column with a 30:1 stream split for cold-trap collection.

Laboratory Assay

The behavioral responses of *D. brevicomis* and *I. paraconfusus* to behavioral chemicals were tested in the laboratory olfactometer developed by Browne et al. (1974) for *I. pini*. However, several modifications were used. The polyurethane foam was removed from the plexiglass manifold to permit faster air flow. The air speed at the level of walking beetles (0 to 2 cm) was 0.9 m/sec.

at the pheromone source and 0.6 m/sec. where the beetles were released. Groups of 10 beetles of one sex were released for each test at 21 ± 2 cm down wind from the source. A positive response was recorded when a beetle walked within a 1 cm radius of the source in the time required for the pheromone in the solvent to evaporate from the 5 ul capillary tube (126.5 ± 10 sec.).

At least 30 beetles of one sex were tested at each concentration of compound(s) dissolved in diethyl ether as well as for an ether solvent control. The percent responding was obtained by dividing the number reaching the source of the behavioral chemical by the number released times 100. The confidence limits were determined from a chart of 95% binominal confidence limits (B.C.L.) for proportions.

Field Assay

The field experiments were conducted during the summer and fall of 1975 and 1976 in the Sierra National Forest near Bass Lake, California, at elevations from 900 to 1200 m. These tests were used to ascertain whether volatile compounds released by I. paraconfusus or D. brevicomis could inhibit the attractive pheromone response of either species. The basic experiment used to determine if inhibition occurs was the comparison of catches on two sticky-traps; both traps containing the same species

while one trap also contained the other species. A reduction in catch at the trap with both species compared to the trap with one species (check) would indicate that inhibition had occured.

Logs with 50 male I. paraconfusus, logs from trees naturally infested with D. brevicomis, and synthetic pheromones were used in various tests. The logs with 50 male I. paraconfusus were prepared by drilling 50 uniformly spaced holes (7 cm spacing) at a 30° angle from the longitudinal log axis so that each hole penetrated the phloem but not the xylem (Wood et al., 1968; Lanier et al., 1972). The logs (approximately 14 cm diameter by 28 cm long) were cut from ponderosa pine trees and stored less than one month at 4° C before drilling. Male beetles were introduced head-first into each hole and the log then was wrapped with aluminum window screen. Control logs were drilled and screened in the same way but without beetles. After a period of 48 hrs. to allow maximum pheromone production (Wood et al., 1966), the log was placed in a "small" sticky-trap (19 cm dia. x 30.5 cm high) supported on a pipe standard 1.2 m above the ground (Bedard and Browne, 1969).

Synthetic pheromones were used in some of the tests to provide a source of attraction or inhibition. The pheromone components of D. brevicomis, exo-brevicomin (> 95%) frontalin (> 95%) and myrcene (> 97%) (E,F,M)

(Chem. Samples Co., Cleveland, Ohio) were released in the field from an elution device (Rodin et al., unpublished). It consisted of an inverted 7.6 cm salt and pepper shaker covered with aluminum foil which contained each compound in separate glass tubes sealed at one end. Exo-brevicomin and myrcene were each placed neat in 5 x 55 mm tubes to a level 30 mm below the opening while frontalin (neat) was placed in a 4 x 65 mm tube to a level 40 mm from the top. Weight losses for each of these compounds averaged 2 mg per day under field conditions (Browne, 1978). Verbenone also was released neat from a glass container (1 cm dia. x 0.5 cm high) inside the salt shaker but at 4 mg per day (Bedard et al., 1978a).

Natural D. brevicomis pheromone substrates were obtained by placing several (E,F,M) elution devices on a healthy ponderosa pine tree so that beetles would initiate the mass-attack (Bedard et al., 1978b). After about 2 days, the tree was felled and sectioned into 0.5 or 0.6 m logs and 2 logs were placed on pipe standards inside "large" sticky-traps. The large sticky-traps were built by criss-crossing 2 (76 cm x 203 cm) vinyl coated fiber-glass screens (5 x 12.7 mm mesh) coated with Stickem Special ® over 4 (1.54 m long) cedar steaks driven vertically into the ground in such a way as to form a 76 cm square box. The vinyl coated screens were originally used by Browne (1978) to trap D. brevicomis.

The specific methods and materials for each type of field test will be described in the appropriate sections.

Interspecific Attraction in the Laboratory Assay

Both sexes of D. brevicomis were tested October 5-7, 1976 for attraction to synthetic I. paraconfusus pheromones (I, II, III) at 10 fold increasing concentrations from 10^{-11}g to 10^{-6}g per µl ether. Both sexes of I. paraconfusus were tested July 29, 1976, for attraction to synthetic D. brevicomis pheromones (E,F,M) at 10 fold increasing concentrations from 10^{-11}g to 10^{-8}g per µl ether. Verbenone also was tested against both sexes of I. paraconfusus on July 28, 1976, at 10 fold increasing concentrations from 10^{-11}g to 10^{-7}g per µl ether.

Inhibition of the Attractive Response of D. brevicomis

Laboratory. The attractive response curves of both sexes of D. brevicomis to their pheromones (E,F,M) were determined so that an attractive dose could be compared to an identical dose with I. paraconfusus pheromone for the possible detection of inhibition. Both sexes of D. brevicomis were tested in the laboratory assay October 8, 1976, for attraction to synthetic D. brevicomis pheromone (E,F,M) at 10 fold concentrations from 10^{-11}g to 10^{-6}g per µl ether. D. brevicomis then was tested on October 11 and 12, 1976, for inhibition

of its attractive pheromone response by adding a 10 fold increasing concentration range of synthetic I. paraconfusus pheromone (10^{-10}g to 10^{-5}g I, II, III per µl) to an attractive dose of E,F,M at 10^{-9}g per µl. Similarly, D. brevicomis also was tested on October 16, 1976, for inhibition by adding a 10 fold increasing concentration series of certain synthetic I. paraconfusus pheromone components (either 10^{-7}g to 10^{-6}g I, III per µl or 10^{-7}g to 10^{-6}g II per µl) to an attractive does of E,F,M (10^{-9}g per µl).

Field. The inhibition of the attractive response of D. brevicomis to either E,F,M or natural pheromone by I. paraconfusus pheromone was tested by comparing the daily number of D. brevicomis caught at 2 sticky-traps. In the first case, one small sticky-trap contained a log with 50 male I. paraconfusus plus an E,F,M elution device (2 mg per day) while the second trap (check) placed 11 to 12 m away contained an uninfested log plus an identical E,F,M elution device. Eight tests were replicated at 7 different locations in the forest and each test was repeated on 2 to 6 successive days during August and September, 1975, a total of 28 day paired comparisons. The 50 male I. paraconfusus log and the uninfested log were interchanged each day of the test period while the E,F,M elution devices and sticky-traps remained stationary.

Variation in beetle catch due to differences in trap position and E,F,M elution rates would be minimized by alternating the suspected inhibitory source (log containing 50 male I. paraconfusus) so that each trap held the treatment and check an equal number of days. D. brevicomis were picked from the traps at the same time each day and placed in mineral spirit solvent to remove the adhesive.

The beetles were sexed and the sex ratio and binomial upper and lower confidence limits (95%) were determined with the following formulas (Spiegel, 1961):

$$\text{sex ratio} = \frac{\text{Number of males}}{\text{Number of females}} = \frac{P}{1-P}$$

$$\text{upper confidence limit} = \frac{Pu}{1-Pu} \; ;$$

$$\text{lower confidence limit} = \frac{P_L}{1-P_L}$$

where:

$$Pu = \frac{P + \frac{Z^2}{2N} + Z\sqrt{\frac{P(1-P)}{N} + \frac{Z^2}{4N^2}}}{1 + \frac{Z^2}{N}}$$

$$P_L = \frac{P + \frac{Z^2}{2N} - Z\sqrt{\frac{P(1-P)}{N} + \frac{Z^2}{4N^2}}}{1 + \frac{Z^2}{N}}$$

Z = 1.96 (95%)

N = Number of males plus females

P = Number of males divided by N

On the first day of a test, the trap with the 50 male I. paraconfusus log was arbitrarily designated position a, and the trap with the check log, position b. Since the traps were paired each day, the non-parametric Wilcoxon test for two paired samples (McCall, 1970) was employed for the comparison of the number and sex caught between the treatments and checks in different traps on the same day. Similarly, the test also was used for comparisons between the treatment and check on successive days but in the same trap (i.e., compare position a, day 1, containing the I. paraconfusus log to position a, day 2, without the log). In the second type of comparison, only day 1 to 2, day 3 to 4, and day 5 to 6 were compared. Finally, the Wilcoxon test was used to determine if one sex was inhibited more than the other. The ratio of the number of male D. brevicomis caught on the trap with the 50 male I. paraconfusus log to the number caught on the check trap was compared to a similar ratio of catch for females.

The inhibition of the attractive response of D. brevicomis to naturally-produced pheromone by the naturally-produced pheromone of I. paraconfusus was tested by comparing the catch of D. brevicomis infested tree parts plus an uninfested log to traps with infested tree parts plus a log infested with 50 male I. paraconfusus. The naturally-infested D. brevicomis tree

parts were obtained by placing 3 E,F,M elution devices on a 30 cm D.B.H. ponderosa pine tree at heights of 2, 6, and 12 m on August 30, 1975. The synthetic pheromone was removed after 2 days and the tree was sectioned into 0.5 m logs. The two large traps as described previously were 11 to 12 m apart and each contained 2 logs of naturally infested D. brevicomis. The I. paraconfusus log and uninfested log were interchanged each day as previously described while the traps and D. brevicomis logs remained stationary. The statistical methods described above were used here also.

Inhibition of the Attractive Response of I. paraconfusus

Laboratory. I. paraconfusus males and females were tested July 29, 1976, for inhibition in the laboratory assay by adding a 10 fold concentration series of E,F,M (10^{-10}g to 10^{-6}g per µl) to a known attractive dose of I, II, III (10^{-9}g per µl). Both sexes of I. paraconfusus also were tested on July 27 and 28, 1976, for inhibition by adding different concentrations of verbenone (10^{-11}g to 10^{-6}g per µl) to an attractive dose of I, II, III (10^{-9}g per µl). Finally, the concentration of II needed to increase the attractive response of female I. paraconfusus to I + III was determined on April 6, 1976, by testing a concentration series of II from 10^{-13}g to 10^{-9}g with I + III at 5 x 10^{-9}g per µl.

Field. The possibility that E,F,M could inhibit the attraction of I. paraconfusus to naturally-produced pheromone was tested by placing two small sticky-traps 11 to 12 m apart with each containing a log infested with 50 male I. paraconfusus and one of the traps also containing an E,F,M elution device. The E,F,M elution device was alternately placed on each trap each successive day during the test (August 30-September 4, 1975). The test consisted of 2 pairs of traps in each of 2 areas of the forest. Statistical tests described above were used.

The possibility that verbenone could inhibit the attractive response of I. paraconfusus to naturally-produced pheromones also was tested. The test consisted of 3 pairs of traps and was conducted from June 25-28, 1976. The test methods were identical to those described above for a test of inhibition of I. paraconfusus by E,F,M except that verbenone and the verbenone elution device were used.

Finally, the possibility that the attraction of I. paraconfusus to natural pheromone substrates could be inhibited by the presence of natural D. brevicomis pheromone substrates was investigated. The naturally-infested D. brevicomis substrates were obtained by placing 2 E,F,M elution devices on a 30 cm D.B.H. ponderosa pine tree at heights of 1.5 and 6.8 m at 8 A.M. on August 24, 1976. Three days later at 8 A.M. August 27,

the tree was sectioned into 0.6 m logs, numbered 1-9, with the first log beginning at the 1.2 m height. Each of 3 replicates consisted of 2 large sticky-traps 11 to 12 m apart both containing a log with 50 male I. paraconfusus and one trap also had 2 of the D. brevicomis logs (#1 + 6; 2 + 5; or 3 + 8). On each day of the test (August 27-30, 1976) the D. brevicomis logs were alternated between paired traps (position a or b) while the I. paraconfusus logs and traps remained stationary. All bark beetle and predator species caught on the traps were collected and analyzed statistically when appropriate.

The progress of the attack, sex ratio and production of pheromone compounds within the hindgut of D. brevicomis were monitored near the start and at the end of the test period by dissecting log #4 at 4 P.M. on August 27 and log #5 (used in the test) at 8 P.M. on August 31. Log #4 was dissected by carefully peeling the bark off with a draw knife, collecting the beetles from the galleries, and recording the number of entrance tunnels and gallery length per tunnel. Log #5 was dissected similarily but the galleries were so extensive that only the total gallery length could be determined.

Identification and Quantification of Pheromones Produced by D. brevicomis in Natural Substrates in the Field

The identification and quantities of pheromones found in the hindguts of D. brevicomis feeding in ponderosa

pine tree parts used in the inhibition test were determined by extraction and gas-liquid chromotography (GLC). The last abdominal segment along with the hindgut and a portion of the midgut were pulled with a fine forceps from 50 males and 50 females collected on the two dates from the dissected logs. The guts were placed in 1 ml Mini-Vials (Applied Science Lab., Inc.) containing 0.1 ml ground glass (crushed 100 ul pipets) and 0.35 ml diethyl ether. All guts then were crushed in the Mini-Vials with a small glass rod. Later the samples were injected into the GLC as described earlier on a 1.83 m x 2 mm i.d. glass column of 10% FFAP on 80/100 Gas Chrom Q with the N_2 carrier gas at 30 ml per min. at 100° C and on a 1.83 m x 2 mm i.d. glass column of 3% Apiezon L on 100/120 Gas Chrom Q with the N_2 flow at 12 ml per min. at 100° C. Quantitative standards of exo-brevicomin, frontalin, trans-verbenol (> 95%) (Glidden Organics, Jacksonville, Florida) and verbenone were compared to the peaks from the hindgut extracts. A portion of each hindgut extract was mixed with comparable quantities of exo-brevicomin (2.5×10^{-8}g per µl), frontalin (2.5×10^{-8}g per µl), trans-verbenol (10^{-8}g per µl) and verbenone (6×10^{-9}g per µl) so that the samples could be coinjected on both columns for maximum sensitivity of separation and identification.

RESULTS

Interspecific Attraction in the Laboratory Assay

Both sexes of D. brevicomis exhibited a moderately attractive response to synthetic I. paraconfusus pheromones at 10^{-9}g I, II, III per µl (Fig. 1). At higher concentrations of I, III, III (10^{-7} - 10^{-6}g µl), however, the response dropped to that of solvent controls (< 7% response). The release rate of pheromones (g per min.) from the 5 µl capillary pipet was estimated to be 2.2 times the g per µl concentration. However, variation due to differential distillation and evaporation must be determined before one can assume that the release rate is identical to the evaporation rate of the compound in a solvent.

In field studies, D. brevicomis was not caught on sticky-traps containing only logs infested with I. paraconfusus males. A few D. brevicomis were collected from these traps (0-2/trap/day) but these probably were missed from the previous day (which contained E,F,M or D. brevicomis infested logs) as none were caught on the first day of any of the tests. In contrast, I. paraconfusus was not attracted to synthetic pheromones of D. brevicomis (E,F,M) or verbenone in the laboratory assay (Fig. 2). Similarly, I. paraconfusus was not collected from traps containing only D. brevicomis infested logs except on occasion as discussed above.

Fig. 1. Response of male and female Dendroctonus brevicomis to synthetic pheromones of Ips paraconfusus (I, II, III) in the laboratory (October 5-7, 1976). Brackets represent 95% binomial confidence limits.

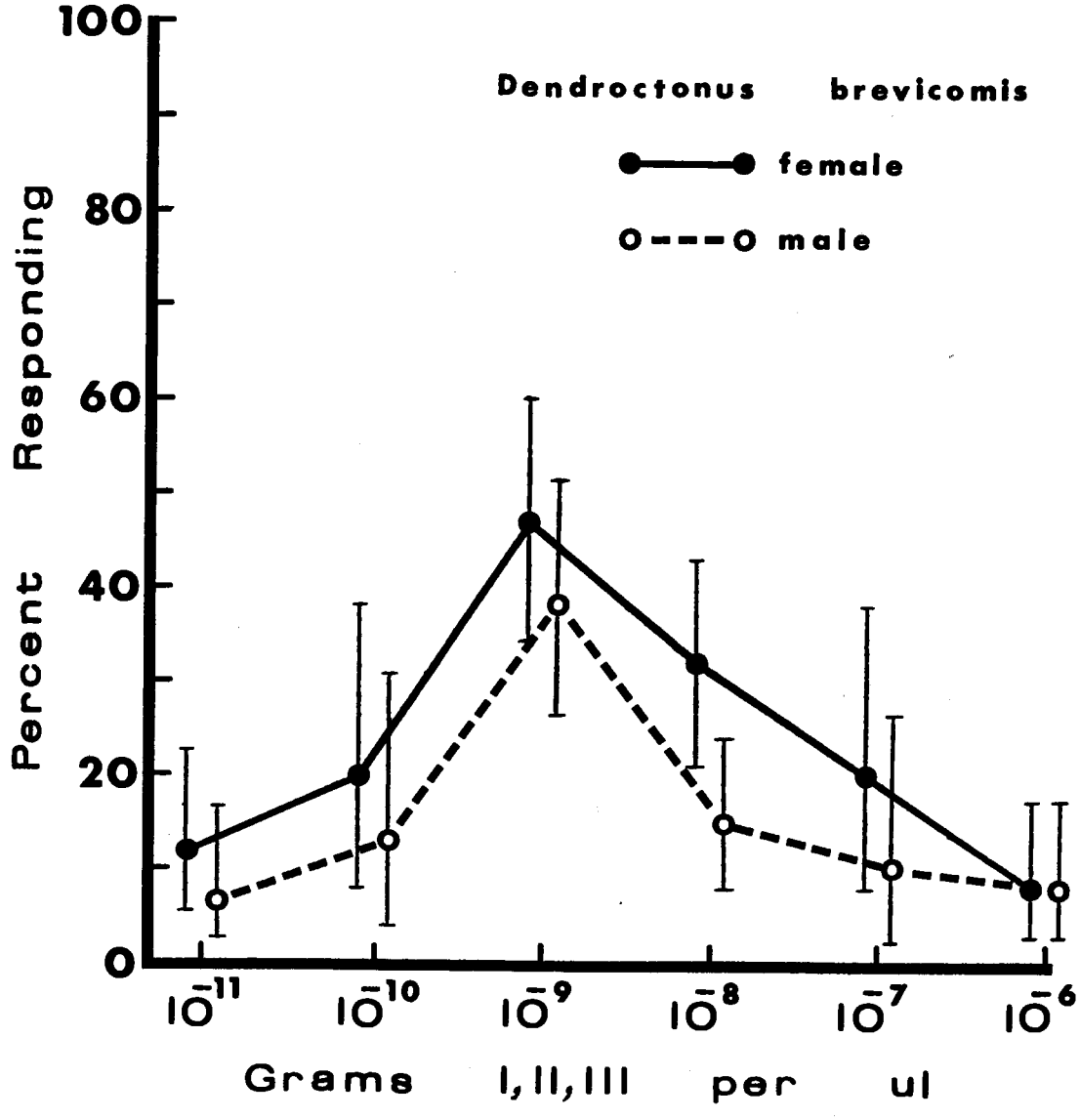
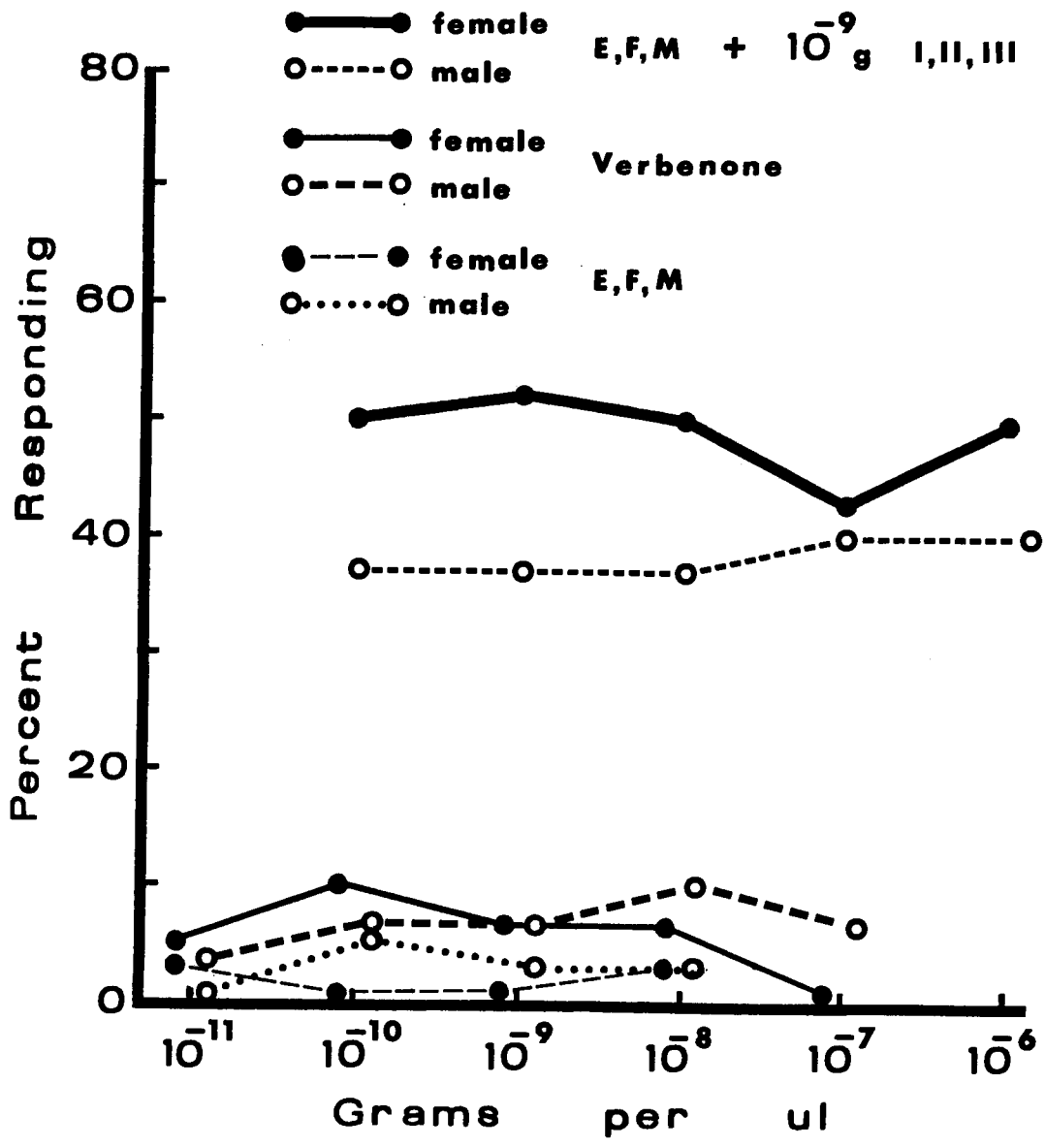

Dendroctonus brevicomis
female
male
Percent Responding
100
80
60
40
20
0
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
Grams I, II, III per ul

Fig. 2. Response of male and female Ips paraconfusus to E,F,M, E,F,M + I, II, III, and verbenone in the laboratory (July 28-29, 1976).


female
male
E,F,M + 10^{-9} g I,II,III
female
male
Verbenone
female
male
E,F,M
80
60
40
20
0
Percent Responding
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
Grams per ul

Inhibition of the Attractive Response of D. brevicomis

Laboratory. Both sexes of D. brevicomis responded similarly to each concentration of their pheromones (Fig. 3) which was consistent with the 1:1 sex ratio caught on field traps (reported later). The attractive response of both sexes of D. brevicomis was inhibited by I. paraconfusus pheromones (I, II, III) at 10^{-6}g per µl or more (Fig. 4). It appears all 3 pheromone compounds of I. paraconfusus are necessary since neither I + III or II can inhibit the response of D. brevicomis to E,F,M (Table 1).

Field. I. paraconfusus pheromones inhibit the attractive response of D. brevicomis to E,F,M (Fig. 5). The traps containing E,F,M plus a log with 50 I. paraconfusus males caught only 35% as many D. brevicomis as traps with E,F,M plus uninfested logs (329 vs. 928). When the individual trap catches were compared between position a and b on each day or between the same position on successive days, the treatment and check were sinificantly different in both comparisons at the 1% level. The logs infested with 50 male I. paraconfusus produced pheromone since an average of 17/trap were caught.

The sex ratio (♂♂/♀♀) for D. brevicomis caught on the E,F,M plus I. paraconfusus log traps was 1.02 with

Fig. 3. Attraction of male and female Dendroctonus brevicomis to E,F,M in the laboratory (October 8, 1976). Brackets represent 95% binomial confidence limits.

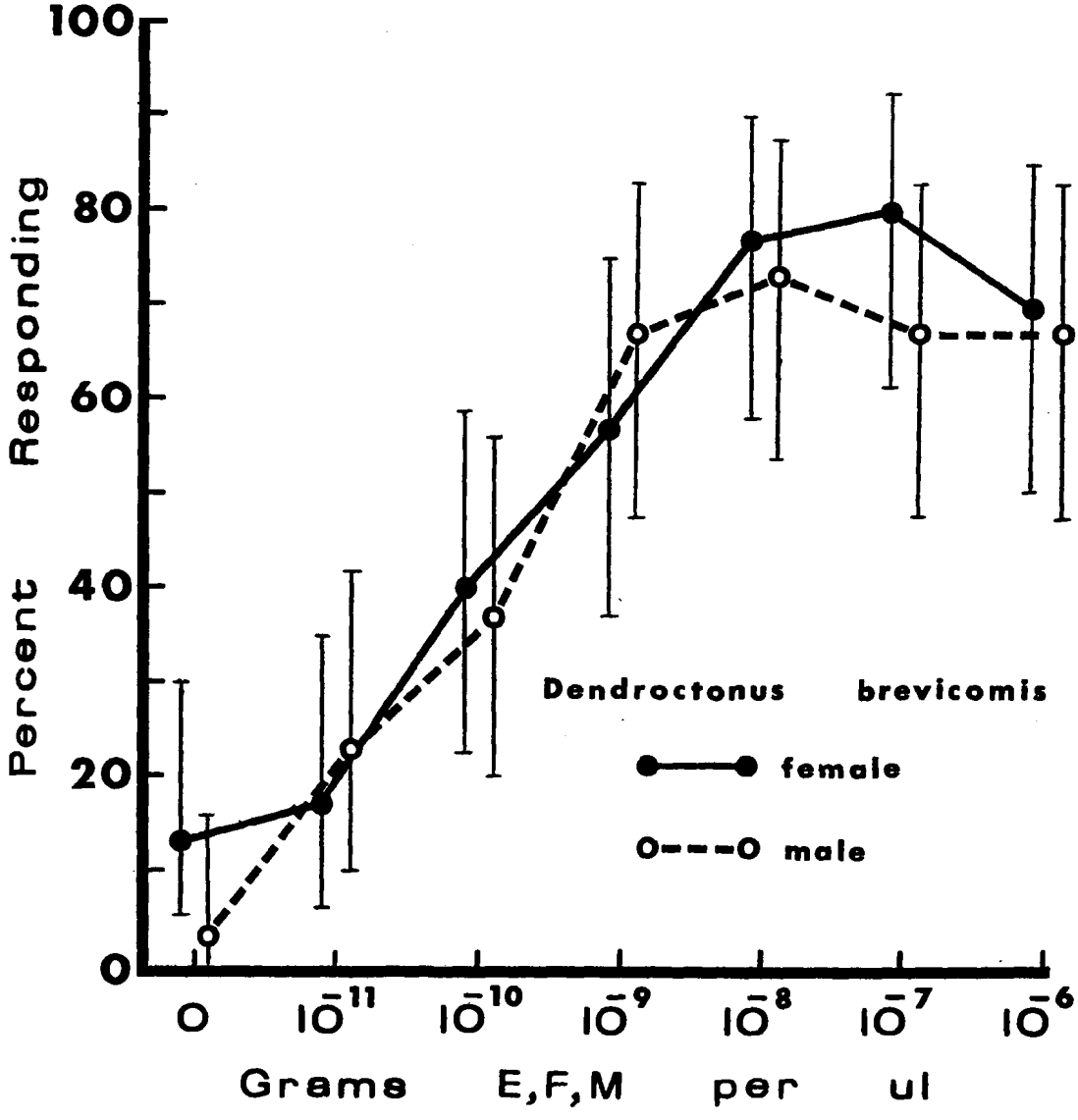

100
80
60
40
20
0
Percent Responding
Dendroctonus brevicomis
female
male
0
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
Grams E,F,M per ul

Fig. 4. Inhibition of the attractive response of male and female Dendroctonus brevicomis to E,F,M by synthetic pheromones of Ips paraconfusus (I, II, III) in the laboratory (October 11-12, 1976). Brackets represent 95% binomial confidence limits.

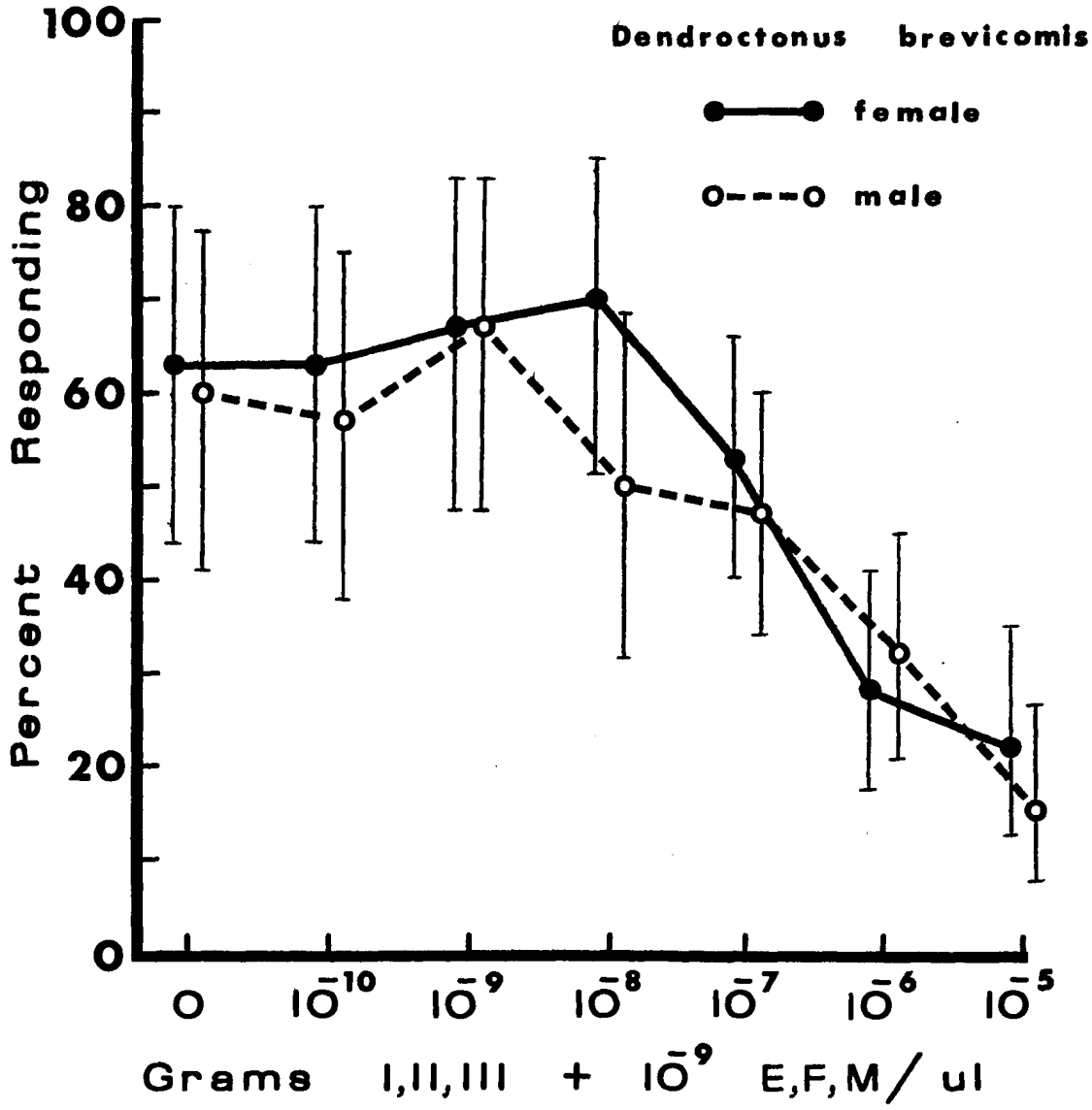
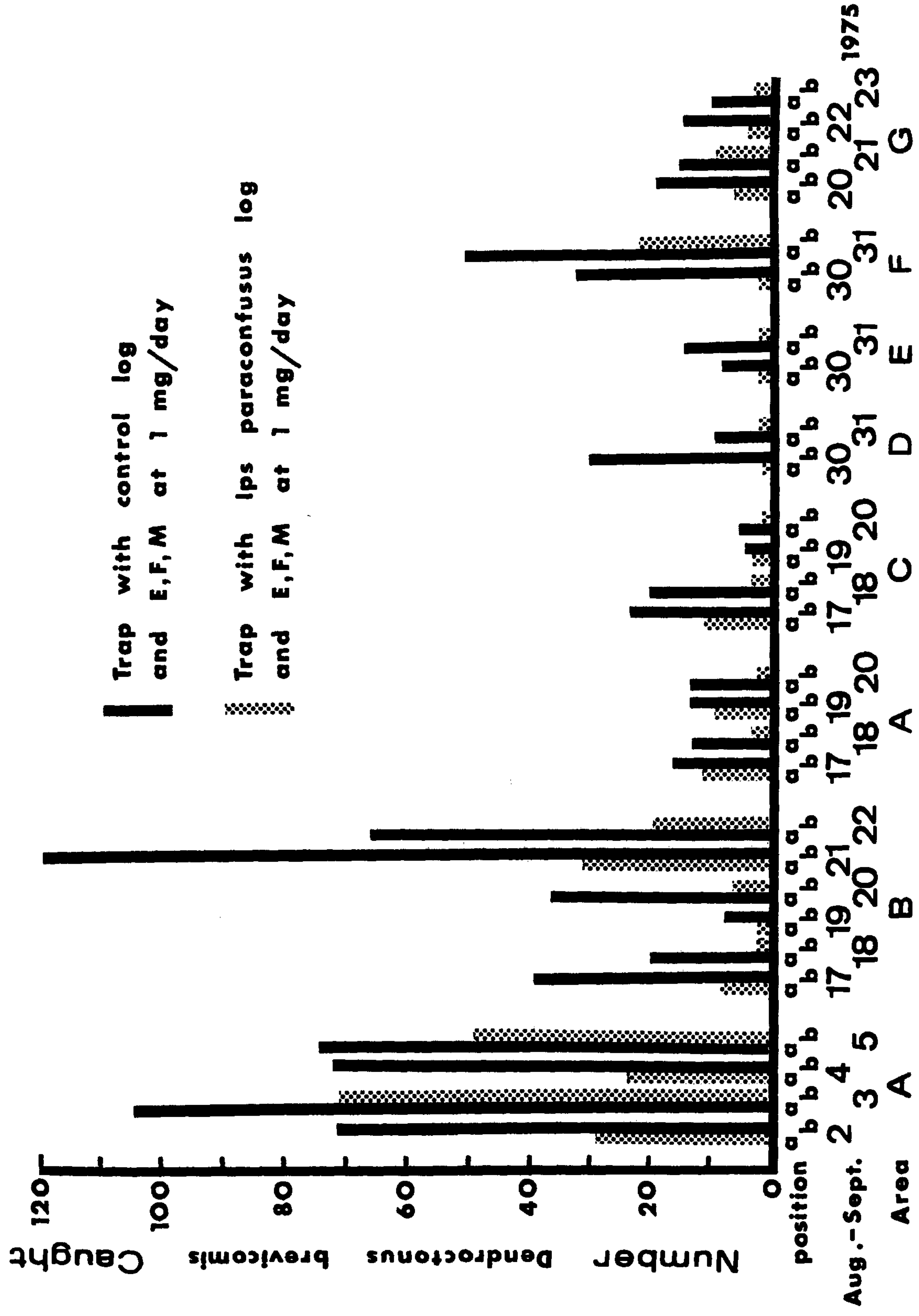

Dendroctonus brevicomis
female
male
Percent Responding
100
80
60
40
20
0
0
10⁻¹⁰
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
Grams I,II,III + 10⁻⁹ E,F,M/ul

Table 1. Inhibition of the attractive response of male and female D. brevicomis to E,F,M by I, II and III in the laboratory (October 16, 1976).

Compounds Tested	Dose	Sex	Number Tested	Percent Responding	Confidence Interval (95%)
E,F,M	10^{-9}g/ 1		90	63	52-73
			120	68	59-76
E,F,M	10^{-9}g/ 1		30	80	61-92
+					
I, III	10^{-7}g/ 1		30	63	43-80
E,F,M	10^{-9}g/ 1		30	87	69-97
+					
I, III	10^{-6}g/ 1		30	83	64-94
E,F,M	10^{-9}g/ 1		30	73	54-88
+					
II	10^{-7}g/ 1		30	73	54-88
E,F,M	10^{-9}g/ 1		60	53	40-68
+					
II	10^{-6}g/ 1		60	60	46-72
E,F,M	10^{-9}g/ 1		90	30	20-41*
+					
I, II, III	10^{-6}g/ 1		90	31	21-42*

*Significantly different from above treatments.

Fig. 5. Inhibition of the attractive response of Dendroctonus brevicomis to E,F,M by naturally-produced pheromone of Ips paraconfusus in the field. Treatments were alternated each day between positions a and b.

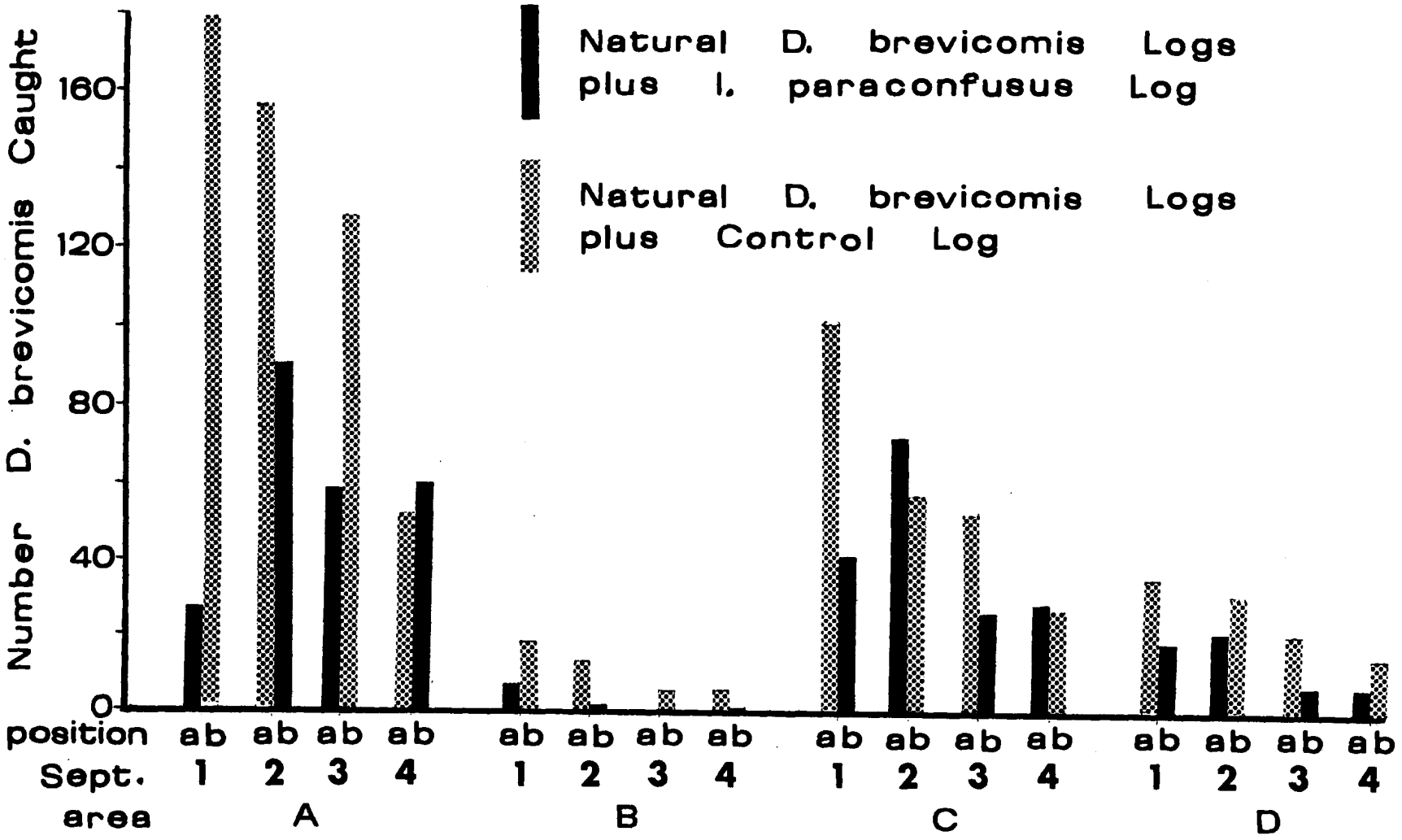

Trap with control log
and E,F,M at 1 mg/day
Trap with Ips paraconfusus log
and E,F,M at 1 mg/day
Number Dendroctonus brevicomis Caught
120
100
80
60
40
20
0
position
Aug.-Sept.
Area
2 3 4 5
A
17 18 19 20 21 22
B
17 18 19 20
A
17 18 19 20
C
30 31
D
30 31
E
30 31
F
20 21 22 23
G
1975

the 95% binomial confidence limits (B.C.L) of 0.82 and 1.26. The sex ratio for the E,F,M only trap was not different at 0.87 (B.C.L. 0.77 and 0.96) from the sex ratio caught on traps containing both species since the confidence intervals broadly overlap. There was no difference between the inhibition of the sexes since the ratios were not significantly different at the 5% level (Wilcoxon test).

In similar experiments, the response of D. brevicomis to naturally-produced pheromone was inhibited by logs infested with 50 male I. paraconfusus (Fig. 6). The logs infested with I. paraconfusus released pheromone since an average of 142 I. paraconfusus were caught per trap/day. The traps with D. brevicomis and I. paraconfusus infested logs caught half as many D. brevicomis as the traps with D. brevicomis infested logs and an uninfested log (463 vs. 896). The comparison of treatment and check at position a or b on each day and the comparison of treatment and check on successive days at the same position were each significantly different at the 1% level.

The sex ratio (♂♂/♀♀) of D. brevicomis trapped at infested logs of both species was 0.84 (B.C.L. 0.7 and 1.0). The sex ratio caught at the D. brevicomis infested logs only was 0.93 (B.C.L. 0.82 and 1.06). The sex ratio values from the treatment and the check were not significantly different from each other or from the

Fig. 6. Inhibition of the attractive response of Dendroctonus brevicomis to natural pheromone by naturally-produced pheromone of Ips paraconfusus in the field. Treatments were alternated each day between positions a and b.


Natural D. brevicomis Logs plus I. paraconfusus Log
Natural D. brevicomis Logs plus Control Log
Number D. brevicomis Caught
0
40
80
120
160
position ab ab ab ab ab ab ab ab ab ab ab ab ab ab ab ab
Sept. 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
area A B C D

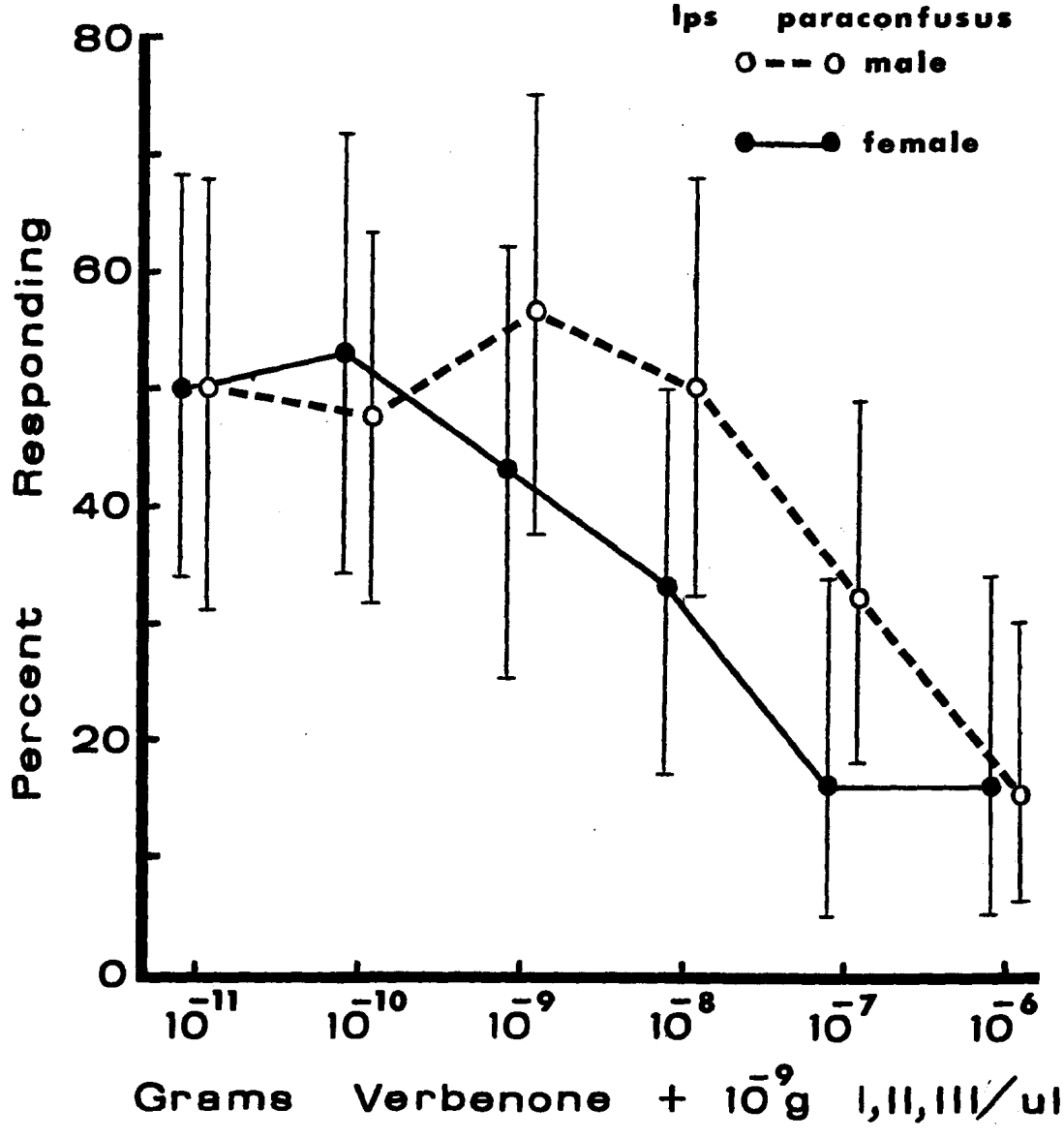
sex ratios obtained in experiments with E,F,M as the attractive source. The Wilcoxon test showed that there was no sifnificant difference between the inhibition of male and female D. brevicomis at the 5% level.

Inhibition of the Attractive Response of I. paraconfusus

Laboratory. The attractive responses of either sex of I. paraconfusus to 10^{-9}g I, II, III per µl was unaffected by E,F,M at concentrations of 10^{-10}g to 10^{-6}g per µl (Fig. 2). However, verbenone (produced by male D. brevicomis) at 10^{-7}g to 10^{-6}g per µl (Fig. 7) was effective in inhibiting the attractive response of both sexes of I. paraconfusus to I, II, III. If verbenone competitively inhibits the acceptor site for II in I. paraconfusus, then the competition must be almost complete since as little as 10^{-10}g II per µl when added to 5 x 10^{-9}g I + III per µl increased the percent response of females from 26% (B.C.L 16% and 38%) to 57% (B.C.L. 38% and 75%).

Field. The E,F,M attractive pheromones of D. brevicomis do not inhibit the attractive response of I. paraconfusus to its naturally-produced pheromone. A total of 1,156 (194♂♂:962♀♀) I. paraconfusus were caught on the traps containing an I. paraconfusus infested log plus E,F,M compared to 1,283 (214♂♂:1,069♀♀) caught on the traps containing only an I. paraconfusus infested

Fig. 7. Inhibition of the attractive response of male and female Ips paraconfusus to I, II, III by verbenone in the laboratory (July 27-28, 1976). Brackets represent 95% binomial confidence limits.

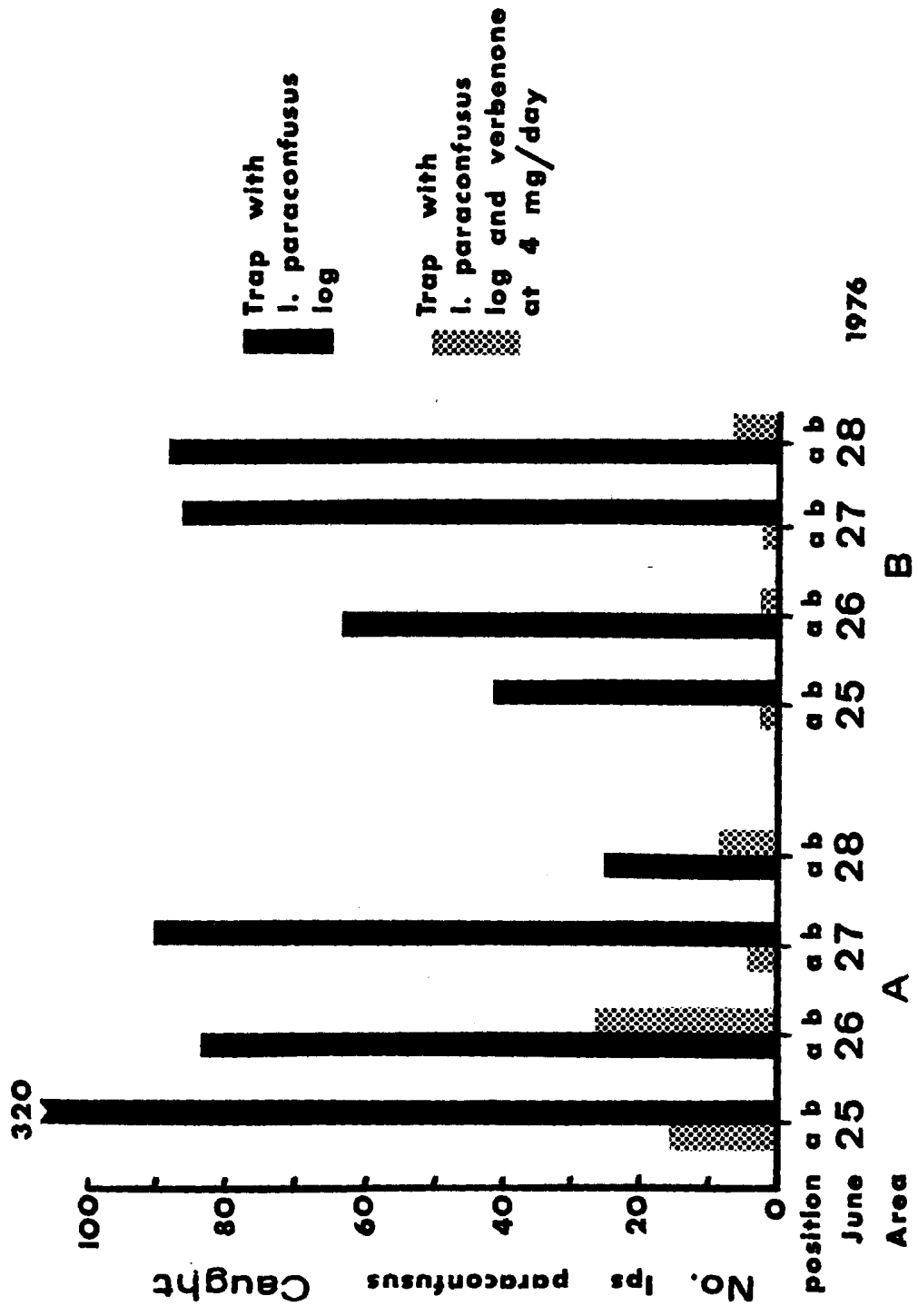

Ips paraconfusus
o--o male
female
Percent Responding
80
60
40
20
0
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
Grams Verbenone + 10^{-9}g I,II,III/ul

log. When the individual trap catches were compared in the Wilcoxon match pair test, the treatment and check were not different at the 5% level. The sex ratio (♂♂/♀♀) of the catch at traps containing the I. paraconfusus long plus E,F,M was 0.20 (B.C.L. 0.17 and 0.24) as was the sex ratio for the catch at the traps containing only the I. paraconfusus log (0.20 with B.C.L. 0.17 and 0.23).

Verbenone, on the other hand, inhibited the attractive response of I. paraconfusus to its naturally-produced pheromone in the field (Fig. 8). Only 67 (17♂♂: 50♀♀) I. paraconfusus were caught on traps containing the I. paraconfusus log plus verbenone (4 mg/day) while 798 (91♂♂:707♀♀) or 92% of the total catch were caught on the traps containing only the I. paraconfusus infested log. When the individual trap catches were compared on the same day in the Wilcoxon test, the treatment was significantly different from the check at the 1% level. The treatment also was different from the check at the 1% level when compared at the same position on successive days.

The sex ratios caught on the treatment and the check also were significantly different since the ratio (♂♂/♀♀) for the catch at the I. paraconfusus infested log plus verbenone was 0.34 (B.C.L. 0.20 and 0.59) while the ratio for the catch at the I. paraconfusus log alone was 0.13 (B.C.L. 0.10 and 0.16). A Wilcoxon comparison

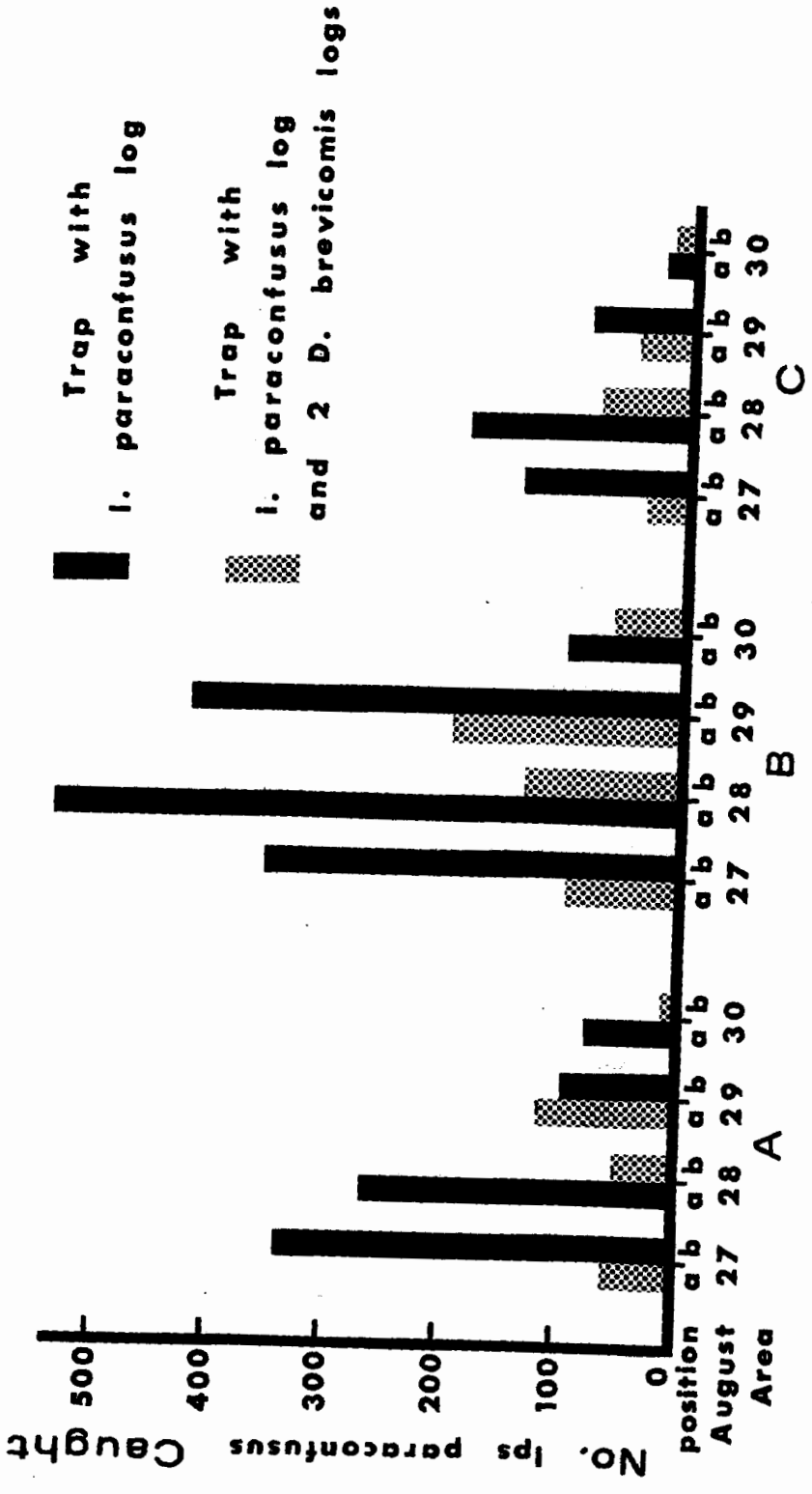
Fig. 8. Inhibition of the attractive response of Ips paraconfusus to natural pheromone by verbenone in the field.


Trap with
I. paraconfusus
log
Trap with
I. paraconfusus
log and verbenone
at 4 mg/day
1976
No. Ips paraconfusus Caught
0
20
40
60
80
100
320
Area
June
position a b a b a b a b a b a b a b a b
25 26 27 28 25 26 27 28
A
B

showed that the number of males caught on the treatment and check were significantly different at the 5% level and the females were different at the 1% level. From the sex ratio one can not determine with confidence that females are inhibited more so than males. However, when the ratios of males caught on the treatment and the check are compared to the ratios of females in the Wilcoxon test, the ratios were significantly different at the 1% level. Although both sexes are inhibited by verbenone, females appear to be inhibited to a greater extent.

Naturally-infested D. brevicomis logs appear to inhibit the attractive response of I. paraconfusus to its natural pheromone in the field (Fig. 9). The inhibition of I. paraconfusus by natural D. brevicomis substrates occured from the first day of the test. Only 899 (305♂♂:594♀♀) I. paraconfusus were caught on the traps with logs of both species or only 34% as many as the number caught on the traps with an I. paraconfusus infested log only (2,622; 651♂♂:1,971♀♀). The Wilcoxon test showed that the treatment and check were different when compared each day at the 1% level or when compared at the same position on successive days at the 5% level. The sex ratio (♂♂/♀♀) of I. paraconfusus caught on the traps containing both species was 0.51 (B.C.L. 0.45 and 0.59) and was significantly different from the 0.33 (B.C.L. 0.30 and 0.36) sex ratio of beetles caught on

Fig. 9. Inhibition of the attractive response of Ips paraconfusus to natural pheromone by naturally-produced pheromone of Dendroctonus brevicomis in the field.


Trap with
I. paraconfusus log
Trap with
I. paraconfusus log
and 2 D. brevicomis logs
No. Ips paraconfusus Caught
500
400
300
200
100
0
position a'b a'b a'b a'b a'b a'b a'b a'b a'b a'b
August 27 28 29 30 27 28 29 30 27 28 29 30
Area
A
B
C

traps with an I. paraconfusus infested log only. The differences between treatment and check were apparently not due to inhibition of one sex only since the Wilcoxon test showed that the treatment was different from the check for both sexes at the 1% level. However, a Wilcoxon comparison of treatment/check ratios of males to ratios of females showed a significant difference at the 1% level indicating that females were inhibited to a greater extent. This is in agreement with the tests with verbenone in the field where both sexes were inhibited, but females to a greater degree.

The D. brevicomis infested log that was dissected at the start of the inhibition test (3½ days after baiting) contained 85 galleries with 85 females and 63 males (only 1 ♂/♀). The average gallery length was 2.93 cm (s.d. 1.34 cm, s.e.$\bar{x}$ 0.15 cm). This is similar to gallery lengths observed by other investigators for D. brevicomis boring a corresponding length of time (Miller and Keen, 1960; Bedard et al., unpublished). The D. brevicomis infested log dissected after termination of the test was screened during the entire period. The log contained 93 galleries with 93 females and 90 males (only 1 ♂/♀). The average gallery length, 20.5 cm, was determined from the total length of galleries (1,910 cm) since individual galleries were impossible to delineate. The logs contained about 166 attacks per m^2 (15.5/ft^2) which is within the

range of attack densities observed earlier (Miller and Keen, 1960). The number of D. brevicomis trapped declined each day over a period of 4 days (r = -0.44; Sy.x = 15.6) after the logs were removed from the tree (Fig. 10). Enoclerus lecontei Wolc. (Coleoptera: Cleridae) was caught in much higher numbers on traps containing both species than on traps with logs infested with I. paraconfusus alone (significant at the 1% level) (Fig. 10).

Identification and Quantification of Pheromones Produced by D. brevicomis in Natural Substrates in the Field

D. brevicomis dissected from the naturally-infested logs used in the inhibition test produced frontalin, exo-brevicomin, trans-verbenol and verbenone (Fig. 11). Males appeared to produce trans-verbenol, frontalin, and verbenone while females produced exo-brevicomin, trans-verbenol and possibly trace amounts of verbenone. When the gut extracts were co-injected with comparable quantities of appropriate standards on 2 different GLC columns, no differences in beetle produced compounds and standards were observed. However, female gut extracts appeared to contain an unidentified compound with a retention time identical to verbenone on the Apiezon L column, but on the FFAP column only a trace amount of compound was observed in the retention area of verbenone indicating that females produced trace,

Fig. 10. Attraction of Enoclerus lecontei and Dendroctonus brevicomis to traps containing logs infested with Ips paraconfusus and to traps containing logs with Ips paraconfusus and logs with Dendroctonus brevicomis in the field.

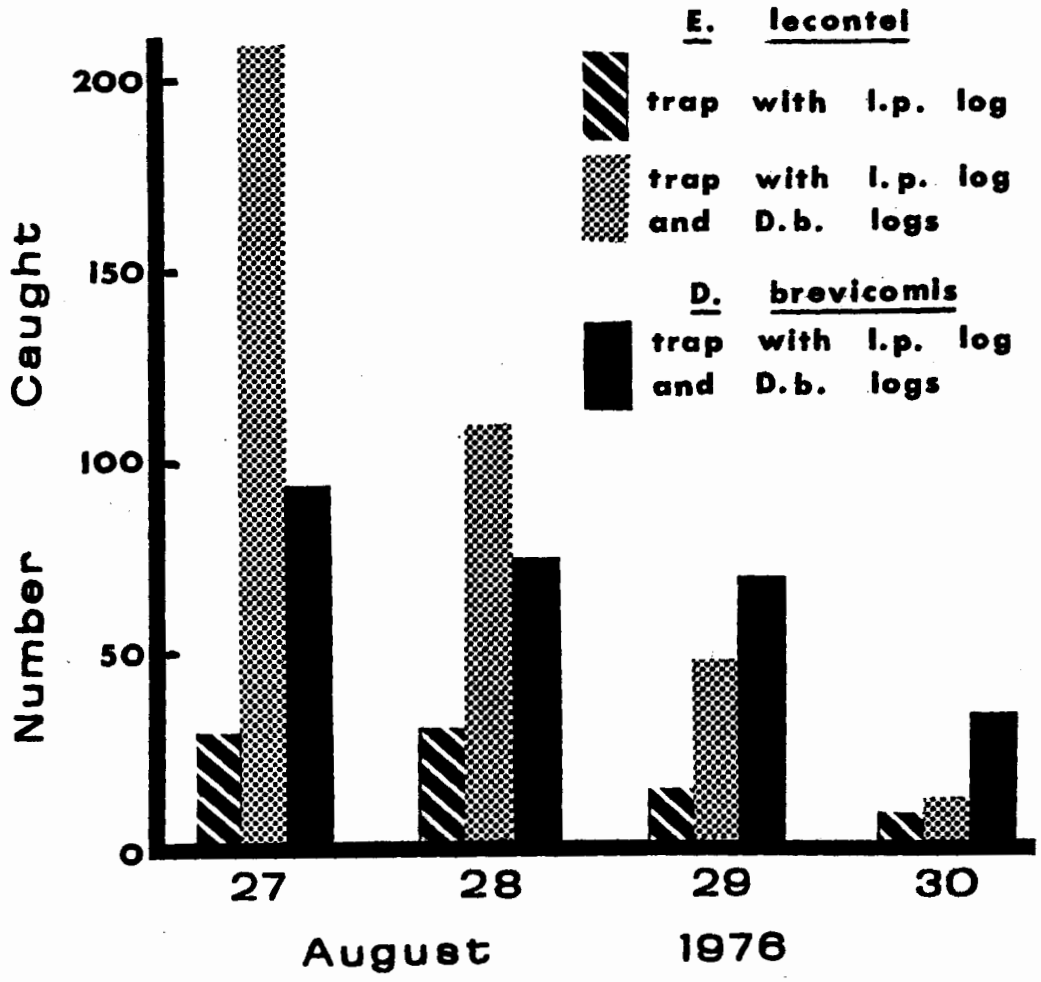
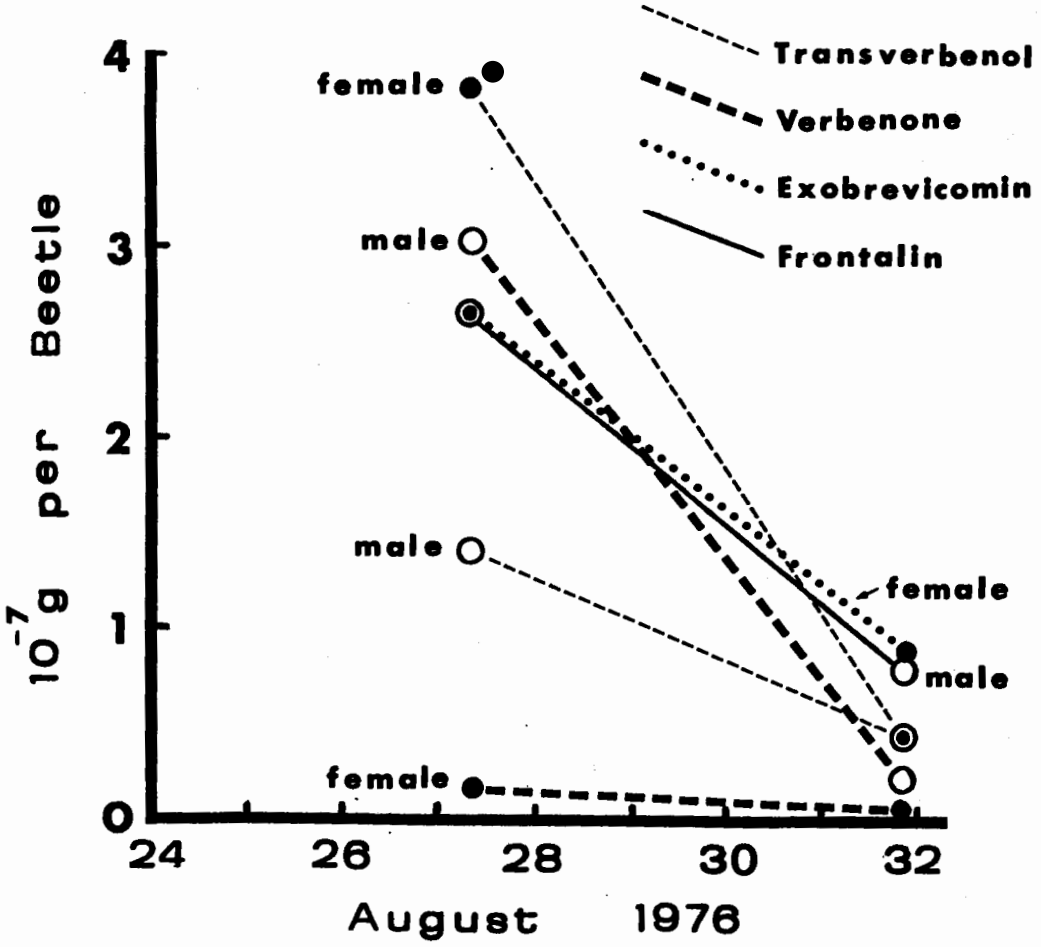

E. lecontei
trap with l.p. log
trap with l.p. log and D.b. logs
D. brevicomis
trap with l.p. log and D.b. logs
Number Caught
200
150
100
50
0
27
28
29
30
August
1976

Fig. 11. Quantities of pheromone compounds (E,F), verbenone, and trans-verbenol found in hindguts of male and female Dendroctonus brevicomis feeding in ponderosa pine logs in the field. The trees were baited with E,F,M on August 24, sectioned into logs on August 27 for use in the inhibition test and the beetles were dissected out of the logs and their hindguts extracted for compounds August 27 and 31, 1976.


Transverbenol
Verbenone
Exobrevicomin
Frontalin
female
male
male
female
male
female
4
3
2
1
0
10^{-7} g per Beetle
24
26
28
30
32
August 1976

if any, amounts of verbenone. The decreasing trend in quantities of exo-brevicomin and frontalin produced by D. brevicomis (Fig. 11) correlates with the apparently decreasing trend in the attraction of D. brevicomis to naturally-infested logs of D. brevicomis (Fig. 10). Male D. brevicomis had only about 1/3 as much trans-verbenol as females (Fig. 11). Verbenone apparently was quantitatively produced only by male D. brevicomis during the time the attractive response of I. paraconfusus to its pheromone was inhibited (Fig. 9).

DISCUSSION

In the laboratory, D. brevicomis was attracted to the mixture of synthetic I. paraconfusus pheromones, I, II, and III. However, as the concentration of I, II, III was increased, the attractive response of D. brevicomis decreased to that of solvent controls (Fig. 1). The phenomenon where lower concentrations are attractive while higher concentrations are less attractive has been reported for D. psuedotsugae Hopk. (Rudinsky et al., 1972). Other reports have shown that attractants at higher concentrations can become repellents (Dethier, 1947; Dethier et al., 1952). On the other hand, I. paraconfusus was not attracted to either the attractive pheromones of D. brevicomis (E,F,M) or to verbenone (Fig. 2). In field studies, D. brevicomis and I. paraconfusus were not caught on traps containing the other

species alone (except when beetles were missed from the previous days trap catch containing both species). This finding is consistent with the absence of any report of interspecific attraction in the literature. The apparent discrepancy between laboratory and field results may be explained by (1) inhibition--as the beetles approach the source of attraction, they fly up a concentration gradient which becomes too strong and inhibits or repels the beetles before reaching the trap, or (2) non-attraction--the beetles are not responsive to naturally-produced I. paraconfusus pheromones or any other beetle associated compounds. Struble and Hall (1955) and Miller and Keen (1960) have summarized several reports indicating that I. paraconfusus may often precede D. brevicomis in the successful attack of ponderosa pine even though I. paraconfusus is considered the less aggressive species. The attraction of D. brevicomis to trees that were top-killed by I. paraconfusus also appeared to be greater than to artificially-topped trees (Miller and Keen, 1960). It is possible that D. brevicomis has increased its susceptible host finding capability and reduced the female mortality by keying on the pheromone produced by I. paraconfusus.

The synthetic I. paraconfusus pheromones (I, II, III) have another behavioral effect on D. brevicomis in the laboratory. As the concentration of I, II, III

was increased relative to a constant dose of synthetic D. brevicomis pheromone, the attractive response of D. brevicomis was inhibited (Fig. 4). The inhibition occured only at the highest concentrations of I, II, III indicating that D. brevicomis in the field may be inhibited only at close range to substrates containing a mixture of these species or I. paraconfusus alone. The laboratory assay also indicated that at least 2 (I + II or III + II) and possibly all 3 synthetic pheromones of I. paraconfusus are necessary to inhibit the attractive response of D. brevicomis to E,F,M (Table 1). Due to the limited quantities of I, II, III available, these compounds were not tested in the field to determine if they could inhibit the attractive response of D. brevicomis to E,F,M or natural pheromone.

One could speculate that the acceptor sites for myrcene on the antennae of D. brevicomis could have evolved into new sites capable of accepting I (2-methyl-6-methylene-7-octene-4-ol) and/or III (2-methyl-6-methylene-2, 7-octadiene-4-ol) which are structurally similar to myrcene (2-methyl-6-methylene-2, 7-octadiene). Similarly, the D. brevicomis acceptor sites for verbenone (4, 6, 6-trimethylbicyclo [3.1.1] hept-3-en-2-one) could have evolved into new sites capable of accepting II (cis-verbenol = 4, 6, 6-trimethylbicyclo [3.1.1] hept-3-en-2-ol) which is structurally similar. An alternative hypothesis

is that I and/or III are competitively inhibiting the acceptor site for myrcene and that II is mimicking the action of verbenone with its known inhibitory effects (Renwick and Vité, 1970; Hughes and Pitman, 1970; McNew, 1970; Wood, 1972; Bedard et al., 1978a).

Field studies showed that the attractive response of D. brevicomis to E,F,M could be inhibited by logs infested with I. paraconfusus (Fig. 5). Other studies indicated that D. brevicomis response to naturally-infested substrates also could be inhibited by logs infested with I. paraconfusus (Fig. 6). In both cases, males and females were inhibited about the same degree which is in agreement with laboratory assays (Fig. 4). The pheromone producing substrates had only one species present in each log so the observed inhibition probably was due to responses of flying beetles rather than possible interactions between feeding beetles which might inhibit pheromone production in one or both species. Furthermore, by separating the 2 species in different logs, the effects of day, position, trap variation, and unequal pheromone release could be uniformly distributed between treatment and check by interchanging the I. paraconfusus infested log and the uninfested log.

The fact that traps containing the same D. brevicomis logs caught significantly different numbers of beetles on successive days shows that the I. paraconfusus

infested logs had an immediate effect on the attractive response, and this effect could be terminated as soon as the log was replaced with a check log. When E,F,M was used as the attractive source, there could be no species interaction on pheromone production. E,F,M might effect I. paraconfusus pheromone production, nevertheless, I. paraconfusus inhibited the D. brevicomis response to E,F,M. The number of D. brevicomis caught on traps with E,F,M was relatively constant each day (Fig. 5) while the number caught on traps with natural pheromone decreased each day (Fig. 6) probably because of a diminution of pheromone production and release (Fig. 11) as has been previously observed (Bedard et al., 1969; Vité and Pitman, 1968; Bedard et al., 1978b).

The attractive response of I. paraconfusus to I, II, III was not inhibited by E,F,M in the laboratory (Fig. 2). Similarly, the response of I. paraconfusus to naturally-produced pheromone was not inhibited by E,F,M in the field. However, verbenone at relatively high concentrations caused inhibition of the attractive response of I. paraconfusus to I, II, III in the laboratory assay (Fig. 7). Based on these results, one can hypothesize that I. paraconfusus pheromone emanating from areas of the tree colonized by both species would probably attract flying I. paraconfusus to the tree but inhibition would occur as the concentration of verbenone from male D. brevicomis became sufficient near the source.

In field studies, the attraction of I. paraconfusus to logs infested with I. paraconfusus males also was reduced by verbenone (Fig 8). Although both sexes were inhibited by verbenone, females appeared to be inhibited more so than males. Female I. paraconfusus are reported to be more responsive to their synthetic pheromone and to naturally-produced frass than male beetles (Wood and Bushing, 1963; Pitman et al., 1965; Borden, 1967) so that inhibition by verbenone might disproportionately affect females. The structure of verbenone is similar to compound II (cis-verbenol) so it is possible that the acceptor site for II evolved into a separate site for verbenone. An alternative explanation is that verbenone competitively inhibits the acceptor site for II, and thus the beetle effectively responds to I + III which is not very attractive (Wood et al., 1967; Wood et al., 1968).

Lanier and Wood (1975) found that I. paraconfusus and I. confusus LeC. were interspecifically attracted when presented infested logs of ponderosa pine containing each species alone (75-100 m apart) in the field. However, significantly more I. paraconfusus females were attracted to nuptial chambers of its own species compared to I. confusus when infested logs of each species were presented 10 or 25 m apart in the field. Similar results were obtained in the laboratory olfactometer since I. paraconfusus preferred its own frass when samples of each species

were presented simultaneously, but I. paraconfusus apparently was equally responsive to frass of each species when presented alone. Young et al. (1973) isolated I, II, and III (I. paraconfusus pheromones) as well as verbenone from both the frass and headspace volatiles of I. confusus feeding in pinyon pine, Pinus monophylla Torr. and Frém. Therefore, the preference of I. paraconfusus for its own frass compared to frass of I. confusus and the significantly lower number of I. paraconfusus found in I. confusus nuptial chambers compared to I. paraconfusus in the field may be due to the release of verbenone from I. confusus substrates.

The attractive response of I. paraconfusus to its naturally-produced pheromone was inhibited by natural pheromone of D. brevicomis in the field (Fig. 9). As in the field studies with verbenone, both sexes were inhibited by natural pheromone of D. brevicomis but females to a greater extent. The ponderosa pine substrates infested with D. brevicomis appeared to be normal in spite of baiting the tree with E,F,M. The attack density (166/m^2), sex ratio 0.86 (♂♂/♀♀), and average gallery length (2.9 cm) were within normal limits for a tree under attack for less than 3½ days (when baited) (Miller and Keen, 1960; Bedard et al., 1978b). The logs infested with D. brevicomis appeared to be releasing E,F,M since D. brevicomis were attracted each day of the inhibition test (Fig. 10).

However, the number of D. brevicomis trapped declined each day over a period of 4 days (r = -0.44; Sy.x = 15.6) after the logs were removed from the tree and starting 3 days after the initial attack. This result supports other studies reported here in Fig. 6 (r = -0.39; Sy.x = 53.1) and by Bedard et al. (1969) but do not support the statement by Vité and Pitman (1968) that "production of pheromone continues only as long as the host resists extensive feeding and gallery construction."

E. lecontei, a predator of both bark beetle species, also was attracted to traps containing I. paraconfusus logs and D. brevicomis infested logs (Fig. 10). E. lecontei has been reported to be attracted to D. brevicomis infested trees (Berryman, 1966; Vité and Gara, 1962) as well as to I. paraconfusus attractive pheromones (Wood et al., 1968). Wood et al. (1968) found that E. lecontei was attracted to I + III or I, II, III and Wood (1972) reported that the beetle did not appear to be attracted to E,F,M. Pitman and Vité (1970) reported some attraction of E. lecontei to several ponderosa pine monoterpenes (such as myrcene) but not to E or F. It appears that E. lecontei may respond to still other compounds because of the increased attraction of E. lecontei to traps with both bark beetle species.

The quantities of F and E found in male and female D. brevicomis respectively (Fig. 11) following dissection

from the logs used in the test showing inhibition of I. paraconfusus indicates that these attractive pheromones were present in the greatest amounts when the catch of D. brevicomis also was highest (Fig. 10).

The production of trans-verbenol in males was only about 1/3 as much as in females. Verbenone was predominantly produced by male D. brevicomis which agrees with previous studies which showed that emerged D. brevicomis males had verbenone and trace amounts of trans-verbenol while emerged females contained trans-verbenol and either undetectable or trace amounts of verbenone (Renwick, 1967; Renwick and Vité, 1968, 1970; Vité and Renwick, 1970; Pitman et al., 1969). In these studies, the amounts of trans-verbenol and verbenone present in emerged beetles were not quantified. The presence of verbenone in feeding males has been reported (Hughes, 1973) and the compound was recently found in headspace air from male/female infested logs (Browne et al., 1978). Verbenone occured in the largest amounts in male D. brevicomis at the same time that the greatest inhibition of the attractive response of I. paraconfusus occured. For example, on the first day of the test the traps containing D. brevicomis infested logs and logs infested with I. paraconfusus caught 23% as much as traps containing logs infested with I. paraconfusus alone. On succeeding days traps with both species caught

27%, 60% and 39%. It appears that verbenone released by male D. brevicomis is responsible, at least in part, for the observed inhibition of the attractive response of I. paraconfusus to its pheromone.

Verbenone and E,F occured in the largest concentrations near the beginning of the attack period (males arrived from 0 to 3 days prior to dissection). Previous theories indicated that E,F,M concentrations would be highest at the beginning of the aggregation phase (Wood, 1972) and that during the termination of the attack phase verbenone then would be produced in large enough quantities to inhibit attractive response (Renwick and Vité, 1970; McNew, 1970). The results (Figs. 10, 11), however, suggest that the gradual reduction in E,F causes the observed reduction in trap catch. Verbenone may play a role in regulation of attack density.

These experiments show that the attractive pheromone response of D. brevicomis and I. paraconfusus to naturally-infested ponderosa pine are mutually inhibited. Verbenone from male D. brevicomis and I, II, III from male and II from female I. paraconfusus appear to cause, at least in part, the observed inhibition in these species. However, inhibition of the attractive response of D. brevicomis to natural substrates by I, II, III has not been tested in the laboratory or field, nor has the test of E,F,M and I, II, III been conducted in the

field. The enantiomer composition of the behavioral chemicals was not known but probably was racemic. It may be expected that behavioral chemicals of a specific enantiomer might have effected behavior of these beetles in ways not observed in the experiments (Wood et al., 1976; Borden et al., 1976).

The function of mutual inhibition appears to help segregate the two species into distinct areas on the tree and to reduce areas of competition for food and space between the two species. Birch and Wood (1975) showed that I. paraconfusus and I. pini are mutually inhibited probably for the same reasons and that compound I inhibited the attractive response of I. pini. Verbenone and I, II, III may prove useful as behavior modifying chemicals that would disrupt the aggregation phase of host colonization of these beetles and perhaps reduce tree mortality.

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PART II

QUANTITATIVE RELATIONSHIPS BETWEEN A HOST PLANT PRECURSOR AND SEX-SPECIFIC PHEROMONE PRODUCTION IN THE BARK BEETLE, *IPS PARACONFUSUS*

INTRODUCTION

Several investigators have indicated that host plant compounds could be converted to attractive pheromones of insects but these studies have not quantified the relationship between the precursor exposure dose and subsequent pheromone production (Edgar et al., 1973; Hughes, 1974; Schneider et al., 1975; Brand et al., 1975; Renwick et al., 1976a; Hughes and Renwick, 1977). Furthermore, none of these studies utilized attractive behavior to substantiate the existence of pheromones. Also, some of the conclusions must remain equivocal since impure precursors were used and inadequate analytical methods (i.e., only GLC retention times) were employed to identify the compounds produced (Hughes, 1974; Hughes and Renwick, 1977).

The results of earlier studies showed that only the males of the bark beetle, Ips paraconfusus Lanier (Coleoptera: Scolytidae) could attract beetles in either field (Wood and Vité, 1961) or laboratory (Wood, 1962; Wood and Bushing, 1963) assays. The pheromone has been shown to accumulate in the hindguts of males (Pitman et al., 1965; Renwick et al., 1966; Vité et al., 1972)

and was first identified from frass as a mixture of I (2-methyl-6-methylene-7-octene-4-ol), II (cis-verbenol), and III (2-methyl-6-methylene-2-7-octadiene-4-ol) (Silverstein et al., 1966a, b).

The objective of this study was to quantify the relationship between host plant precursor dosage and subsequent pheromone production, i.e., each sex and to spectrographically identify the compounds produced.

METHODS AND MATERIALS

Ponderosa pine logging debris containing callow adults was collected on March 29, 1976, near the University of California's Blodgett Experimental Forest (El Dorado, County) at an elevation of 1,200 m. The beetles were reared, collected (Browne, 1972), and sexed (Wood, 1961) when they emerged as mature adults.

A series of increasing concentrations of myrcene vapor was prepared by placing 0 to 200 µl of purified myrcene (99.8%) on 1 x 2 cm strips of glass fiber paper (GF/A Whatman) and inserting the paper into 0.45 l amber-colored glass bottles. The myrcene was purified by gas-liquid chromatography (GLC) on a 3 m x 8 mm i.d. glass column of 3% Apiezon L on 100/120 Gas Chrom Q at 110° C and N_2 flow of 300 ml/min (Varian F.I.D. model 2700). Between 20 and 100 beetles of one sex were added to each bottle with 0.6 g of silane treated glass wool

so that the beetles could disperse and not chew upon each other. Each aluminum lined bottle cap was fitted with a rubber septum (GLC) so that headspace samples could be withdrawn for GLC analysis.

Beetles were exposed to various head-space concentrations of myrcene for 18 ± 1 hrs, at 23° ± 3° C under the natural photoperiod (April 9-May 5, 1976). Samples of headspace were withdrawn from the bottles during the exposure period and the quantity of myrcene per ml was determined by GLC using a 1.8 m x 2 mm i.d. glass column of 10% FFAP on 80/100 Gas Chrom Q at 50° C and N_2 flow of 30 ml/min.

After the exposure period, beetles were removed from the bottles and their mid- and hind-guts were dissected out. The guts were immediately crushed in 0.4 ml of diethyl ether contained in 1 ml Mini-Vials (Applied Science Lab. Inc.). The samples then were analyzed by GLC using 2 columns, a FFAP column previously described but at 100° C and a 1.8 m x 2 mm i.d. glass column of 3% Apiezon L on 100/120 Gas Chrom Q at 100° C and N_2 flow of 12 ml/min. Authentic I and III (Chem Samples Co.) (both > 97%) were compared for retention time and peak area.

Spectroscopic methods were used to determine if these compounds were present in either unexposed beetles or in beetles exposed to myrcene vapors. Groups of 100

beetles were exposed to the vapors from 30 µl of myrcene in the 0.45 l bottles. Three hundred beetles of each sex were exposed and their hindguts extracted with ether. An equal number of unexposed control beetles were treated similarly. GLC analyses of the extracts were compared to the known pheromones, I and III, as well as myrcene, on a Finnigan 9500 GLC using several different columns (DEGS, FFAP, OV-1, and Carbowax 20M). A complete separation of all components was obtained on 3% carbowax 20M, and this column was used for subsequent analyses. The extracts were then analyzed by chemical ionization mass spectrometry (GC CIMS) on a Finnigan 3200 gas chromatograph-mass spectrometer with an interactive 6000 digital data system using methane or isobutane as the reagent gas.

The attractive responses of female beetles were used to confirm the chemical identifications and to detect low levels of pheromones in exposed and untreated beetles. Previous studies have demonstrated that the binary mixtures of I with III and I with II evoke an upwind walking response from both sexes in the laboratory assay (Wood, 1970). Because none of these compounds are active alone, we tested for their presence by assaying mixtures of I or II and III (all racemic) with gut extracts from females exposed to myrcene vapors. The assay was essentially that developed previously (Browne et al., 1974) except that the polyurethane foam was removed from

the plexiglass manifold to permit faster air flow. The air apeed at the level of walking beetles (0-2 cm) was 0.9 m/sec at the pheromone elution source and 0.6 m/sec where the beetles were released. Groups of 10 female beetles were released for each test at 21 ± 2 cm downwind from the source. A positive response was recorded when a female arrived within a 1 cm radius of the source in the time required for the gut extract to elute from a 5 µl capillary tube (126 ± 10 sec).

RESULTS

In other studies where bark beetles have been exposed to terpene hydrocarbons, the concentration of the compounds in the headspace was either not determined or reported to be "saturated," although no analyses were presented (Vité et al., 1972; Hughes, 1973, 1974, 1975; Renwick et al., 1973; Renwick et al., 1976a, b; Hughes and Renwick, 1977). The theoretical quantity of myrcene necessary to saturate the headspace of the 0.45 l bottle can be predicted rom the Perfect Gas Law (Williams and Williams, 1967).

µl myrcene to saturate bottle =

$$\text{M.W. myrcene} \left(\frac{PV}{RT}\right) \times \text{density} = \frac{136.23\text{g}}{\text{mole}} \times \frac{2\text{mm}}{760\text{mm}} \times \frac{.45}{1} \times \frac{\text{mole-deg.}}{.08206 \text{ -atm}} \times \frac{1}{273^\circ + 23^\circ} \times \frac{1.25\ \mu\text{l}}{\text{mg}}$$

$$= 8.3\ \mu\text{l}$$

However, the amount needed to saturate the bottle (Table 1) was found to be at least 11 times higher than the theoretical amount. Experiments in which bottles containing myrcene were repeatedly flushed with N_2, recapped, and headspace samples withdrawn for GLC analysis indicated that adsorption accounted for at least some of this discrepancy.

As the concentration of myrcene was increased to near saturation the mortality/anesthesia of the beetles increased to near 100% (Table 1). High concentrations of myrcene and other monoterpenes have been reported to be toxic to other bark beetle species attacking ponderosa pine (Smith, 1965a, b). Beetles observed as mortality/anesthetized appeared dead prior to dissecting out the guts; however, about half of these individuals recovered at least partial locomotory capability in 5-15 min. Not unexpectedly, as mortality/anesthesia increased (> 24.1 µl of myrcene in the bottle), the amount of I and III production decreased in spite of the increasing concentration of myrcene exposure (Table 1).

The relationship between myrcene exposure dosage and I and III production (Fig. 1) is similar to enzyme reaction rate curves and shows classical saturation effects at the higher concentrations of myrcene (Lehninger, 1970). Both curves have approximately the same km value (myrcene concentration at half maximal rate of I or III production

Table 1: The relationship between the amount of myrcene added to the aeration chamber and the concentration[1/] of myrcene in the headspace and the subsequent production of I and III.

	µl Myrcene Added to Aeration Chamber								
	0	2.1	5.6	9[2/]	12.7	24.1	45.8	74	93[3/]
Concentration of Myrcene in Headspace (10^{-7} g/ml)	-	3.4	6.8	10	13.5	24.5	40.3	57.5	66.1
Percent Mortality-Anesthesia	0	0	0	0	1	3	10	60	95
Amount of I produced per male (10^{-8} g)	-	12.5	21.5	26.2	29.8	37.3	19.4	16.8	15.3
Amount of III produced per male (10^{-8} g)	-	2.2	4.2	5.3	6.1	7.7	4.4	3.7	3.1

[1/] The concentrations of myrcene were calculated from a quadratic least squares regression of 50 data pairs covering the entire range from 0 to 200 mg per chamber ($y = -0.00326x^2 + 1.00069x + 1.31667$).

[2/] Theoretical saturation.

[3/] Near actual saturation.

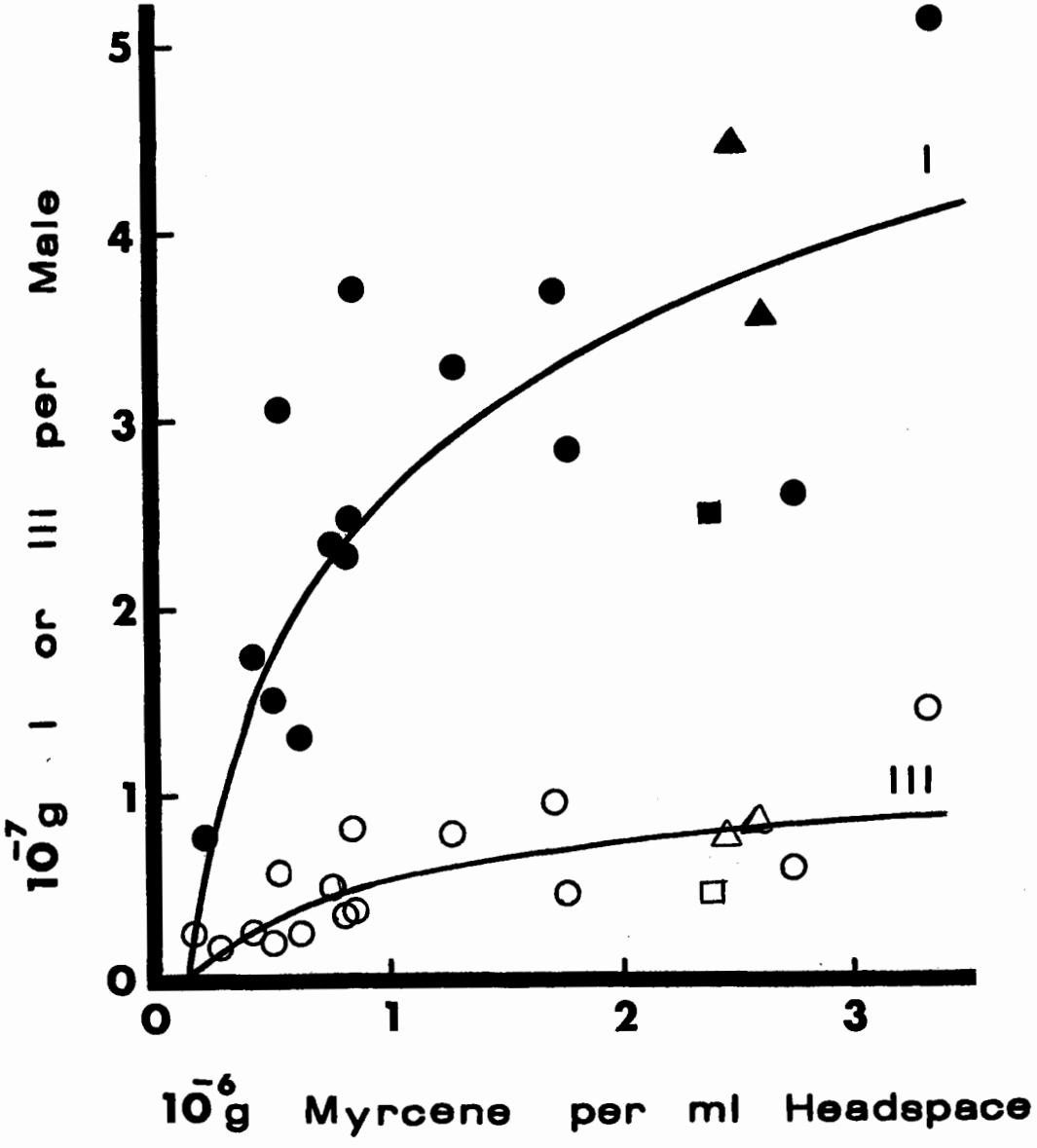
Fig. 1. I and III production in male I. paraconfusus hindguts after exposure to myrcene vapors.

○ Circles represent average of 20 beetles.

□ Squares represent average of 100 beetles.

△ Triangles represent average of 200 beetles.

Equation for logarithmic regression of I is $y = -2.44 + 12.45 \ln x$ and correlation coefficient of 0.81 while equation for logarithmic regression of III is $y = -1.23 + 2.84 \ln x$ and correlation coefficient of 0.71.

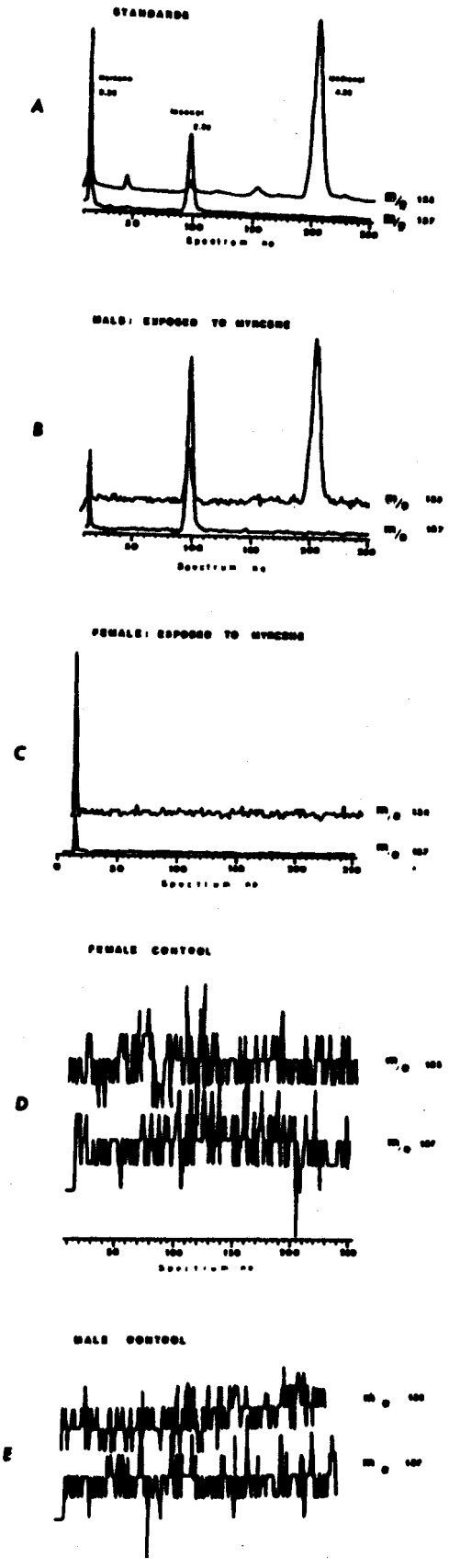

5
4
3
2
1
0
0
1
2
3
I
III
10^{-7}g I or III per Male
10^{-6}g Myrcene per ml Headspace

(ca 0.65) indicating a common rate limiting step. The curves in Fig. 1 also could be caused by saturation of systems for the transport of myrcene across cell membranes.

GC-CIMS analyses of male beetles exposed to myrcene vapors showed three major components (Fig. 2B) having retention times and mass spectra identical to standard myrcene, I and III (Fig. 2A). Female beetles exposed to myrcene contained only myrcene (Fig. 2C). Analyses of both male and female control beetles revealed no volatile compounds (Fig. 2D and 2E). Coinjection of standard I and III with the extracts of myrcene treated males intensified only the appropriate peak in each case. On-column hydrogenation of the male extract revealed a disappearance of the expected I and III peaks with concomitant formation of a new compound having the retention time and mass spectra of a saturated ten carbon alcohol (2,6-dimethyl-octan-4-ol). Standard I and III under the same hydrogenation conditions gave results identical to the male extract. From the above data, it was concluded that I and III are present in myrcene treated I. paraconfusus males and not in females or in unexposed, recently emerged beetles of either sex.

The response of females in the assay to test mixtures of gut extracts from females exposed to myrcene vapors and I or with a mixture of II and III was not different from the response to I or II and III alone

Fig. 2. GC-CIMS spectrograms of compounds in hindguts of male and female I. paraconfusus exposed and not exposed to myrcene vapors.


STANDARDS
A
MALE: EXPOSED TO HYDRAZINE
B
FEMALE: EXPOSED TO HYDRAZINE
C
FEMALE CONTROL
D
MALE CONTROL
E

(Table 2). These assay results confirm the GLC and GC-CIMS data that females do not produce I or III at least at the levels tested in these experiments. Males produce these compounds and they are attractive at levels comparable to synthetic compounds, indicating the absence of inhibitory compounds. The female produced several unidentified compounds which had the same retention time as compounds produced by males. These unidentified compounds apparently are not attractive to I. paraconfusus females, nor can they substitute for I, II, or III since female extracts were not attractive when combined with various pheromone compounds in the bioassay (Table 2).

DISCUSSION

Hughes (1974) found that male I. paraconfusus produced I and III when exposed to one concentration of myrcene vapor. These results are questioned because: (1) the suspected precursor was not purified and could have contained the pheromones; (2) the chemical identifications were based only on one GLC column (FFAP) which cannot separate I or III from other compounds isolated from headspace volatiles from feeding females (linalool) (Young et al., 1973) or known to occur in the beetle (trans-verbenol) (Vité et al., 1972; Renwick et al., 1976a); and (3) assays were not performed to confirm that the isolated compounds were pheromones. Furthermore,

Table 2. Attractive response of female I. paraconfusus to pheromones and to gut extracts from beetles exposed to various treatments in the laboratory.

Extract	Rate of Delivery per Min. per Compound	Percent Females Responding	(C.I.)[1/]	Number of Females
Assay for Presence of I and III Together (April 20, 1976)				
Female Guts (no myrcene)	0.09 gut equivalents	2	(0-8)	60
Male Guts (no myrcene)	0.09 gut equivalents	3	(1-12)	60
Female Guts (myrcene)[2/]	0.09 gut equivalents[3/]	2	(0-8)	90
Male Guts (myrcene)[4/]	0.09 gut equivalents[5/]	59	(48-69)	90
I + III	8.9×10^{-10} g	23	(11-38)	40
I + III	8.9×10^{-9} g	33	(17-52)	30
I + III	8.9×10^{-8} g	57	(38-75)	30
I + III	8.9×10^{-7} g	70	(51-85)	30
Assay for Presence of III Only (July 29, 1976)				
I	8.9×10^{-9} g	13	(4-27)	40
I + III	8.9×10^{-9} g + 1.8×10^{-10} g	50	(32-69)	30
I + Female Guts (myrcene)[6/]	8.9×10^{-9} g + 0.8 gut equiv.[3/]	7	(5-19)	30
I + Female Guts (myrcene)[2/]	8.9×10^{-9} g + 0.8 gut equiv.[3/]	10	(2-27)	30

Table 2 (continued)

Extract	Rate of Delivery per Min. per Compound	Percent Females Responding	(C.I.)[1/]	Number of Females
	Assay for Presence of I Only (November 7, 1977)			
I	8.9×10^{-10}g	10	(2-26)	30
II + III	8.9×10^{-10}g	7	(0-22)	30
I + II + III	8.9×10^{-10}g	43	(25-62)	40
II + III + Female Guts (myrcene)[4/]	8.9×10^{-10}g + 0.08 gut equiv.	10	(2-24)	40
II + III + Female Guts (myrcene)[2/]	8.9×10^{-10}g + 0.08 gut equiv.[3/]	0	(0-12)	30

[1/]Confidence intervals were estimated from a chart of binomial confidence limits (95%) for proportions.

[2/]Exposed to 2.4×10^{-6}g myrcene/ml headspace.

[3/]No I or III detected in gut extract by GCL.

[4/]Exposed to 3.1×10^{-6}g myrcene/ml headspace.

[5/]4.5×10^{-8}g I and 1.3×10^{-8}g III released per min. in gut extract (GLC).

[6/]Exposed to 9.1×10^{-7}g myrcene/ml headspace.

females were not tested so the possibility of sex-specificity in pheromone synthesis from myrcene could not be determined. These shortcomings were overcome in this study, and the results obtained by Hughes (1974) were verified. The quantitative relationship between precursor dosage and subsequent pheromone production was established. The logarithmic correlation between myrcene exposure and pheromone production provides additional evidence for a direct conversion of myrcene. However, alternative mechanisms such as induction of "de novo" synthesis or release of sequestered compounds are still possibilities.

Females exposed to concentrations of myrcene similar to males did not produce I and III although comparable quantities of myrcene were isolated from their hindguts. This indicates that pheromone production must reside in male-specific enzymes and not in the differential penetration of precursor into the hindgut or other body tissues. Recently, Hughes and Renwick (1977) exposed females to a "saturated" myrcene concentration and could not detect pheromones. Again, the same objections can be raised as those mentioned earlier for Hughes (1974).

Linalool has been reported to occur in headspace volatiles from only female I. paraconfusus feeding in ponderosa pine (Young et al., 1973). However, we did not detect linalool using the Apiezon GLC column in

either male or female beetles exposed to myrcene. Of all the major terpene hydrocarbons known from ponderosa pine, myrcene appears to be as likely a precursor for linalool as for I or III.

Spectroscopic methods (GC-CIMS, NMR, IR) are not definitive in proving that an isolated compound is a pheromone because active and inactive enantiomers of the pheromone can be distinguished only by means of a bioassay (Wood et al., 1976; Borden et al., 1976). The gut extracts of males exposed to myrcene evoked an attractive response by females indicating that both I and III were produced. None of the three pheromone components I, II, or III are active by themselves in the laboratory bioassay nor is the mixture of II and III (Wood, 1970; Silverstein et al., 1967). These results were verified in field assays (Wood et al., 1967). Therefore, any one of these compounds or the one binary mixture could have been produced by females and consequently would not have elicited a behavioral response as shown in earlier studies (Wood and Vité, 1961; Wood and Bushing, 1963; Wood et al., 1966; Wood et al., 1968). However, the assay of various mixtures of pheromones shows that females exposed to a range of precursor concentrations did not produce I or III at least in detectable amounts.

Brand et al. (1975) have shown that Bacillus cereus, cultured from the hindguts of both male and female I. paraconfusus could synthesize one of its pheromone components, II, from racemic α-pinene, another common monterpene of host (ponderosa pine) oleoresin. Renwick et al. (1976a) showed that II could be synthesized by male and female I. paraconfusus exposed to vapors of the (-) enantiomer of α-pinene. If I and III also are synthesized by a bacterial enzyme system, then this bacterium (-ia) must be a symbiont specific to the male. Recent evidence indicates that synthesis of I and III may be under hormonal control (Hughes and Renwick, 1977). Gut stretching due to feeding stimulates release of brain hormone which causes juvenile hormone release and then stimulation of pheromone synthesis. However, the extent of this stimulation of pheromone production remains to be quantified. The unequivocal proof that myrcene is a precursor of I and III awaits labelling experiments.

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PART III

SITE AND MECHANISM OF PHEROMONE PRODUCTION IN THE BARK BEETLE, IPS PARACONFUSUS

INTRODUCTION

The male Ips paraconfusus Ianier initiates the entrance tunnel when colonizing one of its hosts, ponderosa pine. Attractive pheromones are produced from 9 to 12 hrs. after the initial penetration of the bark into the phloem/cambium/xylem interface (Wood and Bushing, 1963). It appears that these attractants are not released until host material has passed through the digestive tract (Wood and Bushing, 1963; Vité et al., 1963; Pitman et al., 1965; Wood et al., 1966). The pheromones were isolated and identified from male frass as a mixture of 3 compounds: ipsenol (I), cis-verbenol (II), and ipsdienol (III) (Silverstein and Rodin, 1965; Silverstein et al., 1966a, b; Wood et al., 1966). All 3 compounds are necessary for maximum attraction in the field (Wood et al., 1968). Only compound I shows any activity (low level) in the laboratory while the addition of II, III or both to compound I increases the attractive response (Wood et al., 1967; Wood, 1970).

Pheromone activity (Pitman et al., 1965, 1966; Renwick et al., 1966) as well as the pheromone compounds (Vité et al., 1972) have been isolated in hindguts of males. Males and females produce compound II when exposed

to vapors of the (-) enantiomer of α-pinene which indicates that they would produce II under natural conditions (Renwick et al., 1976). Hughes (1974) reported that when males of I. paraconfusus were exposed to vapors of another host monoterpene, myrcene, it was converted to compounds I and III in the hindgut. Recently, Byers et al. (1978) quantified the relationship between the dose of the myrcene precursor and the subsequent production of I and III, and showed that only the male could synthesize these compounds.

Brand et al. (1975) have suggested that Bacillus cereus, a bacterium isolated from the hindgut of male and female beetles, can convert α-pinene into II. Therefore, in order to determine if the production of I and III from myrcene was influenced by bacteria within male I. paraconfusus and to substantiate the results of Brand et al. (1975), antibiotics were fed to males in a ground phloem-powdered cellulose diet. Loss of the capability to produce I, II, and III during exposure to myrcene and (-) α-pinene would indicate a symbiotic relationship between microorganisms and the beetle. The site of accumulation of pheromone also was investigated.

METHODS AND MATERIALS

I. paraconfusus were obtained by collecting naturally-infested ponderosa pine logging debris in or

adjacent to the University of California's Blodgett experimental forest at an elevation of 1,220 m. The logging debris containing late larvae and callow adults was placed in emergence cages for rearing and collection of adult beetles (Browne, 1972). The beetles were sexed by the method of Wood (1961). The emerged adults were stored from 2 to 20 days at 4° C on moist paper toweling until used in the experiments.

It was necessary to group beetles that had emerged over several days in order to obtain a sufficient number for a particular experiment. Therefore, the beetles were randomized so that certain treatments and controls would not contain a disproportionate number of beetles from any one date. The beetles of one sex were randomized by taking individuals that could walk from each day's collection and allowing all of them to disperse on a crumpled paper towel in one container.

Bacteria Associated with Pheromone Production

A semi-synthetic diet was developed consisting of 62 ml water, 34.5 g powdered cellulose (Alphacel ®, ICN Pharmaceuticals, Inc.), 8 g sucrose, and 22 g ground phloem which caused male *I. paraconfusus* to feed and defecate fecal pellets of undigested diet (Byers, 1978). The ground phloem was prepared by cutting strips of phloem from a ponderosa pine tree (cut September 14, 1976

and stored at 4° C) and triturating the strips with dry ice in a stainless steel Waring blender at high speed. The phloem and dry ice homogenate was sieved through a nylon screen such that phloem particles less than 0.5 mm dia. were obtained. The dry ice was allowed to sublimate until the ground phloem reached room temperature whereupon it was premixed with the powdered cellulose. The sucrose dissolved in water then was added and mixed.

The resulting diet displaced about 225 ml volume and was evenly distributed among three 10 cm petri dishes. The diet then was tamped in each dish to an approximate volume of 50 ml so that air pockets were less than 1 mm in dia. One set of 6 petri dishes containing the diet was prepared and 21 males were released in each dish (November 5, 1976). Each dish of diet has 21 indentations, 3 mm dia. x 8 mm deep, to promote entry of beetles into the diet. Wire spacers were used to keep the petri dish cover separated about 1.5 mm above the lower dish to allow some ventilation. A second set of 6 petri dishes and beetles was prepared with the same diet mixture except that 1 mg of the antibiotics streptomycin sulfate (Sigma), penicillin-G (1667 units/mg, Sigma), chloramphenicol (Sigma), and tetracycline (Sigma) were added per ml of water.

The beetles were allowed to bore and feed for 48 hrs. at 21° ± 1.7° C under natural photoperiod

conditions. The beetles then were dissected out of the diets, dropped on the table to remove any particles of diet still adhering to the body, and 20 were placed in a 3.2 x 3.2 x 1.3 cm aluminum screen cage (7/cm mesh) inside a chamber for subsequent exposure to pheromone precursors. The aeration chamber consisted of a 70 ml tubular glass jar with a wide mouth lid lined with teflon. The percent mortality was noted at this time, as well as at the end of the aeration period, to determine if differences existed in mortality during feeding or aeration due to the presence of antibiotics in the diet. The beetles were exposed to purified myrcene (Chem. Samples Co.) and purified (-) α-pinene ($[\alpha]^{22}$ - 47.5°, Aldrich) by placing 5 µl of each monoterpene each divided between two 5 µl capillary tubes in the bottom of the aeration chamber. The myrcene and (-) α-pinene were purified (> 99.8%) by gas-liquid chromatography (GLC) on a 3 m x 8 mm i.d. glass column of 3% Apiezon L on 100/120 Gas Chrom Q at 110° C and N_2 flow of 300 ml/min. (Varian f.i.d. model 2700).

Twenty males from the antibiotic diet and 20 from the non-antibiotic diet were exposed to (1) purified myrecene (2) purified myrcene and (-) α-pinene and (3) not exposed to any precursors. The beetles were aerated for 18 ± ½ hrs. at 21 ± 1.7° C under the natural photoperiod. The concentration of monoterpenes (per ml)

volatilized in the aeration chamber was determined by withdrawing headspace samples with a gas-tight syringe for GLC analysis. The quantities of myrcene and (-) α-pinene in headspace samples were estimated (1.8 m x 2 mm i.d. glass column of 3% Apiezon L on 100/120 Gas Chrom Q at 100° C and N_2 flow of 12 ml/min.) to be 23.3 x 10^{-7}g myrcene/ml (s.d. 3.5 x 10^{-7}g/ml) and 61.4 x 10^{-7}g (-) α-pinene/ml (s.d. 9.4 x 10^{-7}g/ml). During the aeration period the males void their guts and the fecal material falls through the aluminum screen cage to the floor of the chamber. Therefore, a comparison of the number of fecal pellets between the antibiotic and non-antibiotic diet was made to indicate relative amounts of feeding in the respective diets.

After the aeration period, beetles were removed from the chambers and their mid-and hindguts were dissected out. The guts were immediately crushed in 0.3 ml of diethyl ether contained in 1 ml Mini-Vials (Applied Science Lab. Inc.). The hindgut extracts then were analyzed by GLC using the 1.8 m x 2 mm i.d. glass column of 3% Apiezon L with the same conditions as described above. Authentic I, III (both > 97%) and II (> 95%) (Chem Samples Co.) and (-) myrtenol (> 99%) ($[\alpha]^{22}$ - 47.5°, Aldrich) were compared to gut extracts for retention time and peak area. Compounds "B" and "H" were unidentified and thus their peak areas were quantified in terms of authentic I.

In the second experiment (November 17, 1976) the diets were prepared in the same way as in the first experiment. A new sample of ground phloem was prepared from the same tree cut September 14, 1976. The non-antibiotic diet remained identical to that described in the first experiment, however, in the antibiotic diet only streptomycin sulfate and penicillin-G were added each at concentrations of 1 mg/ml and 10 mg/ml water. Sufficient amounts of the 3 test diets were prepared to fill six 10 cm petri dishes with non-antibiotic diet and 3 petri dishes each with the low and high dosage antibiotic diets. The concentrations of antibiotic in the low and high dosage diet were about 0.41 and 4.1 µg of each antibiotic per µl of diet. In this experiment the beetles were allowed to feed for 96 hrs. before they were removed and aerated for 18 hrs. in purified myrcene plus purified (-) α-pinene as described for the first experiment. The headspace concentrations of myrcene and (-) α-pinene were determined as in the first experiment (21.1 x 10^{-7}g myrcene/ml, s.d. 3.4 x 10^{-7}g/ml; 47.1 x 10^{-7} g (-) α-pinene/ml, s.d. 7.8 x 10^{-7}g/ml). Ten replicates of 10 males each from the non-antibiotic diet were aerated in the precursors and 2 replicates of 10 males each were not exposed to precursors. Five replicates of 10 males from each of the two concentrations of antibiotic diet were aerated in the precursors

while one group of 10 males from each of the antibiotic diets were not aerated with precursors.

The percent mortality after feeding and aeration and the number of fecal pellets defecated during the aeration period were noted. Gut extracts were prepared and analyzed as in the first experiment. A chi square test with Yates' correction (McCall, 1970) was used to compare treatment and control mortality and the t-test was used to compare the non-antibiotic and antibiotic treatments for fecal pellets and for pheromone production.

Site of Pheromone Production and/or Accumulation

Two groups of 15 beetles of each sex were each aerated in the 70 ml chambers for 18 ± ½ hrs. with 5 µl of purified myrcene and 5 µl of purified (-) α-pinene as described above (November 19, 1976). The headspace concentration of (-) α-pinene (Apiezon L column at 100° C) was 55.6×10^{-7} g/ml (s.d. 7.2×10^{-7} g/ml) and the myrcene concentration was 24.2×10^{-7} g/ml (s.d. 2.9×10^{-7} g/ml). After the aeration period, the mid- and hindguts of 17 beetles of each sex were dissected out and divided into 3 pieces and separately extracted and analyzed as described before. Each mid-hindgut was laid on a glass slide which had been previously sprayed with a fine mist of water. The fine droplets of water insured that the gut remained moist while sectioning and thus retain structural

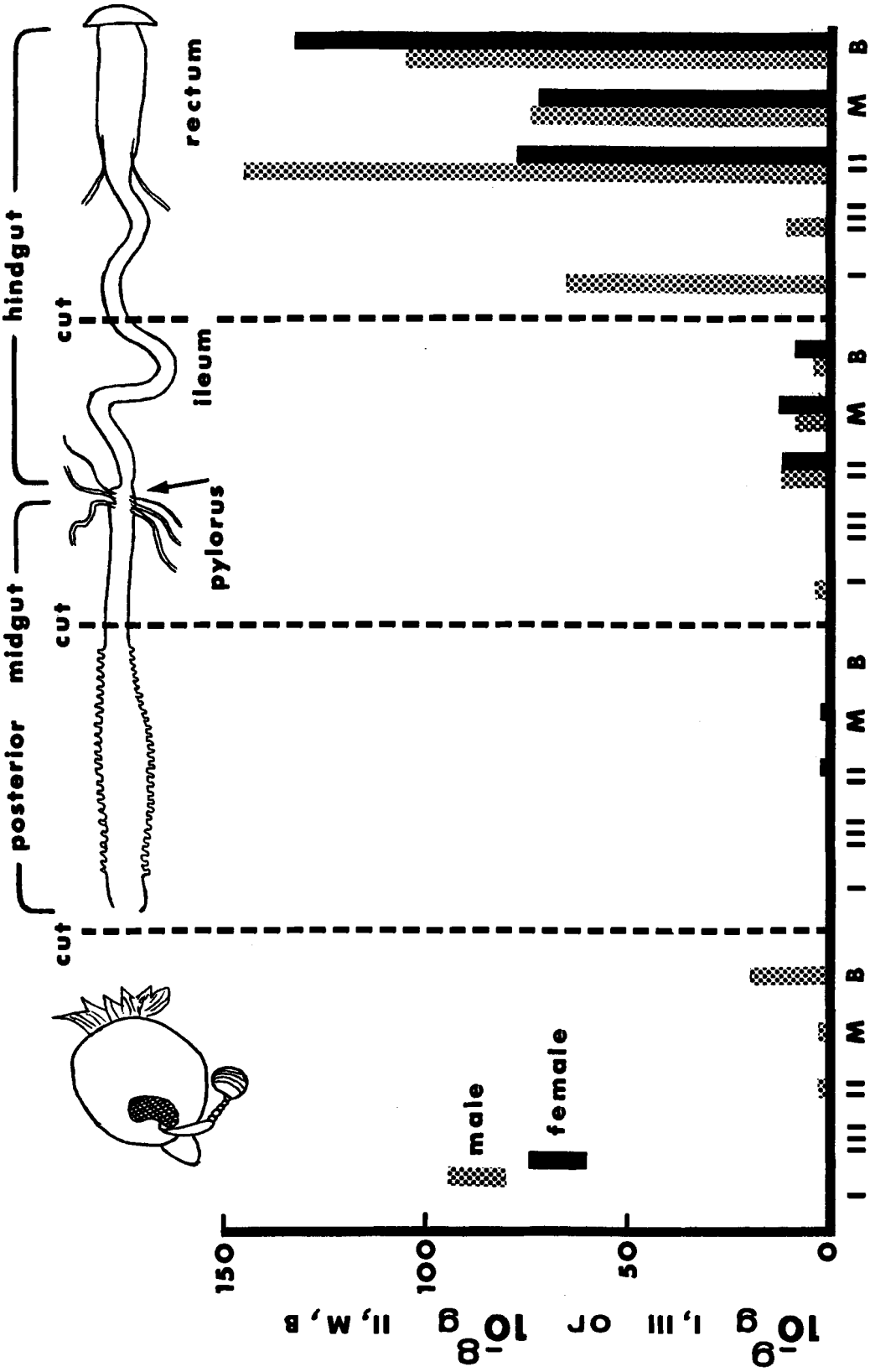
integrity to retard pheromone evaporation. The sparcity of water droplets should have minimized extraction of pheromones or rupturing of epithelial cells by hypoosmotic water since hemocoel fluids adhering to the gut would buffer the small volume of water used. Unused portions of a razor blade were used to slice each gut into 3 sections. The posterior section consisted of the anus and rectum and a portion of the hindgut to about midway between the anus and the pyloris. The mid section contained the pyloris and often a few short strands of malpighian tubules and ended just posterior to the posterior ventriculus which has the parallel rows of short gastric papillae. The anterior section consisted of the posterior ventriculus and an indefinite but short portion anterior to the posterior ventriculus (Fig. 1). The heads and cervical muscles of the male beetles whose guts were sectioned also were extracted as a comparison to hindguts. The fresh and dry weight of 10 male heads were compared to the corresponding weights of 10 male guts by weighing the samples and then reweighing after heating the samples at 77° C for 1 hr.

RESULTS

Bacteria Associated with Pheromone Production

In the first experiment, the males feeding for 48 hrs. in the antibiotic diet sustained 3% mortality

Fig. 1. Accumulation of pheromones and associated compounds in various portions of the gut of male and female I. paraconfusus following aeration in myrcene and (-) α-pinene (November 19, 1976). Female heads were not extracted.


posterior midgut hindgut
cut
cut
cut
pylorus
ileum
rectum
male
female
0
50
100
150
I III II M B
I III II M B
I III II M B
I III II M B
10^{-9} g I, III or 10^{-8} g II, M, B

while the males in the control diet sustained 4% mortality, and the percentages were not significantly different at the 5% level. Mortality did not occur during the 18 hr. aeration for males that had fed in either type of diet. The number of fecal pellets voided by males during the aeration period which had fed on antibiotic diet averaged 7.5 fecal pellets/male while males that had fed on non-antibiotic diet averaged 7.4 pellets/male (no difference at the 5% level).

The hindguts of male beetles exposed to myrcene and (-) α-pinene contained compounds I, III, II, "H," myrtenol, and "B" which had relative retention times on the Apiezon L column of 1.00, 1.19, 1.37, 1.54, 1.9, and 3.59 respectively (Table 1). The compounds H and B were unidentified derivatives of myrcene (Byers, unpublished data). Compound II had a small side peak (probably trans-verbenol from the (+) enantiomer impurity in (-) α-pinene) which was ignored. A few other compounds eluting before compound I and after B were observed but not analyzed. The males which fed in either the antibiotic or antibiotic-free diets but which were not aerated in host precursors (myrcene and (-) α-pinene) did not contain any detectable compounds 18 hrs. after feeding, indicating that the ground phloem diet did not contribute sufficient precursors for detection. A t-test showed that only the amounts of compounds I and III were reduced in the antibiotic treated

Table 1. Production of pheromone compounds in male I. paraconfusus after feeding in semi-defined diets with or without antibiotics and then aerated in myrcene and (-) α-pinene (November 5, 1976).

	Amount of Pheromone Compounds (10^{-8}g) per male					
	I	II	III	H[1/]	Myrtenol	B[1/]
	No Antibiotic					
mean	40.6	267.5	7.4	14.0	104.1	341.5
s.d.	11.6	48.4	1.0	0.4	12.3	13.0
s.e.x	5.8	34.3	0.5	0.3	8.7	6.5
	Antibiotic					
mean	21.1[2/]	195.1[3/]	3.3[4/]	13.1[3/]	70.8[3/]	316.9[3/]
s.d.	10.7	6.1	1.8	0.2	9.9	68.7
s.e.x	5.4	4.3	0.9	0.15	7.0	34.3

[1/] H and B quantified in equivalents of compound I.

[2/] A t-test showed mean was different than mean for no antibiotic diet at the 5% level.

[3/] A t-test showed mean was not different than mean for no antibiotic diet at the 5% level.

[4/] A t-test showed mean was different than mean for no antibiotic at the 1% level.

males while all other compounds were unaffected. This result indicates that bacteria may be involved in the conversion of myrcene to I and III.

In the second experiment, after feeding 96 hrs. in the respective diets, 3% died in the antibiotic-free diet, 5% in the low concentration antibiotic diet, and 7% in the higher concentration antibiotic diet. A Yates chi square test indicated no differences in mortality at the 5% level. Similarly, the mortality during aeration for the 2 treatments and control was not significantly different at the 5% level. Fecal pellet production during the aerations was not significantly different (5% level) at 8.2, 8.5, and 7.5 pellets per male in the non-antibiotic, low concentration antibiotic diet, and high concentration antibiotic diet, respectively.

It appears that feeding on the high concentration antibiotic diet containing streptomycin sulfate and penicillin-G (10 mg each/ml water) caused almost complete inhibition of synthesis of the pheromones I and III from myrcene in males (Table 2). The less concentrated antibiotic diet, in contrast to the first experiment, did not affect synthesis of I and III probably because the dosage is near the threshold causing incipient inhibition. Production of compounds derived from (-) α-pinene (II, myrtenol) and myrcene (B) were not affected by the antibiotics (5% level). As in the first experiment, unaerated

Table 2. Production of pheromone compounds in male I. paraconfusus after feeding in semi-defined diets with or without antibiotics and then aerated in myrcene and (-) α-pinene (November 17, 1976).

	Amount of Pheromone Compounds (10^{-8}g) per Male				
	I	II	III	Myrtenol	B[1/]
			No Antibiotic		
mean	9.5	183.9	3.9	60.0	381.1
s.d.	5.5	78.9	1.7	24.5	84.5
s.e.$\bar{x}$	1.8	25.0	0.6	7.8	26.7
			Antibiotic (1 mg/ml)		
mean	11.5[2/]	125.8[2/]	2.7[2/]	49.6[2/]	340.6[2/]
s.d.	7.8	41.0	0.9	15.0	65.6
s.e.$\bar{x}$	3.5	18.3	0.4	6.7	29.3
			Antibiotic (10 mg/ml)		
mean	< 0.1[3/]	216.1[2/]	< 0.1[3/]	77.2[2/]	292.1[2/]
s.d.	-	72.2	-	21.1	46.8
s.e.$\bar{x}$	-	32.3	-	9.4	27.0

[1/] B quantified in equivalents of compound I. In experiment I (Table 1), H was detected because some males were only exposed to myrcene. Compound II from (-) α-pinene elutes close to H and thus causes the masking of H.

[2/] A t-test showed mean was not different than mean for no antibiotic diet at the 5% level.

[3/] A t-test showed mean was different than mean for no antibiotic diet at the 1% level.

males that had fed on diets did not produce detectable amounts of any compounds, indicating that the ground phloem diet did not contribute significant quantities of myrcene and α-pinene to the production of compounds observed in aerated males.

Site of Pheromone Production and/or Accumulation

Trans-verbenol, myrtenol, II and an unidentified compound made from myrcene (B) accumulated and perhaps were produced in the posterior part of the hindgut of both males and females (Fig. 1). Only the males produced compounds I and III which also accumulated and perhaps were synthesized in the posterior part of the hindgut (Fig. 1). The male head and cervical muscles contain about twice as much water/volatiles (4.9×10^{-4}g) as the 3 gut sections combined (2.3×10^{-4}g).

DISCUSSION

Most bacteria are not killed by antibiotics which affect protein synthesis but are prevented from multiplying whereupon they eventually die due to natural causes (Brock, 1970). Therefore, a relatively long period of feeding on antibiotics was felt to be necessary to affect the beetle's hypothetical symbiotic bacteria and thus reduce pheromone production. On the other hand, penicillin-G could affect pheromone production in a relatively short time because it inhibits the enzyme that cross-links

peptides of adjacent polysaccharide chains in the cell walls of certain kinds of bacteria (usually Gram-positive, Brock, 1970). Inhibition of cell-wall synthesis then is often followed by cell lysis and death due to the unchecked action of autolytic enzymes normally functioning in cell wall growth (Brock, 1970). The ground phloem-powdered cellulose diet stimulated feeding and defecation while containing lower amounts of precursor than host substrates so that the subsequent aeration in precursors accounted primarily for the compounds observed in the beetle.

After introducing antibiotics into the digestive system, the ability of the insect to synthesize pheromone compounds then was challenged by supplying constant amounts of precursor. Aerating the males in constant amounts of myrcene and (-) α-pinene circumvents any control the beetle may have over pheromone production as could be the case in a feeding system. Aeration also allows the accumulation of II and associated compounds (Renwick et al., 1976) which are not easily detected in the hindgut during feeding (Vité et al., 1972; Byers, unpublished data).

If the antibiotics had reduced feeding stimulation this would have been reflected in the production of fecal pellets during the aeration period, however, no differences were measured. Furthermore, reduced feeding would have

minimized any differences in pheromone production between the treatments and the control since the antibiotics would not have contacted the hypothesized internal bacteria. If all compounds observed in the hindgut were reduced in beetles feeding in antibiotic diets compared to the control, then one might conclude that the antibiotics somehow damaged the beetle and thus indirectly affected pheromone production. However, the only harm that antibiotics appeared to have on males was to inhibit the biosynthetic system that converts myrcene to pheromones I and III (Tables 1 and 2). The health of the beetle did not appear to be adversely affected by the antibiotics since the mortality, fecal pellet production, and synthesis of II and other associated compounds were not reduced from the controls (Tables 1 and 2).

Brand et al. (1975) reported the isolation of Bacillus cereus from the lumen of I. paraconfusus hindguts. The bacterium was able to convert recemic α-pinene into compound II and trans-verbenol. It is surprising that the B. cereus were not affected by the antibiotics especially since penicillin-G is very effective against Gram-positive bacteria such as B. cereus. The dosage of antibiotics may not have been sufficient to affect B. cereus especially if they are located within the hemocoel, although this possibility was not indicated by Brand et al. (1975). The site of production studies also

do not support the hypothesis that B. cereus produces compound II in areas other than the hindgut.

Recent evidence by Hughes and Renwick (1977) indicated that the production of I and III in I. paraconfusus may be under hormonal control. Gut stretching due to feeding stimulates release of brain hormone which causes juvenile hormone release and subsequent stimulation of pheromone synthesis (Hughes and Renwick, 1977; Borden et al., 1969). The extent of this stimulation remains to be quantified. However, the enhancement of pheromone production due to gut stretching must be rather subtle since a comparison of I, II, III production between groups of males fed on the non-antibiotic diet and unfed males each aerated in myrcene and (-)α-pinene showed no quantitative differences (Byers, unpublished data). If hormones are responsible for increasing the synthesis of pheromones, then these hormones would have to interact (directly or indirectly) with the suspected bacteria. It is expected that many other Ips species which attack pine species and use I and III as attractants (Vité et al., 1972) may also prove to have bacteria which convert myrcene to these pheromones. The results of this study indicate for the first time in insects and other organisms that a sex-specific bacterium(a) is probably responsible for the unique conversion of the host monoterpene, myrcene, into pheromone components of I. paraconfusus.

Studies by Wood (1962) showed that males of I. paraconfusus that had fed in ponderosa pine logs contained the attractive pheromone. Other studies reported the presence of pheromone activity in fecal pellets (Wood and Bushing, 1963; Vité et al., 1963; Wood et al., 1966). These results are supported by several other studies which have shown that the hindguts of various bark beetle species feeding on host substrates contain pheromone activity (Pitman et al., 1965; Zethner-Møller and Rudinsky, 1967; Schneider and Rudinsky, 1969), suspected pheromone compounds (Renwick et al., 1966; Pitman et al., 1966; Renwick and Vité, 1972) or identified compounds known to be pheromones (Pitman et al., 1969; Vité et al., 1972; Hughes, 1973, 1975). The host monoterpenes myrcene (Hughes, 1974) and (-) α-pinene (Renwick et al., 1976) were shown to be converted to the pheromone compounds (I, III) and (II), respectively, and to accumulate in the hindguts of I. paraconfusus. Many of these studies, however, have not specifically investigated feeding beetles of the opposite sex (Vité et al., 1963; Pitman et al., 1965; Zethner-Møller and Rudinsky, 1967; Schneider and Rudinsky, 1969; Vité et al., 1972; Hughes, 1974). In none of these studies were the distribution of pheromone compounds throughout the body determined.

Feeding might tend to cause accumulation of pheromone in the rectum due to passage of host material

through the digestive tract, while on the other hand, aeration in pheromone precursors might allow a more precise localization of pheromone production. In this study only male I. paraconfusus produce the pheromones I and III from myrcene and these pheromones accumulate primarily in the posterior section of the hindgut (Fig. 1). Other compounds produced from myrcene (B) and (-) α-pinene (II and myrtenol) also accumulate in the posterior hindgut and appear to be similarily distributed in both sexes.

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