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AGGREGATION PHEROMONES IN *Dryocoetes affaber* (Mann.) (COLEOPTERA: SCOLYTIDAE): STEREOISOMERISM AND SPECIES SPECIFICITY

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Abstract—Chemical analysis of whole body extracts and volatiles produced by feeding males *Dryocoetes affaber* (Mann.) disclosed (+)-*exo*-brevicomin and (+)-*endo*-brevicomin [(+)EXOB and (+)ENDOB], as the major insect-produced potential pheromones. Laboratory bioassays and field-trapping experiments demonstrated that (+)ENDOB is the main pheromone component, and (−)ENDOB has an inhibiting effect. EXOB either as (+) or (±) appears to be a multifunctional pheromone. It has a synergistic effect in blends of EXOB and ENDOB in ratios up to 1:1, and it is inhibitory at higher ratios. (−)EXOB was inactive. The most attractive blend for *D. affaber* was a 1:2 blend of (+)EXOB and (+)ENDOB. When this blend was compared with a 9:1 blend, the best known blend for *Dryocoetes confusus* Swaine, the responses by beetles of each of the two species were highly specific, providing evidence for pheromonal exclusion between the two congenerics. We conclude that the combined effect of chirality and the ratio of geometrical isomers of brevicomin determines both the level of response and the species-specificity of the chemical signal in *D. affaber*.

Key Words—Semiochemicals pheromones, *Dryocoetes affaber*, *Dryocoetes confusus*, Coleoptera, Scolytidae, enantiomers, diastereoisomers, *exo*-brevicomin, *endo*-brevicomin.

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INTRODUCTION

Dryocoetes affaber (Mann.) infests *Picea* spp., and has been reported from *Abies*, *Pseudotsuga*, *Larix*, and *Pinus* spp. It is the most widespread member of the genus in North America, ranging from Alaska and eastern Canada to New Mexico and North Carolina (Bright, 1963, 1976). The males are polygynous and are the first to attack the bole of weakened trees (Keen, 1952; Furniss and Carolin, 1977). The life cycle is poorly known, but in Colorado it appears to have one generation per year and overwinters as adults (McCambridge and Knight, 1972).

Furniss et al. (1976) observed attraction of *D. affaber* and *D. autographus (Ratzeburg)* to uninfested spruce logs and to one or more semiochemicals (*trans*-verbenol, verbenol, seudenol, and frontalin), produced by spruce beetles, *Dendroctonus rufipennis* (Kirby). Evidence for secondary attraction in other *Dryocoetes* spp. has been reported (Nilssen, 1979; Stock and Borden, 1983). European male *D. autographus* produce *exo*- and *endo*-brevicomin (7-ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]octane); (+)-*endo*-brevicomin was attractive to females in field tests, while the antipode was inactive (Kohnle and Vité, 1984; Kohnle, 1985). (Herein, we use EXOB for *exo*-brevicomin and ENDOB for *endo*-brevicomin; blends are referred to as EXOB:ENDOB, in that sequence). Males of the western balsam bark beetle, *Dryocoetes confusus* Swaine, produce EXOB and ENDOB, mostly as (+) enantiomers (Schurig et al., 1983); *trans*-verbenol, verbenone, and myrtenol have also been identified from males in this species (Borden et al., 1987; Stock, 1991).

D. confusus is sympatric with *D. affaber* in the subalpine forests of British Columbia, wherein mixtures of (+)EXOB and (+)ENDOB at a 9:1 ratio optimally attract *D. confusus*. At a 1:1 ratio, either as (±) or (+) enantiomers, *D. affaber* was attracted rather than *D. confusus*, which suggested the existence of an isolation mechanism based on the chirality and the ratio of EXOB and ENDOB (Camacho et al., 1993).

Our objectives were: (1) to isolate and identify the major volatiles produced by feeding male *D. affaber*, (2) to determine the most attractive combination of these insect-produced volatiles, (3) to investigate the role of chirality in the system, and (4) to elucidate mechanisms of pheromone-based species specificity between *D. confusus* and *D. affaber*.

METHODS AND MATERIALS

Collection of Insects and Hosts. Bolts of Engelmann spruce, *Picea engelmannii* Parry, both healthy and infested with *D. affaber*, were obtained from trees felled near Merritt, British Columbia. The infested bolts were placed in

screened cages in the laboratory at 20–21°C. Emerging beetles were sexed and kept on moistened paper at 5°C until used in laboratory bioassays.

Collection and Analysis of Volatiles. Groups of male or female beetles were placed individually in preformed entrance holes in fresh spruce logs. The beetles were allowed to bore into the phloem tissue for three days and were then excised from the phloem. Extracts were prepared by crushing whole beetles in pentane held over Dry Ice; the liquid fraction was recovered and stored at −29°C. Another batch of spruce logs was infested in the laboratory with male or female *D. affaber* and placed in aeration chambers. The emanating volatiles were captured on Porapak-Q and recovered by extracting it with pentane (Pierce et al., 1981).

The whole-body extracts and the volatiles from infested logs were analyzed by gas chromatography (GC) using Hewlett Packard 5830A and 5880A instruments equipped with capillary inlet systems, flame ionization detectors, and open tubular glass columns (30 m × 0.5 mm ID) coated with SP-1000 (Supelco, Bellefonte, Pennsylvania). The temperature program was 70°C for 2 min, then 4°C/min to 180°C, and holding for 20 min. The enantiomeric composition of EXOB and ENDOB was determined by analysis of the volatiles from feeding-male whole-body extracts on a Chirasil-Dex (8) column (25 m × 0.25 mm ID) (V. Schurig, University of Tübingen, Germany). Coupled gas chromatography–mass spectrometry (GC-MS) was performed with a Hewlett Packard 5895A GC-MS fitted with a fused silica column (30 m × 0.33 mm ID) coated with SP-1000 (J&W Scientific, Inc., Folsom, California). Helium was the carrier gas for the GC and GC-MS.

Synthetic Pheromones. (±)EXOB (96.3% pure with 2.5% ENDOB) and (±)ENDOB (96.4% pure with 0.4% EXOB), were obtained from Phero Tech Inc., Delta, British Columbia. Optically pure brevicomins were synthesized by B.D. Johnston (Department of Chemistry, Simon Fraser University), according to the procedures developed by Johnston and Oehlschlager (1982) and Oehlschlager and Johnston (1987); formulations included (+)ENDOB (98.8% and 90.15% chemically and optically pure, respectively), (−)ENDOB (97.8% and 91% chemically and optically pure, respectively), and (−)EXOB (96.4% and 92.6% chemically and optically pure, respectively). For field experiments conducted in 1992, we also employed (+)EXOB (98.79% and 94.0% chemically and optically pure, respectively). The Sharpless asymmetric dihydroxylation (Sharpless et al., 1991) was used for the synthesis (E.K. Czyzewska, Department of Chemistry, Simon Fraser University, unpublished). Blends of EXOB and ENDOB were prepared by weight, and ratios referred to below are on a weight-to-weight basis.

Determination of Ratio in Vapor Phase. The ratio in vapor phase was determined for the 1:2 formulation of (±)EXOB:(±)ENDOB. Two glass cap-

illary tubes (1.0 mm ID) sealed at one end, containing 12 μl of the 1:2 blend, were kept inside open 400-μl polyethylene tubes at 24–26°C. Vapor-phase samples were taken from the plastic tube at 24, 40, 48 and 70 hr after formulation and analyzed by GC as above.

Laboratory Bioassays. Experiments on responses to EXOB and ENDOB and their blends were performed using walking beetles in an open arena olfactometer (Wood and Bushing, 1963; Stock and Borden, 1983). Groups of 10 beetles of either sex were exposed for 2.5 min. to an airstream (500 ml/min) containing volatile stimuli applied in 10 μl of pentane to a filter paper wick. The solvent was used as a control. Room temperature was 20–21°C and room lighting was diffuse and of low intensity (22.57 lux). A series of 1-pg stimuli consisting of (+)EXOB, (+)ENDOB, and binary blends of these compounds at ratios of 3:1 (the natural ratio), 2:1, 1:1 [reported attractive (Camacho et al., 1993)], 1:2, 1:3, and 1:6 were tested. We tried to cover a wide range of possible combinations excluding those with high content of (+)EXOB, reported attractive for *D. confusus* (Camacho et al., 1993).

Field Experiments. Trapping experiments were conducted in a forest of Engelmann spruce and subalpine firs, *Abies lasiocarpa* (Hook.) Nutt., 40 km west of Merritt, British Columbia. Multiple funnel traps (Lindgren, 1983) (Phero Tech Inc.), were placed 15 m apart in randomized complete blocks, with 9–20 replicates per experiment. The release rates, either as single compounds or as blends, were approximately 0.2 mg/24 hr at 27°C from each glass capillary (10. mm ID), as determined in the laboratory (Stock et al., 1990). In the forest, the release rates are temperature dependent.

Experiment 1, conducted in 1992, tested blends of (+)EXOB and (+)ENDOB at the following ratios: 3:1 (found in the aerations of male infested logs) 1:1 [attractive for *D. affaber* (Camacho et al., 1993)], 1:2 (from the results of laboratory bioassays), 1:10 (attractive to *D. affaber* in other field experiments, J.H. Borden, unpublished), and an unbaited control.

Experiments 2–4 investigated the question of enantioselectivity. In 1991, experiment 2 tested the attractiveness of (±), (−), and (+)ENDOB, and blends of EXOB:ENDOB in the following combinations of enantiomers (±):(±), (±):(−) and (±):(+). Experiments 3 and 4 in 1992, tested EXOB:ENDOB blends of (−):(+), (+):(+), and (±):(+) (experiment 3), and (−):(−), (+):(−) and (±):(+) (experiment 4). The (±):(+) combination was always present in experiments 2–4. In all cases, blends of EXOB:ENDOB were in a 1:1 ratio.

Experiment 5 utilized the best blends of EXOB:ENDOB established in previous experiments for *D. affaber* and *D. confusus* and challenged their capacity to maintain species specificity. We used (+)EXOB:(+)ENDOB at a 9:1 ratio for *D. confusus* (Camacho et al. 1993) and (±)EXOB:(+)ENDOB at a 1:1 ratio (i.e., a 1:2 ratio of (+) enantiomers) for *D. affaber*.

Statistical Analysis. Laboratory bioassay results were analyzed by one-way

analysis of variance (ANOVA) and the Ryan-Einot-Gabriel-Welsch multiple F or "REGWF" test (Schlotzhauer and Littell, 1987) utilizing percentages of positive responders converted to $p' = \text{arcsine } \sqrt{p}$, to approximate a normal distribution (Zar, 1984). Percent values of 0% were recorded as $1/4\,n$ to improve the transformation (Bartlett, 1937). For field trapping experiments we used two-way ANOVA and the REGWF test on numbers of beetles captured transformed by $x' = \log(x + 1)$, to remove heteroscedasticity (Zar, 1984). In all cases $\alpha = 0.05$. Treatments with zero catches were excluded from statistical analyses. All analyses employed SAS computer software (SAS Institute, 1990).

RESULTS AND DISCUSSION

Identification of Candidate Pheromones. (+)EXOB and (+)ENDOB in a 1.7:1 ratio were conspicuous insect-produced compounds found by GC-MS analysis in whole-body extracts of males. Volatiles emanating from male-infested logs also contained (+)EXOB and (+)ENDOB; in this case the ratio was 3.04:1 (Figure 1). Small amounts of EXOB of undetermined chirality were detected from feeding females in logs.

In laboratory bioassays, blends of (+)EXOB:(+)ENDOB in the ratio range between 2:1 and 1:2 elicited the highest levels of response from female *D. affaber*; males showed some preference for blends in the ratio range between

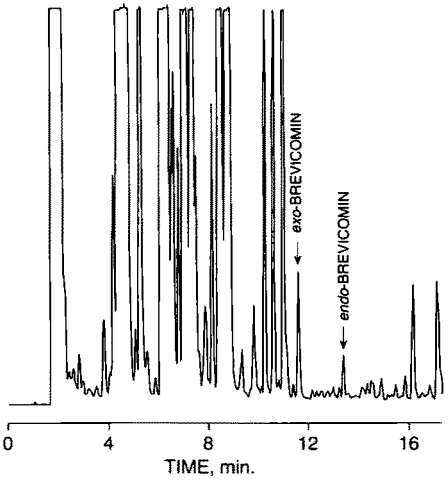

FIG. 1. Gas–liquid chromatogram of Porapak Q-trapped volatiles produced by male *Dryocoetes affaber* feeding in fresh bolts of *Picea engelmannii*, showing *exo*-brevicomin and *endo*-brevicomin in a 3:1 ratio.

3:1 and 1:2 (Figure 2). Both sexes showed the highest numerical response to the 1:2 ratio. The response to (+)EXOB or to (+)ENDOB presented individually was very low.

Ratio in Vapor Phase. The 1:2 blend of (+)EXOB:(±)ENDOB formulated by weight was confirmed by GLC analysis. The ratios ($\bar{X}$ ± SD) determined by GLC analyses of vapor phase samples after 24, 40, 48, and 70 hr were: 1:1.3 ± 0.015, 1:1.7 ± 0.03, 1:1.6 ± 0.015, and 1:1.7 ± 0.015, respectively. Differential volatility of EXOB and ENDOB caused only minor changes from the formulated ratio in the proximity of the release device. Modifications of this ratio are to be expected at further distances from the release point as a result of diverse environmental factors (e.g., temperature, turbulence) that affect the pheromone plume in the forest (Murlis et al., 1992).

Field Experiments. The highest response from both males and females in

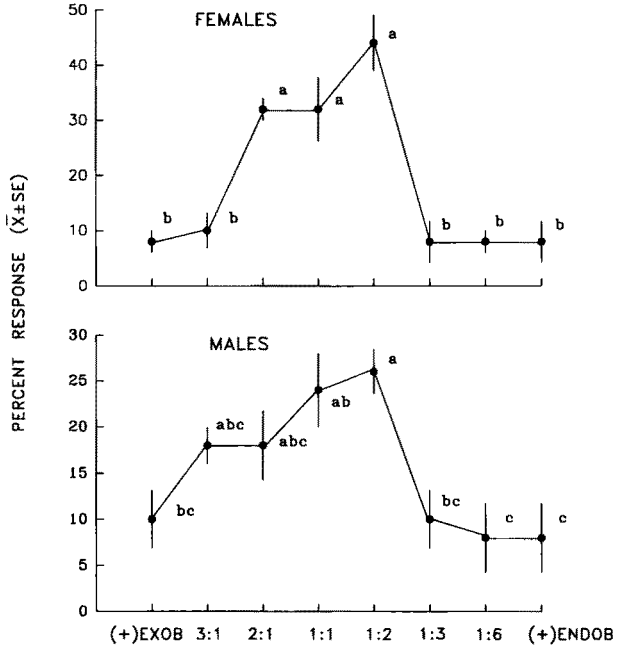

FIG. 2. Response of *Dryocoetes affaber* in laboratory bioassays to 1 pg stimuli of (+)-*exo*-brevicomin (EXOB), (+)-*endo*-brevicomin (ENDOB) and six blends of the two isomers at different ratios. Fifty beetles of each sex tested per stimulus. Response to pentane controls: male 4%, females 6%. Percents with the same letter are not significantly different, Ryan-Einot-Gabriel-Welsh multiple F test, $P < 0.05$.

experiment 1 was elicited by the (+):(+) blend at the 1:2 ratio (Figure 3). As in the laboratory (Figure 2), the natural 3:1 blend was poorly attractive. Blends of 1:1 and 1:10 (+)EXOB:(+)ENDOB attracted significantly less *D. affaber* of both sexes than did the 1:2 blend.

For Lepidoptera, it is generally accepted that optimum blends of pheromone components closely approximate the natural ratio emitted by the producing sex. However, production and reception genes are not linked (Roelofs et al., 1987). Response to different blends could indicate missing elements in the chemical message (Baker, 1989). It is possible that environmental factors could alter the pheromone plume from the natural 3:1 ratio to more attractive ratios. Other effects such as geographical and individual variation (Miller et al., 1989), physiological changes due to manipulation and storage, or mechanisms of avoidance

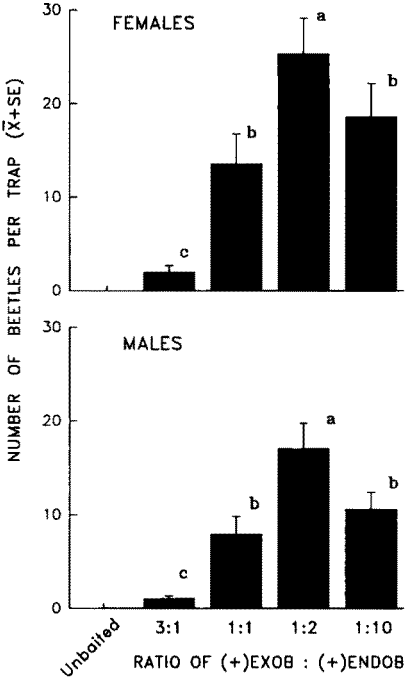

FIG. 3. Numbers of *Dryocoetes affaber* caught in experiment 1 in traps baited with blends of (+)-*exo*-brevicomin (EXOB) and (+)-*endo*-brevicomin (ENDOB) in four ratios; 10 replicates, July 7 to August 5, and 10 replicates August 5–20, 1992. Bars with the same letter are not significantly different, Ryan-Einot-Gabriel-Welsh multiple F test, $P < 0.05$.

of competition for pheromonal channels, could help to explain the observed difference between pheromone production and response in *D. affaber*.

Our results suggest that in *D. affaber* there is considerable tolerance to variation in ratios of pheromone components, as reported for other bark beetles (Schlyter et al., 1987; Byers, 1988) and moths (Linn and Roelofs, 1989). This plasticity could be of selective advantage for secondary bark beetles. For *D. affaber*, plasticity would be restricted to EXOB:ENDOB blends that comprised >50% ENDOB.

There was a decrease in response to ENDOB by both sexes in experiment 2, from (+) to (±) and finally (−), indicating that the (+) enantiomer is the active component; response to the blends indicates that the antipode is inhibitory (Figure 4). The 1:1 blend of (±)EXOB:(+)ENDOB was the most attractive, indicating that synergism occurs between EXOB and ENDOB, but not disclosing which enantiomer of EXOB is active. This question was resolved by experiment

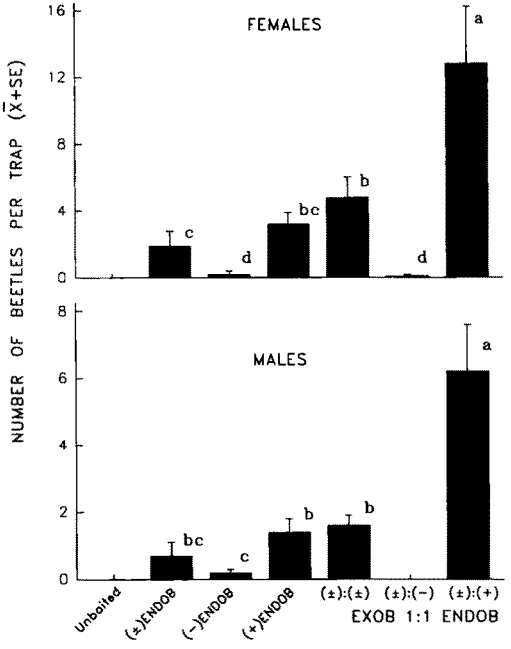

FIG. 4. Numbers of *Dryocoetes affaber* caught in experiment 2 to traps baited with (+), (−), and (±)-*endo*-brevicomin (ENDOB) and blends of (±)-*exo*-brevicomin (EXOB) and (ENDOB) mixed in a 1:1 ratio, in three enantiomeric combinations, nine replicates, August 9 to September 25, 1991. Bars with the same letter are not significantly different, Ryan-Einot-Gabriel-Welsh multiple F test, $P < 0.05$.

3, in which (+) or (±) EXOB in combination with (+)ENDOB elicited the highest levels of response (Figure 5). These results indicate that (+)EXOB is active and that (−)EXOB is inactive. The partial activity of the (−):(+) blend (Figure 5) can be attributed to the 7.4% (+)EXOB impurity. In experiment 4, binary blends containing only (−)ENDOB were not attractive (Figure 6), confirming that (+)ENDOB is the active enantiomer.

To facilitate interpretation of enantioselectivity, the pooled results of experiments 2–4 (all conducted in the same forest stand) were plotted as proportions (percentages), with response to the most attractive treatment [(±)EXOB:(+)ENDOB in a 1:1 ratio] normalized to 100% (Figure 7). It should be noted that a 1:1 ratio of (±)EXOB:(+)ENDOB results in a 1:2 ratio of the active (+) enantiomers, the most attractive ratio of geometrical isomers found in experiment 1 (Figure 3). Figure 7 shows evidence for the

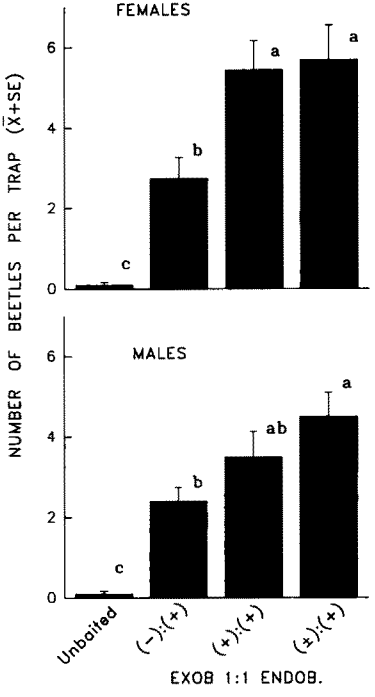

FIG. 5. Numbers of *Dryocoetes affaber* caught in experiment 3 to traps baited with chiral combinations of *exo*-brevicomin (EXOB) and (+)-*endo*-brevicomin (ENDOB) all in a 1:1 ratio, 10 replicates June 16 to July 7, and 10 replicates, July 7–23, 1992. Bars with the same letter are not significantly different, Ryan-Einot-Gabriel-Welsh multiple F test, $P < 0.05$.

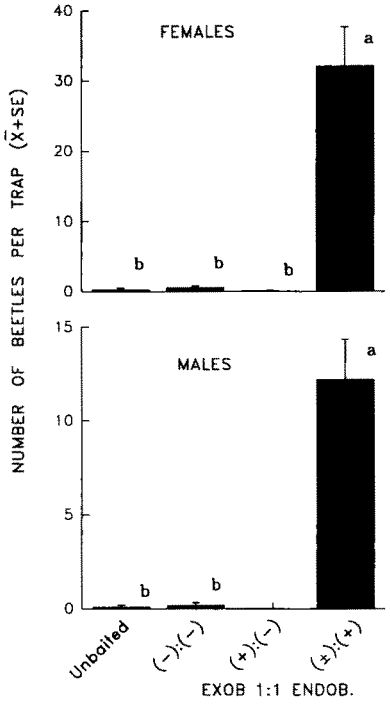

FIG. 6. Numbers of *Dryocoetes affaber* caught in experiment 4 to traps baited with enantiomeric combinations of *exo*-brevicomin (EXOB) and *endo*-brevicomin (ENDOB) all in a 1:1 ratio, 10 replicates, July 22 to August 20, 1992. Bars with the same letter are not significantly different, Ryan-Einot-Gabriel-Welsh multiple F test, $P < 0.05$

combined effect of optical and geometrical isomerism. (+)ENDOB (top row) is revealed to be the major component in the chemical signal. The capacity for (−)ENDOB to cause an inhibition of response to its antipode is shown in the second row from the top (Figure 7).

Synergism between (+)EXOB and (+)ENDOB, either as (+):(+) or (±):(+) blends, is disclosed in the two columns on the left of Figure 7. (+)EXOB is multifunctional, as at high ratios it is inhibitory (Figure 2), just as (+)ENDOB is for *D. confusus* (Camacho et al., 1993).

Cooccurrence of the (−) enantiomers of EXOB and ENDOB has an inhibitory effect for *D. confusus* (Camacho et al., 1993) and the European *D. autographus* (Kohnle and Vité, 1984). It is probable that a similar effect occurs in *D. affaber* (Figure 7).

When the best blends of EXOB:ENDOB for *D. confusus* (Camacho et al.,

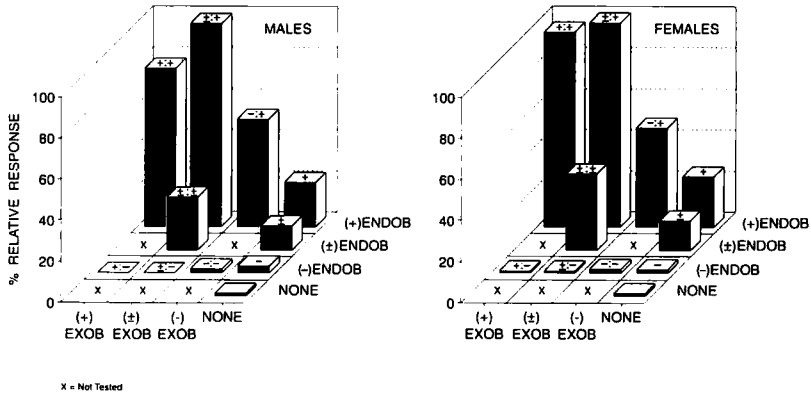

FIG. 7. Summary of pooled results obtained in experiment 2–4. Data normalized so that 100% response occurs to the blend of (+)-*exo*-brevicomin:(+)-*endo*-brevicomin in a 1:1 ratio.

1993) and *D. affaber* (Figures 2–7) were tested in the same location in experiment 5, there was a very clear demonstration that the responses of the two sympatric *Dryocoetes* spp. to blends of EXOB:ENDOB were highly species-specific (Figure 8). The numbers of *D. confusus* captured in response to the 1:2 blend, and of *D. affaber* attracted by the 9:1 blend were not statistically different from the captures obtained with unbaited traps (Ryan-Einot-Gabriel-Welsh multiple F test, $P < 0.05$). Our results support the hypothesis of semiochemical-based reproductive isolation advanced by Camacho et al. (1993), and we conclude that a mechanism of pheromonal exclusion based on the ratio of EXOB:ENDOB and on discrimination of enantiomers exists between *D. affaber* and *D. confusus*.

D. affaber is not sympatric with *D. confusus* over much of its range (Bright, 1963, 1976). The evolutionary forces operating in the development of fine tuning of pheromone channels when closely related species are in sympatry would not exist in allopathy. Therefore, we hypothesize that character displacement of pheromones could occur in *D. affaber* where it is sympatric with *D. confusus*. Studies on *D. affaber* pheromones in other areas might well disclose considerable variation in the production of and response to pheromones.

Practical Implications. Our results reaffirm that if attractive semiochemicals are to be used efficiently against *D. confusus*, e.g., to contain and concentrate infestations prior to logging (Stock et al., 1993), regulation of the composition and chirality of semiochemical baits is critical. Conversely, the evidence that ENDOB is a multifunctional pheromone for *D. confusus* (Camacho et al., 1993) is now stronger; it is involved in attraction at low ratios and is repellent at higher

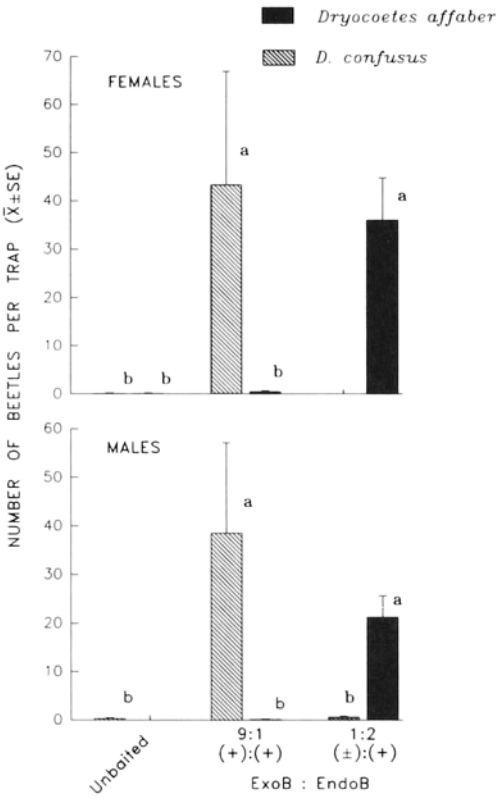

FIG. 8. Numbers of *Dryocoetes affaber* and *D. confusus* caught in experiment 5 to traps baited with optimal blends for each species of *exo*-brevicomin (EXOB) and *endo*-brevicomin (ENDOB), 10 replicates, June 16 to July 7, 1992. Bars with the same letter within each species are not significantly different, Ryan-Einot-Gabriel-Welsh multiple F test, $P < 0.05$. Note that a 1:1 ratio of (±)EXOB:(+)ENDOB results in a 1:2 ratio of (+) enantiomers.

ratios with EXOB (Figure 8). Thus according to our results, the use of ENDOB to prevent or deter attack by *D. confusus* in high hazard stands (Stock et al., 1990; Stock, 1991), requires a formulation with appropriate enantiomeric composition released at adequate rates.

Borden (1992) proposed some novel tactics for the use of semiochemicals, among them the use of pheromone-induced competitive displacement of tree-killing bark beetles by secondary species. This method of biological control was proposed for mountain pine beetles (MPB), *Dendroctonus ponderosae* Hopkins.

MPB can be effectively displaced by pine engravers, *Ips pini* (Say), which rapidly utilize all available phloem tissue (Rankin and Borden, 1991). *D. affaber* is often present in large number in hosts infested by the spruce beetle, *Dendroctonus rufipennis* (Kirby), and is involved in the mortality due to interspecific competition (McCambridge and Knight, 1972). If it can displace *D. rufipennis*, as occurs between *I. pini* and MPB, biological control of the spruce beetle through semiochemically induced competitive displacement with *D. affaber* may be possible.

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