ELECTROANTENNOGRAMS BY MOUNTAIN PINE BEETLES, *Dendroctonus ponderosae* HOPKINS, EXPOSED TO SELECTED CHIRAL SEMIOCHEMICALS

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Abstract—Electroantennograms (EAGs) were recorded from *D. ponderosae* to the enantiomers of the terpenoid bark-beetle pheromones *trans*-verbenol, *cis*-verbenol, verbenone, and the bicyclic ketals frontalin, *exo*-brevicomin, and *endo*-brevicomin. Male and female responses to enantiomers of the terpenoids differed significantly only at the two highest concentrations. No sex differences were seen in response to the bicyclic ketals. Significantly different responses to the enantiomers of all the chemicals, except frontalin, were noted over at least part of the dosage–response ranges tested. The negative antipode for all of the terpenoids elicited higher responses, while for the bicyclic ketals, the positive antipode effected the largest responses except for the two highest concentrations of *exo*-brevicomin.

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INTRODUCTION

Several species of pine trees in western North America are susceptible to the highly destructive attack of the mountain pine beetle (MPB) *Dendroctonus ponderosae* Hopkins. Colonization of the host tree depends on the beetles' responses to a complex blend of aggregation pheromones and host-tree kairomones (Vité and Gara, 1962; McCambridge, 1967; Pitman and Vité, 1969). *trans*-Verbenol, identified as the main female-produced pheromone (Pitman et al., 1968), is ineffective by itself (Pitman and Vité, 1969; McKnight, 1979), but when combined with the host-tree terpenes α-pinene or, especially, myrcene (Merrifield, 1972; Billings, 1974; Billings et al., 1976; Pitman et al., 1978), it becomes an effective aggregation pheromone. The addition of small quantities of *exo*-brevicomin to *trans*-verbenol plus myrcene (Borden et al., 1983a,b; Conn et al., 1983) produces a highly effective blend capable of attracting both sexes and mediating mass attack on several hard-pine species.

Investigations of chiral sensitivity in bark beetles began with *Scolytus multistriatus* when its aggregation pheromone was found to contain a three-component blend of (−)-enantiomers (Lanier et al., 1976). Since then, chiral discrimination has been shown in a number of other species of bark beetles, as well as in many other insects (Silverstein, 1979).

Research on optical isomers of pheromones of the mountain pine beetle began when significant differences were found in the behavior of the MPB to several semiochemicals, especially *trans*-verbenol (McKnight, 1979). Racemic verbenone and (−)-verbenone, but not (+)-verbenone, were found to have antiaggregative properties in both field and lab tests of beetles associated with lodgepole pine, *Pinus contorta* var. *latifolia* Engelmann (Ryker and Yandell, 1983). However, both enantiomers were active when tested on beetles from ponderosa pine, *Pinus ponderosa* Dougl. ex Laws. Male MPB from Oregon and British Columbia were found to produce predominantly (+)-*exo*-brevicomin and (+)-*endo*-brevicomin (Schurig et al., 1983). MPB in white pine in Idaho and lodgepole pine in Oregon responded best to (−)-*trans*-verbenol (Libbey et al., 1985).

Electroantennograms to racemic semiochemicals were recorded from MPB (Whitehead, 1986). The purpose of this paper is to report results of an investigation of antennal olfactory responses of the MPB to specific chiral pheromones, most of which have been determined to be important in the behavior of the insect.

METHODS AND MATERIALS

Adult beetles were obtained from infested bolts of lodgepole pine, *Pinus contorta* var. *latifolia* Engelmann, cut in Logan Canyon, Utah. After emergence, the beetles were sexed (Lyon, 1958) and placed on moist strips of filter

paper in Petri dishes at 5°C until use within 30 days after emergence. No differences in response were detected from beetles of different ages.

Electroantennogram (EAG) techniques were described previously (Whitehead, 1986) and were modified from those reported by Dickens et al. (1983). Briefly, EAGs were recorded with Ag–AgCl capillary electrodes filled with insect saline (Yamasaki and Narahashi, 1959). The recording electrode was placed into the distal end of the antennal club following prepuncture with an electrolytically sharpened tungsten needle. The indifferent electrode was placed into the head via the insect's mouth. EAG signals were amplified by a Grass P-16 amplifier and plotted on graph paper by an Apple II microcomputer. Chemicals were diluted in n-pentane in decade steps. A 10-μl sample was placed on a 20- $\times$ 6-mm filter-paper strip and inserted into an 80- $\times$ 5-mm glass cartridge. The stimulus was made by orienting the cartridge toward the preparation, the tip being about 1 cm away, and by passing a 1-sec flow of air through the cartridge at 1.7 liter/min. Samples were presented from lowest to highest concentrations. Five replicates (different beetles) for each sex were made at each concentration. At least 4 min were allowed between stimuli, except at higher concentrations, when 5 min were allowed (Dickens et al., 1983; Whitehead, 1986). Racemic frontalin (10 μg on filter paper) was used as a standard and was applied between every set of two stimulations of enantiomeric chemicals. EAGs were measured for negative amplitude and were shown as a percent of the mean of the two bracketing standards.

The semiochemicals used in this study are listed in Table 1. The optical purities of *trans*- and *cis*-verbenol and verbenone were determined using a chiral NEB column fitted gas chromatograph (Bradshaw et al., 1987). Optical purities of *exo*- and *endo*-brevicomin and frontalin were published by Mori (1974, 1975, 1985) and confirmed by chiral gas chromatography. The chemical purities of all the compounds were determined by gas chromatography. The temperature program initial value was set at 40°C for 2 min, increasing at a rate of 4°C/min until 150°C final temperature was reached (Whitehead, 1986).

Response to 10 μl of n-pentane on filter paper was measured but not subtracted from the responses to the other chemicals. Responses to air were subtracted from the test-chemical reaction. We did a repeated-measures ANOVA (Myers, 1979) to control for the differences in measuring between beetles and across the same beetle.

RESULTS

EAGs obtained in response to the test chemicals were similar to those recorded previously from the MPB (Whitehead, 1986) and those from other species of beetles (Angst and Lanier, 1979; Grant and Lanier, 1982; Dickens, 1981; Dickens, et al., 1983). Increases in stimulus concentrations yielded increases in response amplitude (Figures 1–3). Saturation occurred at 100 μg

TABLE 1. SOURCES AND PURITIES OF CHEMICALS

Compound	Chemical purity (%)	Optical purity (%)	Source[a]
Bicyclic ketals			
(+)-endo-Brevicomin	100	100	A
(−)-endo-Brevicomin	100	100	A
(+)-exo-Brevicomin	100	97.1	A
(−)-exo-Brevicomin	100	100	A
(+)-Frontalin	100	>98	A
(−)-Frontalin	100	>98	A
(±)-Frontalin[b]	99.6	—	C
Terpenoids			
(+)-cis-Verbenol	98.4	93.5	B
(−)-cis-Verbenol	99.1	95.4	B
(+)-trans-Verbenol	94.7	85.8	B
(−)-trans-Verbenol	93.5	91.6	B
(±)-Verbenone	93.8	92.9	B
(−)-Verbenone	91.8	95.5	B

[a] A = K. Mori; B = H. Wieser and E.A. Dixon, Department of Chemistry, University of Calgary, Alberta, Canada; C = Albany International, Willoughby, Ohio.
[b] Used for standard.
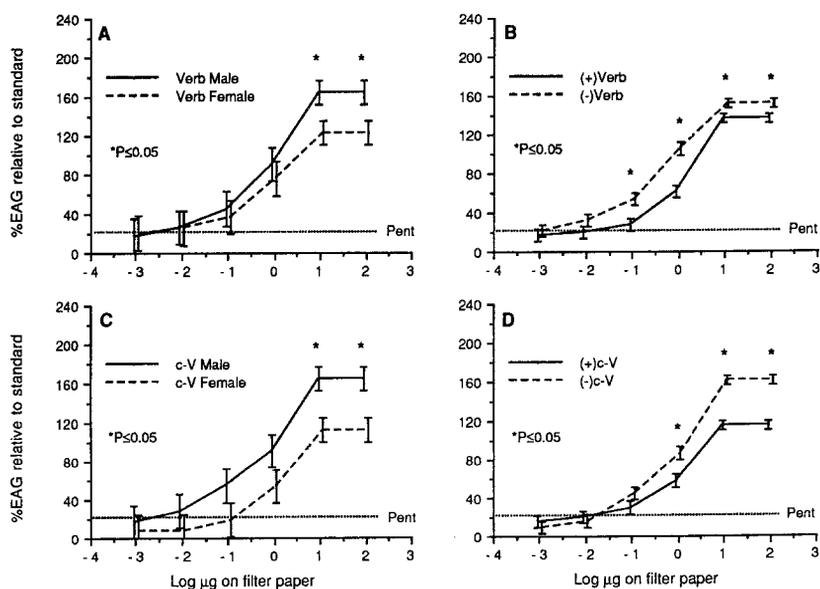
FIG. 1. (A,C) EAG-derived curves of MPB males (solid lines) and females (dashed lines) to serial dilutions of verbenone and *cis*-verbenol (enantiomeric responses averaged) and EAG-derived curves (responses from sexes averaged) to serial dilutions of (+)-enantiomer (solid lines) and (−)-enantiomer (dashed lines) of verbenone (B) and *cis*-verbenol (D). $N = 10$; error bars indicate ±SEM. *Significant difference between responses at 0.05 level. Verb = verbenone, c-V = *cis*-verbenol. Horizontal dotted line = pentane response.
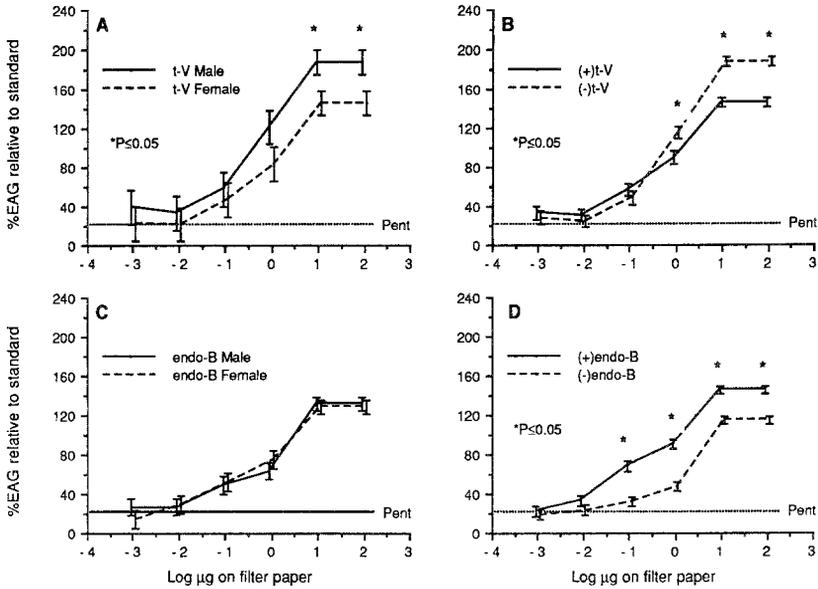
FIG. 2. (A,C) EAG-derived curves of MPB males (solid lines) and females (dashed lines) to serial dilutions of *trans*-verbenol and *endo*-brevicomin (enantiomeric responses averaged) and EAG-derived curves (responses from sexes averaged) to serial dilutions of (+)-enantiomer (solid lines) and (−)-enantiomer (dashed lines) of *trans*-verbenol (B) and *endo*-brevicomin (D). $N = 10$; error bars indicate $\pm$SEM. *Significant difference between responses at 0.05 level. t-V = *trans*-verbenol, endo-B = *endo*-brevicomin. Horizontal dotted line = pentane response.

for all chemicals except frontalin where, because of the limited amount of product on hand, the highest concentration tested was 10 μg. The pentane control produced responses of 21.8 $\pm$ 15.14% (SE) of the frontalin standards (Figures 1–3). There were no significant differences between the male and female responses. The frontalin standard yielded 0.48 $\pm$ 0.01 mV (SE) from males and 0.49 $\pm$ 0.01 (SE) mV responses from females, which were also nonsignificant. Few responses to air were noted.

The analysis of the terpenoids showed that the males and females responded differently at each concentration level (Figures 1A,C and 2A). As the concentration of the terpenoids increased, there was usually a greater separation between the points on the curves. The analysis of the bicyclic ketals showed no differences by sex (Figures 2C and 3A,C) but did show a chemical by enantiomer interaction not found in the terpenoids.

Figures 1–3 indicate that the magnitude of the responses were dependent upon the chemical, its concentration, and its enantiomer. There were statistically different responses between the sexes at the two highest concentrations of all the terpenoids (Figures 1A,C and 2A). In all cases where sex differences were noted, the males showed the largest responses.
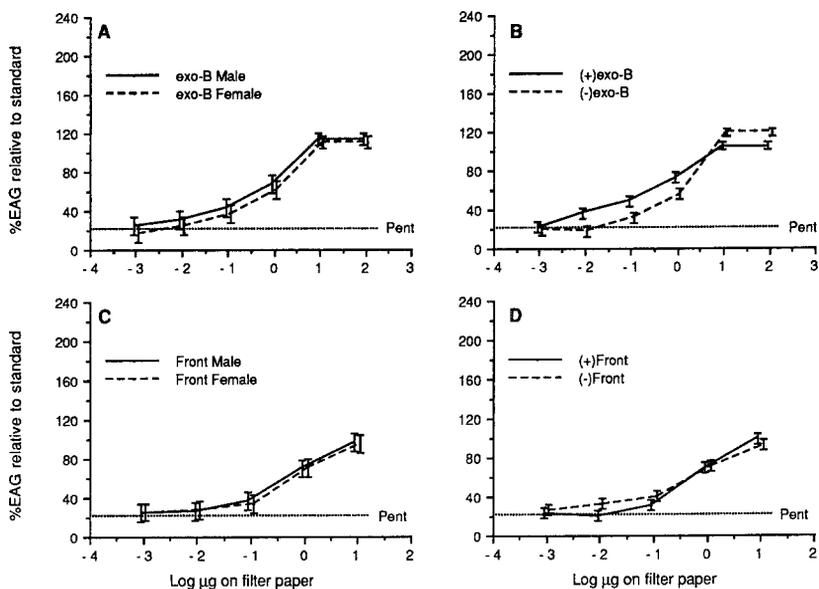
FIG. 3. (A,C) EAG-derived curves of MPB males (solid lines) and females (dashed lines) to serial dilutions of *exo*-brevicomin and frontalin (enantiomeric responses averaged) and EAG-derived curves (responses from sexes averaged) to serial dilutions of (+)-enantiomer (solid lines) and (−)-enantiomer (dashed lines) of *exo*-brevicomin (B) and frontalin (D). $N = 10$; error bars indicate $\pm$SEM. exo-B = *exo*-brevicomin, Front = frontalin. Horizontal dotted line = pentane response.

Figure 4A,B shows the values of the response averaged across concentration for sex, chemicals, and chirality. Figure 4A shows that frontalin was not significantly different from *exo*-brevicomin; however, both compounds were different from *endo*-brevicomin. The responses to the bicyclic–ketal enantiomers were statistically different from each other. Figure 4B, on the other hand, shows that both the sex and chirality factors for the terpenoids were different but that the overall responses to verbenone and *cis*-verbenol were not different from each other, while the response to *trans*-verbenol was different from those to verbenone and *cis*-verbenol when averaged across concentration. However, because of the interactions, these results could change for a given concentration level, e.g., in Figure 2B the responses to enantiomers were the same for the lowest concentrations but different for the highest concentrations.

Responses to the enantiomers of the terpenoids and the bicyclic ketals were more pronounced than the influence of the sex of the beetle. However, the analysis showed that no significant differences existed between responses to the frontalin enantiomers (Figure 3D). On the other hand, (+)-*exo*-brevicomin (Figure 3B), (+)-*endo*-brevicomin (Figure 2D) and (−)-verbenone (Figure 1B)
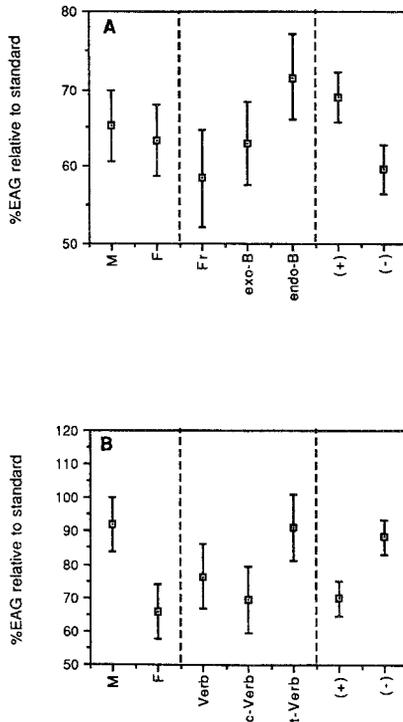
FIG. 4. Estimated means of the EAG responses by sex, chemicals, and enantiomers averaged over concentration. M = male, F = female, Fr = frontalin, exo-B = *exo*-brevicomin, endo-B = *endo*-brevicomin, Verb = verbenone, c-Verb = *cis*-verbenol, t-Verb = *trans*-verbenol, (+) = (+)-enantiomer, (−) = (−)-enantiomer. N = 180 (sex), 120 (chemical), 180 (enantiomers). Vertical dashed lines indicate that the enclosed averaged responses should not be compared to the responses within the other dashed lines.

had the largest responses over most of their concentration ranges. (−)-*cis*-Verbenol (Figure 1D) and (−)-*trans*-verbenol (Figure 2B) showed significantly greater responses than their antipodes at the three highest concentrations. In general, responses to (−)-enantiomers were greater for the terpenes; while, by comparison, responses to (+)-enantiomers were greatest for the bicyclic ketals.

DISCUSSION

Our results confirm some of the findings of previous workers investigating the effects of chiral semiochemicals on the MPB both in laboratory and in field studies. McKnight (1979) found that the MPB responded significantly better to

(−)-*trans*-verbenol than to the (+)-antipode in white pine stands in Idaho and lodgepole pine stands in Oregon. This was supported by Libbey et al. (1985), who determined that males responded better to the (−)-antipode while females were attracted equally. Borden et al. (1987) indicated that both (±)- and (−)-*trans*-verbenol caused significant attraction of both sexes, while the (+)-enantiomer did not, and they determined by gas chromatographic analysis that females from British Columbia contained 65–87 % of (−)-*trans*-verbenol.

Ryker and Yandell (1983) determined in field trapping that both sexes of the MPB showed significantly reduced attraction to the attractive mixture of *trans*-verbenol, myrcene, and α-pinene when exposed to either (−)- or (±)-verbenone in both ponderosa and lodgepole pine stands. However, their laboratory bioassay demonstrated that (±)-verbenone was effective in reducing the arrestment of male MPB and the number emitting attracting chirps, while either enantiomer would reduce arrestment. On the other hand, only the (−)-antipode was effective in reducing arresting chirps. They concluded that the antiaggregative effect of (−)-verbenone is not changed by the presence of its antipode in the racemic mixture. Verbenone is now recognized as an inhibitor of aggregation behavior in several bark beetle species (Borden, 1982). Borden et al. (1987) confirmed that verbenone was inhibitory to MPB males at several release rates in field studies.

The role of *cis*-verbenol is unclear in the biology of the MPB. Pitman et al. (1969) failed to find major quantities in either sex; however, it has been identified in female frass (Ryker and Rudinsky, 1982; Pierce et al., 1987). It was significantly more effective in eliciting EAG responses in male MPB than was *trans*-verbenol but not in females (Whitehead, 1986); however, in our current study, the opposite occurred, wherein males were slightly more sensitive to *trans*-verbenol than to *cis*-verbenol. The reasons for this disparity are unclear. Possibly the relative impurity of compounds (90 %) used in the previous study (Whitehead, 1986), the low numbers of test animals ($N = 5$), or errors in recording in both studies could be contributing factors. In *Dendroctonus pseudotsugae*, *cis*-verbenol stimulates more generalized synergist cells (Dickens et al., 1984). The MPB may have similar cells, and if *cis*-verbenol fills the receptor sites on these cells in a similar fashion to *trans*-verbenol, then more or less equal responses may be expected from both compounds.

exo-Brevicomin is a female attractant at low concentrations (Conn et al., 1983; Rudinsky et al., 1974), but high release rates resulted in inhibition of male responses while intermediate doses yielded partial inhibition (Borden et al., 1987), thus suggesting that *exo*-brevicomin may be a multifunctional pheromone (Rudinsky et al., 1974). Analysis of male MPBs from Oregon and British Columbia showed that they produce predominantly (+)-*exo*-brevicomin and (+)-*endo*-brevicomin (Schurig et al., 1983). Their findings correlate positively with our observations that MPB antennal sensilla are more sensitive to (+)-*exo*-brevicomin than its antipode. However, field tests showed that the beetles seem

to be insensitive to chirality, in that they responded to racemic *exo*-brevicomin in a similar fashion as to the enantiomers (Borden et al., 1987).

The role of *endo*-brevicomin in the biology of the beetle is also unclear. It has been reported as a male-produced pheromone that acts as an antiaggregant similar to *exo*-brevicomin (Rudinsky et al., 1974). Ryker and Rudinsky (1982) suggested that high levels of both racemic *exo*- and *endo*-brevicomin may act as antiaggregative pheromones in lodgepole pine infestations. However, Borden et al. (1987) report that their unpublished experiments have failed to confirm any attractive or antiaggregative properties for *endo*-brevicomin in lodgepole pine forests. Our results showed that the (+)-enantiomer elicited significantly higher EAGs than its antipode and the enantiomers of *exo*-brevicomin. No other known field or laboratory studies have been carried out to date on the effects of *endo*-brevicomin enantiomers.

At low release rates, frontalin has been implicated recently as an aggregation pheromone in females, and at high release rates as an antiaggregative pheromone; furthermore, neither enantiomer was attractive at low rates, but they were inhibitory at high rates compared to the racemate (Borden et al., 1987), suggesting an additive effect of both enantiomers (Dickens et al., 1985). However, our results suggest that the beetle is incapable of detecting differences between the enantiomers at any concentration, and also, contrasting with the field data, both sexes had about the same sensitivity to frontalin (Whitehead, 1986) (Figure 3C).

A new series of experiments, being undertaken using extracellular recordings from individual antennal sensilla, may clarify some of the unanswered questions regarding semiochemical detection by the MPB.

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