

ENANTIOMER PREFERENCE OF *Trypodendron lineatum*
AND EFFECT OF PHEROMONE DOSE AND TRAP
LENGTH ON RESPONSE TO LINEATIN-BAITED TRAPS
IN INTERIOR BRITISH COLUMBIA

S. E. R. HOOVER,¹ B. S. LINDGREN,^{1,*} C. I. KEELING,²
and K. N. SLESSOR²

¹College of Science and Management
University of Northern British Columbia
Prince George, BC V2N 4Z9, Canada

²Department of Chemistry
Simon Fraser University
Burnaby, BC V5A 1S6, Canada

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Abstract—Both enantiomers of the aggregation pheromone lineatin were obtained in very high enantiomeric purity by preparative chiral liquid chromatography on microcrystalline cellulose triacetate. Catches of the striped ambrosia beetle, *Trypodendron lineatum*, in multiple-funnel traps baited with lineatin enantiomer ratios (plus/minus) of 100:0, 75:25, 50:50, 25:75, and 0:100, demonstrated that (+)-lineatin is the only active enantiomer in interior British Columbia, where two other *Trypodendron* species are sympatric. In additional experiments using (±)-lineatin, catches of both sexes increased significantly with trap length, either at a constant pheromone dose per trap, or a constant dose per four-funnel unit, up to 16 funnels per trap. When trap length was held constant at eight funnels, increasing the lineatin dose fourfold had no effect on trap catches.

Key Words—Sex ratio, multiple-funnel traps, preparative chiral liquid chromatography, microcrystalline cellulose triacetate, Coleoptera: Scolytidae.

INTRODUCTION

Damage by ambrosia beetles, particularly the striped ambrosia beetle, *Trypodendron lineatum* (Olivier), continues to be a major source of timber degradation

*To whom correspondence should be addressed.

in British Columbia sawmills (Lindgren, 1990; Orbay, 1994). Economic losses result from direct damage, import bans of Canadian lumber by foreign nations, and remanufacturing costs (McLean, 1985; Orbay, 1994). Because ambrosia beetles feed exclusively on fungi introduced to the host tree, they are able to exploit a variety of host species (Nijholt, 1978). Staining caused by the fungus may also cause extensive damage (Liu and McLean, 1993).

Ambrosia beetles play an important role in maintaining healthy forest ecosystems (Lindgren, 1990). They are among the first invaders of dead and dying trees, and their galleries may provide infection courts for saprophytic fungi, resulting in breakdown of the wood and nutrient recycling. It is only when high volumes of suitable host material become concentrated in one area that population levels become problematic, e.g., at harvesting sites, timber processing areas, or sawmills.

Considerable efforts have been made to devise effective mass trapping programs for ambrosia beetles in timber storage and processing areas (McLean and Borden, 1979; Lindgren and Borden, 1983; Lindgren and Fraser, 1994). Lineatin (3,3,7-trimethyl-2,9-dioxatricyclo[3.3.1.0^{4,7}]nonane), the aggregation pheromone of *T. lineatum*, attracts both sexes (Borden, 1979), and in North America it is synergized by the host volatile ethanol (Shore and Lindgren, 1996). In coastal British Columbia and in England, (+)-lineatin was responsible for attracting beetles, while the (-)-enantiomer appeared to be inert (Borden et al., 1980a; King et al., 1983).

In interior British Columbia, *T. lineatum* is sympatric with two other conifer-infesting *Trypodendron* species, *T. rufitarsus* (Kirby) and *T. retusus* (LeConte), as well as one hardwood-infesting species, *T. betulae* Swaine (Bright, 1976). The pheromones of these species are unknown. If one or more of them utilize lineatin as an aggregation pheromone, reproductive isolation between the species could be achieved through enantiospecificity, as occurs for two other sympatric ambrosia beetles, *Gnathotrichus sulcatus* (LeConte) and *G. retusus* (LeConte) (Borden et al., 1980b). If this is the case, *T. lineatum* could be expected to respond to specific ratios of lineatin enantiomers in areas where it is sympatric with the other species.

All methods developed for the synthesis of chiral lineatin (Mori and Sasaki, 1980; Slessor et al., 1980; Mori and Uematsu, 1983; Kandil and Slessor, 1985; Mori and Nagano, 1991) involve considerable effort to produce each enantiomer, and in some cases require optical resolution of an intermediate. Since (\pm)-lineatin is commercially available and many chiral stationary phases are available (Francotte and Junker-Buchheit, 1992), we reasoned that it might be possible to separate the enantiomers of racemic lineatin directly by preparative chiral liquid chromatography.

Considerable research has been done to investigate optimal pheromone concentrations (Bakke, 1983), attractant combinations (Salom and McLean, 1990a),

trap type and color (Lindgren, 1983; Lindgren et al., 1983; Shore and Lindgren, 1996), and trap location (Paiva, 1982; Salom and McLean, 1990b). However, the behavior of *T. lineatum* with respect to interaction between the pheromone and the traps is not yet fully understood.

In this study we tested the following null hypotheses in the interior of British Columbia: (1) catches of *T. lineatum* are independent of lineatin enantiomer ratio; (2) catches and sex ratio of *T. lineatum* are independent of trap length when pheromone release is constant; (3) catches and sex ratio of *T. lineatum* are independent of pheromone release rate when trap length is held constant; and (4) catches and sex ratio of *T. lineatum* are independent of trap length when pheromone release is held constant relative to trapping surface (number of funnels per trap).

METHODS AND MATERIALS

Enantiomer Synthesis. We chose microcrystalline cellulose triacetate as the stationary phase because it was available inexpensively in bulk and has proven effective for many preparative chiral separations including a spiroacetal pheromone component with some similarities to lineatin (Isaksson et al., 1984). A glass column (2.5 × 100 cm) was slurry-packed with about 200 g (dry wt) of microcrystalline cellulose triacetate (25–40 μm, EM Science) that had been swollen for 30 min in boiling ethanol–water (90 : 10). The column was connected to a liquid chromatograph (Varian model 5000) and a fraction collector (Waters). Initial trials on a 0.9- × 59-cm column suggested that higher temperatures and very low flow rates were required, in agreement with Bevan and Mutton (1992), and that ethanol–water at 90 : 10 gave better separation than the 95 : 5 ratio normally used with microcrystalline cellulose acetate. Therefore, the column was water-jacketed at 50° and preparative runs were completed at 0.2 ml/min ethanol–water at 90 : 10 after conditioning the column overnight at 1 ml/min.

Fractions were collected every hour and analyzed by gas chromatography (HP 5890, split, FID) with a chiral capillary column (J&W Scientific Cyclodex-B, 30 m × 0.25 mm ID × 0.25 μm, head pressure 135 kPa, 105°C, retention times 16.23 and 16.68 min). The enantiomers eluted from the cellulose triacetate column after 40 and 46 hr. Chromatographic parameters were as follows: $\alpha = 1.15$, $R = 0.7$, $N = 522$ and 339. Fractions with similar enantiomeric purity were combined, diluted with 3% aqueous NaHCO₃ and extracted with pentane. Polarimetry (Rudolph Research Autopol II Polarimeter) of the extracted peaks identified the first eluting peak from both gas chromatography and preparative liquid chromatography to be (+)-lineatin.

Four injections of 117 mg each of neat racemic lineatin (Phero Tech Inc.,

Delta, British Columbia) yielded 80 mg of >99.98% enantiomeric excess (ee) and 89 mg of 97.6% ee (+)-lineatin and 39 mg of 99.65% ee and 111 mg of 97.4% ee (-)-lineatin. To improve the enantiomeric purity of the (-)-lineatin, 58 mg of 97.4% ee (-)-lineatin was reinjected to yield 35 mg of >99.98% ee purity.

Preparation of Enantiomer Ratios and Release Devices. Release devices were prepared in the laboratory to provide a pentane control plus the (+)/(-) enantiomer ratios of 0:100, 25:75, 50:50, 75:25, and 100:0. The 100:0 and 0:100 ratios were prepared using the purest enantiomers of lineatin. Enantiomeric mixtures with 75:25 and 25:75 ratios were prepared as pentane solutions by mixing appropriate amounts of 97.6% ee (+)-lineatin and 97.4% ee (-)-lineatin, followed by chiral gas chromatographic analysis, and then concentrated. Racemic lineatin provided the 50:50 ratio.

Release devices for the enantiomer experiment were prepared from 25- μ l capillary tubes (Microcaps, Drummond Scientific Co.) flame sealed at one end and shortened to 18 mm. Using a 10- μ l syringe, 2 μ l of lineatin was placed in each capillary tube. Each tube was placed in a 0.4-ml microcentrifuge tube and centrifuged briefly to disperse any bubbles and droplets on the inside walls of the capillary tube. The meniscus was 10 mm below the opening of the capillary tube for all treatments. As part of the release device, the microcentrifuge tube was capped and two 3-mm-diam. holes were made near the top for air exchange. These devices released a constant 38 μ g/24 hr for 50 days at room temperature. For the trap length/pheromone release rate experiments, we used Hercon laminated dispensers (Phero Tech Inc.) which had been cut in half, giving a release rate of racemic (\pm)-lineatin at room temperature of about 50 μ g/24 hr.

Field Bioassays of Enantiomer Mixes. For experiment 1, three experimental sites were located along the Blackwater Road south of Baldy Hughes, approximately 40, 55, and 60 km, respectively, southwest of Prince George, British Columbia. A fourth site was on the University of British Columbia campus in Prince George. Each site differed in tree species composition and harvesting. Site A consisted of mature lodgepole pine, *Pinus contorta* Dougl. ex Loud.; Douglas fir, *Pseudotsuga menziesii* (Mirb.) Franco; hybrid spruce, *Picea glauca* \times *engelmanni* (Parry) Engelman; and a minor component of subalpine fir, *Abies lasiocarpa* (Hook.) Nutt.; trembling aspen, *Populus tremuloides* Michx.; and paper birch, *Betula papyrifera* Marsh. Site B was dominated by trembling aspen, with a minor hybrid spruce and pine component, and was surrounded on three sides by a clear-cut. Site C was a mature lodgepole pine-hybrid spruce stand along the edge of an area recently sanitation-harvested for the mountain pine beetle *Dendroctonus ponderosae* (Hopkins). Site D was dominated by paper birch and trembling aspen, with minor components of lodgepole pine, hybrid spruce, and Douglas fir. At each site, two randomized complete blocks, each with six eight-unit multiple-funnel (Lindgren) traps (Phero Tech Inc.) with treatments assigned

randomly, were set up on May 1, 1998. Traps were checked weekly until June 2, 1998. Captured beetles were transferred to labeled Ziploc bags, and stored at -40°C until the beetles were counted and their sex determined.

Trap Length and Lineatin Release Rate Experiments. All trap length and release rate experiments (Experiments 2–6) were conducted along the edges of the sanitation-harvested area at site C. Each of five experiments consisted of 10 randomized complete blocks of four traps approximately 5 m apart, with replicates at least 25 m apart. All traps were placed just inside the forest margin (Salom and McLean, 1991) around the harvested area.

The five experiments (Table 1) tested: four trap lengths, each with equal release rate (experiment 2); four trap lengths with increasing number of lures at two intensities with increasing trap length, i.e., constant release rate relative to trap surface area (experiments 3 and 4); and a constant trap length with 1–4 (experiment 5) or 2–8 (experiment 6) lures per trap. The combined pheromone plume from all four traps of each treatment was assumed to act as a long-range beetle attractant (Lindgren et al., 1983a), while the individual treatments (trap length or pheromone concentration) determined landing behavior (Salom and McLean, 1990a).

At the end of each experiment, trapped beetles were collected, sorted to species, counted, and their sex determined in the laboratory. *T. lineatum*, with pale legs, was initially separated from *T. rufitarsus* and *T. retusum* based on the black or darkly infuscated or rufous legs of the latter two species. Determinations were then confirmed by characteristics given in Wood (1982) and Bright (1976). In samples with large numbers of *T. lineatum*, numbers caught were estimated by measuring the total volume collected (Lindgren et al., 1983). Sex ratio was determined on all beetles in a 1-ml subsample from these. The total numbers of beetles of each sex collected per treatment were extrapolated from the subsample count.

To achieve homogeneity of variances, the number of beetles of each sex

TABLE 1. DESIGN SPECIFICATIONS FOR FIVE EXPERIMENTS EXAMINING INTERACTION BETWEEN AMBROSIA BEETLE CATCH, TRAP LENGTH, AND PHEROMONE PLUME CONCENTRATION

Experiment/date	Trap lengths ^a	Lures (<i>N</i>)
2. May 6–12, 1997	4, 8, 12, 16 funnels	2 lures per trap
3. May 12–21, 1997	4, 8, 12, 16 funnels	1 lure per 4 funnels
4. May 21–27, 1997	4, 8, 12, 16 funnels	2 lures per 4 funnels
5. May 30–June 10, 1997	8 funnels	1, 2, 3, 4 lures per trap
6. June 10–17, 1997	8 funnels	2, 4, 6, 8 lures per trap

^aOne funnel \approx 9 cm.

were transformed by $\log_{10}(x + 1)$ and proportions of females were transformed by $\arcsin\sqrt{x}$, before analysis by ANOVA using SYSTAT (SPSS Inc., Chicago, Illinois). Means were separated by Tukey's test (Zar, 1984). In all cases $\alpha = 0.05$.

RESULTS

Field Bioassays of Enantiomer Mixes. In experiment 1, catches in traps baited with the (-)-enantiomer were not significantly different from those in unbaited control traps (Table 2). All of the treatments containing the (+)-enantiomer differed significantly from the control and the treatment with no (+)-lineatin, but they did not differ from each other (Table 2).

Trap Length and Lineatin Release Rate Experiments. Longer traps in experiment 2 caught greater numbers of *T. lineatum* than shorter traps (Table 3). The proportion of females in the catch was independent of trap length. In experiments 3 and 4 the greatest numbers of beetles captured again increased with trap length, while sex ratio was independent of trap length (Table 4). In experiment 5 and 6 catches within each sex did not vary with pheromone release rate (Table 5). Regression analyses of the data for males and females, respectively, showed that trap length explained 75.5 and 72.5% of the variation in experiment 2, 74.7 and 77.6% in experiment 3, and 58.3 and 46.9% in experiment 4 (all $P < 0.001$). In experiment 6 the proportion of females tended to decrease with increased pheromone release rate, but was not significant (Table 5, $P = 0.054$). Throughout all experiments, the percentage of females in the catches remained relatively constant for *T. lineatum* at between 30.9 and 39.5%.

TABLE 2. EXPERIMENT 1: RESPONSE OF *Trypodendron lineatum* TO 5 ENANTIOMER RATIOS OF LINEATIN AND A CONTROL IN LINDGREN FUNNEL TRAPS^a

Enantiomer ratio (+)/(–)-lineatin	Beetles captured (Mean \pm SD) ^b	
	Male	Female
Blank control	0.08 \pm 0.408 a	0.0 \pm 0.0 a
0:100	0.04 \pm 0.204 a	0.0 \pm 0.0 a
25:75	28.17 \pm 29.19 b	5.67 \pm 6.63 b
50:50	42.71 \pm 40.64 b	7.21 \pm 7.47 b
75:25	44.17 \pm 37.68 b	7.83 \pm 8.83 b
100:0	44.21 \pm 35.88 b	8.04 \pm 8.89 b

^aLineatin was released at 38 $\mu\text{g}/24$ hr, Prince George, British Columbia, May 1–June 2, 1998.

^bWithin each column, means followed by the same letter not significantly different, analysis of variance ($\alpha = 0.05$) followed by Tukey's test for mean separation.

TABLE 3. EXPERIMENT 2: EFFECT OF TRAP LENGTH WITH CONSTANT LINEATIN RELEASE PER TRAP ON CATCHES OF *Trypodendron lineatum*^a

Trap length	Beetles captured (mean \pm SD) ^b		
	Male	Female	Female (%)
4 funnels	341.1 \pm 163.0 a	210.3 \pm 111.6 a	38.0 \pm 4.5
8 funnels	733.4 \pm 294.2 b	457.1 \pm 144.2 b	39.0 \pm 3.8
12 funnels	1345.5 \pm 490.4 c	876.1 \pm 332.4 c	39.5 \pm 3.0
16 funnels	1782.2 \pm 593.4 d	1233.5 \pm 662.2 c	39.3 \pm 9.7

^aEach lure releases about 50 $\mu\text{g}/24$ hr of racemic lineatin. Prince George, British Columbia, May 6–12, 1997. $N = 10$.

^bWithin each column, means followed by the same letter not significantly different, analysis of variance ($\alpha = 0.05$) followed by Tukey's test for mean separation.

TABLE 4. EXPERIMENTS 3 AND 4: EFFECT OF CONSTANT LINEATIN RELEASE PER TRAP SURFACE AREA (1 OR 2 LURES PER 4 FUNNELS) ON CATCHES OF *Trypodendron lineatum* IN LINDGREN FUNNEL TRAPS OF VARYING LENGTH^a

Trap length	Beetles captured (mean \pm SD) ^b		
	Male	Female	Female (%)
1 lure per 4 trap-funnels			
4 funnels	79.3 \pm 33.9 a	42.3 \pm 25.1 a	33.5 \pm 5.4
8 funnels	216.7 \pm 94.6 b	118.3 \pm 48.6 b	35.3 \pm 3.5
12 funnels	459.1 \pm 319.9 c	228.9 \pm 111.0 c	34.5 \pm 5.1
16 funnels	1294.9 \pm 1721.6 d	392.9 \pm 222.5 d	31.2 \pm 9.4
2 lures per 4 trap-funnels			
4 funnels	25.3 \pm 10.9 a	13.7 \pm 7.8 a	34.1 \pm 10.2
8 funnels	58.7 \pm 17.9 b	27.1 \pm 12.0 b	30.9 \pm 4.5
12 funnels	103.1 \pm 40.9 c	51.3 \pm 25.6 bc	32.3 \pm 6.5
16 funnels	133.0 \pm 79.4 c	78.0 \pm 65.0 c	33.9 \pm 6.5

^aEach lure releases about 50 $\mu\text{g}/24$ hr of racemic lineatin. Prince George, British Columbia, May 12–21, 1997. $N = 10$.

^bWithin each column, means followed by the same letter not significantly different, analysis of variance ($\alpha = 0.05$) followed by Tukey's test for mean separation.

DISCUSSION

Our data confirm the findings by Borden et al. (1980a) in coastal British Columbia that the (+)-enantiomer was responsible for attraction of *T. lineatum*. Borden et al. (1980a) caught significantly more beetles with the (–)-enantiomer than the blank control, whereas in our experiment the (–)-enantiomer did not differ from the blank control. This difference in results may be explained by

TABLE 5. EXPERIMENTS 5 AND 6: EFFECT OF PHEROMONE DOSE WITH CONSTANT TRAP LENGTH (8 FUNNELS) ON CATCHES OF *Trypodendron lineatum* IN LINDGREN FUNNEL TRAPS^a

Lures ^b	Beetles Captured (mean \pm SD) ^c		
	Male	Female	Female (%)
Experiment 5			
1 lure	56.3 \pm 31.1	35.4 \pm 24.7	37.7 \pm 4.5
2 lures	70.0 \pm 35.6	43.4 \pm 26.7	37.0 \pm 5.8
3 lures	74.7 \pm 44.4	45.7 \pm 34.1	36.3 \pm 3.9
4 lures	62.6 \pm 29.5	33.6 \pm 17.2	34.7 \pm 5.1
Experiment 6			
2 lures	75.0 \pm 30.5	46.4 \pm 12.4	39.4 \pm 7.3
4 lures	72.0 \pm 45.3	41.2 \pm 25.4	36.4 \pm 4.1
6 lures	102.3 \pm 45.9	48.9 \pm 19.6	33.1 \pm 4.2
8 lures	73.9 \pm 54.1	36.1 \pm 24.5	32.5 \pm 7.0

^aPrince George, British Columbia, May 21–27, 1997. $N = 10$.

^bEach lure releases about 50 $\mu\text{g}/24$ h of racemic lineatin.

^cNo significant difference among means within columns, analysis of variance ($\alpha = 0.05$).

the relatively higher optical purity achieved by our method of preparation of the enantiomers. Because the total release rate of the two lineatin enantiomers was held constant, the amount of the active enantiomer released would have been roughly proportional to its proportion of the mix. However, the response to lineatin by *T. lineatum* was independent of dose or enantiomer ratio, i.e., it was simply a function of presence or absence of (+)-lineatin. Therefore, to achieve reproductive isolation based primarily on pheromone specificity, sympatric *Trypodendron* species must produce and respond only to the (–)-enantiomer, utilize a multicomponent blend that has a component that is repellent to *T. lineatum*, or must have a completely different pheromone. *T. retusum* and *T. rufitarsus*, which both fly too early in the year to have been captured in the enantiomer experiment, were captured in relatively high numbers in the trap-length experiments in 1997, possibly indicating a response to lineatin, as in sympatric European *Trypodendron* spp. (Klimetzek et al., 1981).

An alternative explanation for the lack of ratio response is that the release rates used in our study were too high to discriminate between the different ratios, i.e., the release rate of (+)-lineatin from the 25 : 75 ratio treatment would have been approximately 1/4 of the release rate of the 100% (+)-lineatin treatment, or close to 10 $\mu\text{g}/24$ hr. This release rate may have been sufficiently high in the forest environments where these experiments were carried out (Lindgren et al., 1983).

The positive regression of ambrosia beetle catches as a function of trap length may simply reflect the greater surface area available for capture. Alternatively, the beetles may perceive a long, vertical silhouette as representative of

a large host tree, resistant to drying out, and thus a more suitable habitat for the ambrosia beetle than a small tree. The lack of any effect of trap length on the sex ratio of the captured beetles indicates that both sexes respond in a similar fashion to the aggregation pheromone and to visual stimuli.

Our results are consistent with the ecology of ambrosia beetles, which can occupy their three-dimensional host at very high densities (Borden, 1988). Only beyond extreme population densities would it be advantageous for females to find a new host, rather than boring into an already heavily attacked host. Male beetles searching for mates would be attracted to the pheromone even at high concentrations.

In Europe, Bakke (1983) found that increasing ethanol–lineatin ratios resulted in greater proportions of females in the sample. However, European populations of *T. lineatum* appear to be more dependent on host volatile cues than western North American populations (Borden et al., 1982). The host volatiles α -pinene and ethanol were shown to have no effect for western North American *T. lineatum* by Salom and McLean (1988), while Shore and Lindgren (1996) found that α -pinene increased catches, and ethanol altered sex ratios. Thus, it is not certain that the addition of host volatiles in our experiments would have led to altered sex ratios. The role of host volatiles needs to be investigated further, particularly as it relates to reproductive isolation among the sympatric *Trypodendron* species found in interior British Columbia.

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