ROLE OF FUNGAL PATHOGEN *Ophiostoma novo-ulmi* IN SEMIOCHEMICAL-MEDIATED HOST SELECTION BY THE NATIVE ELM BARK BEETLE, *Hylurgopinus rufipes* (COLEOPTERA: SCOLYTIDAE)

by

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Role of fungal pathogen *Ophiostoma novo-ulmi* in semiochemical-mediated host selection by the native elm bark beetle, *Hylurgopinus rufipes* (Coleoptera: Scolytidae)

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ABSTRACT

Dutch elm disease (DED) is caused by the fungal pathogen *Ophiostoma novo-ulmi* which is transmitted by the native elm bark beetle, *Hylurgopinus rufipes*. We have found that four semiochemicals [(−)-β-pinene, (−)-α-cubebene, (+)-spiroaxa-5,7-diene and (+)-δ-cadinene] from diseased American elms, *Ulmus americana*, synergistically attract *H. rufipes*, and that their emission is up-regulated in elm trees inoculated with *O. novo-ulmi*. The fungus thus manipulates host trees to enhance their apparency to foraging beetles, a strategy that increases the probability of transportation of the pathogen to new hosts.

DEDICATION

This thesis is dedicated to the memory of my grandfather John Neville Webb who passed away May 15th, 2003, shortly after I began my research. Not a day passed that he didn't cross my mind. He may be gone but is certainly not forgotten.

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INTRODUCTION

1.1 Plant-Insect-Pathogen Interactions

Insect-associated pathogens are common, but their role in the life history of insects can be quite variable. Woodwasps or horntails (Sircidae) are phytophagous Hymenoptera that infest various conifer and angiosperm tree species around the world. Development of woodwasp larvae is highly dependent on *Amylostereum* and *Stereum* basidiomycete fungi that transform host tree tissue into readily assimilable forms (Madden, 1988). Without such transformation, woodwasp larvae could not feed, grow and develop in host tissue. Female sircid larvae acquire and store the arthrospores of the fungus within abdominal hypopleural organs. These arthrospores are re-acquired following each molt, ultimately ending up lodged and nurtured within paired intersegmental, mycangial sacs at the base of ovipositors in adult females (Madden, 1988). Inoculation of host trees during oviposition then ensures that hosts will be suitable for development of sircid larvae

The most familiar plant-insect-pathogen relationships are those of various bark beetle species and associated ascomycete fungi. The evolutionary success of bark beetles can be attributed, in part, to their symbiotic relationship with fungi (Raffa, 1988). There are four theories as to how bark beetles benefit through a association with fungi: Fungi introduced by bark beetles into host trees (1) aid in overcoming host resistance (Whitney, 1982); (2) play a role in larval nutrition (Hubbard, 1897, Kok, 1979, Barras, 1973); (3) debilitate trees and render them susceptible to further attack (Castello, 1976), and; (4) modify the semiochemical profile emanating from trees (Borden, 1989).

1.2 Biology and Ecology of *Hylurgopinus rufipes*

1.2.1 Distribution and Classification

The native elm bark beetle, *Hylurgopinus rufipes,* is the primary vector of Dutch elm disease (DED) in the Prairie Provinces (Ives & Wong 1988; Westwood 1991). In Canada, the range of *H. rufipes* closely approximates the natural range of *Ulmus americana* (Bright, 1976), reaching as far west as Manitoba (Wood, 1982) and Saskatchewan (Ives & Wong, 1988). Because of elm reforestation programs in western Canada this range may well extend further west (McIntosh, pers. comm., 2003).

Adult *H. rufipes* are small (~ 2.5 x 1.1 mm), cylindrical, brownish-black insects with clubbed, elbowed antennae and short stiff yellow hairs covering the body. Unlike females, males have stridulatory scrapers on abdominal tergite 8. Tergite 8 of females is completely covered by tergite 7. The larvae are legless, wrinkled, subcylindrical, and curved with a convex dorsum (Kaston, 1936). The body is creamy white and soft, capable of significant contraction and expansion as well as a change in curvature. Fully-grown larvae are ~ 3.5 - 4 x ~1.4 mm, and pupae are ~ 3 x 1.5 mm in size. Pigmentation and sclerotization continue up to and after emergence of adults.

Ulmus spp. are the preferred hosts of *H. rufipes*, but the beetles will occasionally colonize *Fraxinus, Prunus* and *Tilia* spp. (Kaston, 1936). Infestations in these alternate or occasional hosts may be due to a lack of elms during periods of high beetle population densities.

1.2.2 Life History

Overwintering adult beetles emerge from the base of elm trees in late April or May to fly in search of living elm trees, recently cut logs, upper crown and twig crotches of moribund trees or other broodwood, where they feed before breeding. To initiate breeding, females create entrance holes, typically under bark scales or in crevices, with tunnels penetrating directly into the cambial layer of the host elm. Male courtship of females occurs around gallery sites (Swedenborg, 1988). While aggregation to host trees may be host-mediated (Millar, 1986), there is evidence suggesting that females produce a short-range pheromone to guide males to female galleries (Lanier, 1982). Male-produced stridulatory signals exhibited in pre-mating behaviour may help affect mate-choice by females (Swedenborg, 1988).

Females enter the bark to form a biramous brood chamber perpendicular to the grain of the wood (Agrios, 1988). Eggs are laid within the gallery on both sides of the walls. Larvae hatch and feed on the cambium perpendicular to the main gallery, usually following the grain of the wood. Larvae develop through five to six instars during 40-50 days. Larval development may be complete within 29 days if conditions are favourable (Bright, 1976). Pupation occurs at the ends of the larval mines and lasts 8-12 days. Adults emerge in September and fly to healthy tree canopies to feed prior to overwintering. Following this feeding period, the beetles fly or crawl to the base of host trees where they bore into the lower 1 m of the tree trunk to overwinter.

1.3 Biology and Ecology of *Ophiostoma novo-ulmi*

1.3.1 Introduction and Evolution of *Ophiostoma novo-ulmi*

Plant disease epidemics resulting from the introduction of exotic fungal pathogens are well known (Brasier, 2001). Introduction of aggressive pathogens to hosts with limited resistance can result in explosive outbreaks of disease. In their endemic locations, plant pathogens tend to be subject to routine selection constraints, favouring the maintenance of a relatively stable population dynamic over time. Introduction of a plant pathogen to a new environment will often result in novel selection, reflecting sudden exposure to new biotic and abiotic influences. These may include new host populations, vectors, or competitors, or a different climate (Brasier, 2001).

The first recorded incidence of DED caused by *Ophiostoma ulmi* (Ascomycetes) occurred in northwest Europe around 1910 (Brasier, 2001). The disease rapidly spread east into southwest Asia and eventually into the United Kingdom and North America in around 1927. That DED had spread to North America was first noted in Ohio because wooden crates were made with infected elm wood. During the 1940's in Europe, the first epidemic unexpectedly declined after 10 - 40% of elms were lost in most European countries (Peace, 1960). This decline may have resulted from the spread of deleterious viruses in the *O. ulmi* population (Mitchel, 1994). A similar decline did not occur in North America, and elms have been virtually eliminated in most of their range.

A second outbreak of DED occurred in Britain in the 1960's - 70's. This outbreak was caused by the previously unknown *Ophiostoma novo-ulmi.* Intensive surveying of the northern hemisphere indicated that this second outbreak actually began in the 1940's in two different regions: the Moldova-Ukraine region (EAN pathogen form) and the

southern Great Lakes area in North America (NAN pathogen form) (Brasier, 1999). *Ophiostoma novo-ulmi* is characteristically very aggressive. In regions where the two pathogen strains overlap, *O. novo-ulmi* rapidly replaces *O. ulmi* at a rate of about 10% per annum (Brasier, 1986). The steady spread of the North American form of *O. novo-ulmi* in the 1940's destroyed over half the remaining elm trees in eastern Canada and the US. By the 1970's to 1980's, *O. novo-ulmi* had reached both coasts of North America, leaving only 34 million elm trees (Hubbes, 1999). In Canada, DED has continued to spread westward following the natural range of the native elm, killing elms throughout Ontario, Manitoba and Saskatchewan. The first documented case of DED in Saskatchewan was reported in 1981 when a single diseased American elm was discovered in the City of Regina. Efforts to keep the disease in check in the province of Saskatchewan have been successful. To date, a single case reported in the province of Alberta is suspected to be from transport of firewood, not from natural movement of *H. rufipes* carrying the pathogen (McIntosh, pers comm., 2003).

1.3.2 Biology and Classification of *Ophiostoma novo-ulmi*

The fungus *O. novo-ulmi* is the pathogen causing wilt disease and death in elm trees. *Ophiostoma novo-ulmi* lives in xylem vessels of *O. novo-ulmi* infected elms, as well as in the wood and bark of trees already killed by the disease. When beetles carrying fungal spores select, and bore into standing elm trees or logs, they deposit fungal spores along the breeding gallery walls. Once deposited, the spores germinate and grow into the xylem tissue and produce short hyphal branches.

Ophiostoma novo-ulmi is a heterothallic fungal species that requires two sexually compatible mating types for sexual reproduction to occur (Agrios, 1988). Frequently only one mating type is found in large areas in nature; therefore, sexual reproduction is rare (Brasier, 2001). When sexual compatibility is present and sexual reproduction has occurred, perithecia develop (Agrios 1988). These perithecia are black with a globose base and long neck terminating at an ostiole. Within the bodies of perithecia, many asci develop, each housing eight ascospores. The asci disintegrate as they mature, leaving the ascospores free in the perithecial cavity. These are then exuded through the neck, accumulating as a sticky droplet at the ostiole. Because sexually compatible strains are geographically isolated, reproduction usually occurs asexually (Agrios, 1988). Hyphae group together in erect dark solid stalks known as coremia or synnemata. Conidia form droplets at the ends of these stalks. Within the brood galleries, hyphae are actively growing on the nutrient rich phloem tissue. As *O. novo-ulmi* is developing, so too are beetle larvae feeding in the galleries. When mature adult beetles exit the tree to disperse, they walk through a mat of synnemata covered with sticky fungal spores. Through the beetle vectors, the spores are dispersed to new host trees.

Ophiostoma novo-ulmi can also be transferred from one tree to another through root grafts. This is only possible between elm trees that have root contact. In the absence of a vector, this is the only means of dispersal of the pathogen. If the population of elms is clustered or geographically isolated, dispersal of the pathogen is therefore limited. Thus, beetle vectoring is important for distribution of *O. novo-ulmi,* but the presence of the pathogen is not essential to the existence of *H. rufipes.*

Ophiostoma novo-ulmi kills elm trees by closing off the vascular tissue and restricting water flow to the upper portions of the tree. Wilt disease ensues and eventually kills off infected portions of the trees. Fungal spores are able to pass through the rest of the tree, eventually killing it. Sometimes, however, elms compartmentalize the disease by closing off vascular tissue and restricting movement of the pathogen (tyloses) (Agrios, 1988; Ouellette, 1979).

1.4 Research Objectives

Pioneering work done by Millar et al. (1986) suggested that elm bolts infected with *O. novo-ulmi* are significantly more attractive to *H. rufipes* than healthy elm bolts. The objectives of my research were to: (1) identify the semiochemicals from *O. novo-ulmi* infected elms that mediate attraction of *H. rufipes*; and (2) determine whether these semiochemicals are produced by *O. novo-ulmi* or the host tree.

II
IDENTIFICATION OF SEMIOCHEMICALS FROM MORIBUND ELMS THAT MEDIATE ATTRACTION OF *Hylurgopinus rufipes*

2.1 Introduction

Host location by bark beetles is paramount to their survival. Bark beetles select a particular host tree species based upon preferences for mating and oviposition sites. Larvae of the developing brood are likely to become conditioned to the host in which they developed (Baker, 1971). Larval mortality may also be lowest in the appropriate host tree species, allowing beetles that have completed development in such trees to locate new hosts and pass along their genes.

Anti-herbivore chemicals produced by host plants have played a significant role in the co-evolution of beetles and their associated host trees (Sturgeon, 1982). Some insects evolved the ability to overcome the toxic effects of these defence chemicals, and were able to feed in the absence of competition from other herbivorous insects (Sturgeon, 1982). Some toxic plant chemicals became cues to foraging insects enabling them to locate their hosts (Sturgeon, 1982). This may also be true for bark beetles that have evolved the ability to hone in on volatile metabolic by-products of host trees (Harbourne, 1973). For example, monoterpenes form in gymnosperms as metabolic by-products in the production of abscisic and gibberellic acids and carotenoid pigments (Harbourne, 1973), and secondarily may have become host recognition cues for bark beetles, which seem to have learned in evolutionary times to detoxify monoterpenes. As different species of bark beetles evolved, the selective pressures on the trees possibly led to the development of other chemical defences (Sturgeon, 1982).

The evolutionary responses of bark beetles and their hosts appear reciprocal (Sturgeon, 1982). The beetles influence the chemical composition of their host trees and the trees, in turn, affect the genetic composition and morphology of the beetle population.

Hylurgopinus rufipes being a secondary bark beetle cues in on dead and dying elm trees (Allen, 2001). Millar et al. (1986) demonstrated that elm bolts infected with *O. novo-ulmi* were significantly more attractive to male and female *H. rufipes* than were healthy elm bolts.

My objectives were to: (1) obtain behavioural evidence that *H. rufipes* are attracted to host volatiles derived from *O. novo-ulmi* infected tissue; (2) identify the candidate semiochemical(s); and (3) demonstrate behavioural response of beetles to the candidate semiochemical(s) in laboratory and field experiments.

2.2 Methodology

2.2.1 Collection of Host Tree Materials and Volatiles

Tissue samples from trees with symptoms of DED in and near the City of Regina were taken from June to August 2002. Considering that *H. rufipes* utilizes different niches within elm trees, tissue samples were taken from leaves, twig branches, upper mid and lower crown, as well as the lower bole of trees. Under permit of the Canadian Food Inspection Agency (CFIA), tissue samples were shipped to the CFIA-authorized Global Forest quarantine facility at Simon Fraser University (SFU), and stored at 5°C. To initiate collection of semiochemicals, tissue was ground into sawdust (2.72 kg) and placed into a Pyrex glass aeration chamber (15 cm in diameter x 20 cm in length). Charcoal filtered air (1L/m) was drawn through the chamber and a glass tube (14 mm OD

x 200 mm) downwind containing Porapak Q. Volatiles were adsorbed on Porapak Q for 96 hours and desorbed with 2 ml of redistilled pentane.

Porapak Q volatile extracts were analyzed by gas chromatography to determine potential commonalities among samples. Sample extracts were then combined for analyses and behavioural bioassays.

2.2.2 Collection and Rearing of *Hylurgopinus rufipes*

Beetle-infested logs were collected at two different times. On 30 April 2003, elms in the Silica Sands forest east of Hudson Bay (Saskatchewan) were visually inspected for evidence of beetle-derived boring dust indicating the presence of overwintering beetles. Such trees were felled, the bark and portions of the wood from the lowest 1 m trunk sections were shaved off and placed into Rubbermaid® containers (61 x 40 x 42cm) with moist towels. Containers were sealed for transport and shipped to SFU's Global Forest quarantine facility.

To initiate emergence of beetles, bark/wood tissue was transferred from storage at 5°C to a rearing room in the quarantine facility. The temperature within the rearing room ranged between 22-30°C with a photoperiod of 16L:8D. Emergent beetles were collected daily and separated by sex (Kaston, 1936). Beetles were also observed to re-enter the bark/wood tissue to initiate gallery systems.

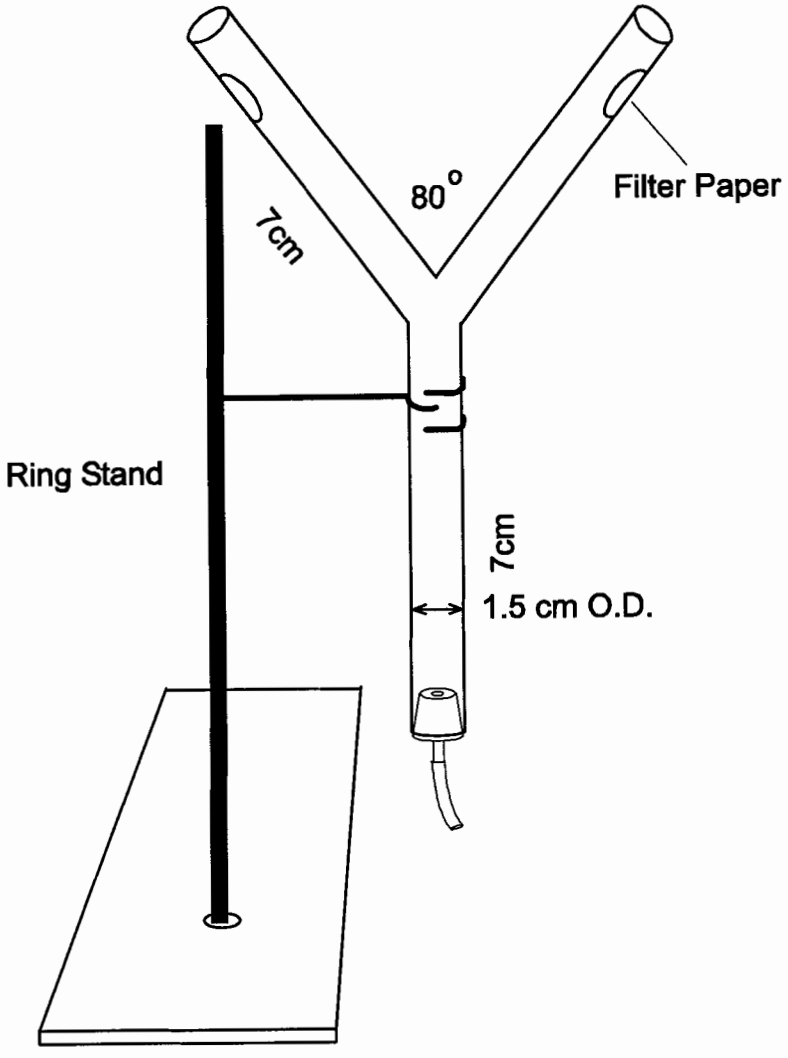
The second collection of beetles occurred on 25 June 2003. In the Silica Sands elm forest, smaller-diameter trees were examined for evidence of brood galleries by removing small pieces of bark tissue. Trees with galleries were felled, cut into small pieces, and placed in Rubbermaid® containers with moistened towels for transport. At

SFU, the cut ends of log sections were waxed and the logs stored at 5°C. With insects in either the larval or pupal stage, emergence of beetles took between 3 – 6 weeks once the logs were transferred into a warm rearing room (see above).

2.2.3 Laboratory Bioassays

Response of *H. rufipes* to aliquots of Porapak Q extract of diseased elm wood volatiles was tested in Y-tube olfactometer (Delury, 1999) (Figure 1) at 22-26°C and 40-44% relative humidity. To standardize visual cues, the olfactometer was enclosed on three sides with white poster board. The olfactometer was illuminated by two overhead light tubes (fluorescent GE Plant and Aquarium F40PL/AQ Wide Spectrum and Sylvania Daylight Deluxe F40DX 40W). Treatment and control odour sources were micro-pipetted onto Whatman No. 2 filter paper (13.5 mm diameter) assigned near the orifice of side arms (Figure 1). For each replicate, a new male or female beetle, a clean (Sparkleen-washed and oven-dried) Y-tube, and new filter papers were used, with test stimuli assigned randomly to one of the side arms. Air was drawn through the olfactometer at a rate of 1 litre/min with a water-driven aspirator. Volatile-laden air from odour sources was drawn down through the stem of the Y-tube. Thirty seconds after placement of stimuli, a beetle was released into the entrance of the olfactometer. Beetles walking up wind that reached a filter paper emanating host-derived odour or pentane as the control stimulus were classed as responders. Beetles that failed to reach filter paper within 5 minutes after initiation of the replicate were classed as non-responders, and were not included in statistical analyses.

Figure 1. Pyrex glass Y-tube olfactometer used for testing behavioural response of *Hylurgopinus rufipes* to test stimuli.


80°
Filter Paper
7cm
Ring Stand
7cm
1.5 cm O.D.

Experiment 1 tested the response of beetles to aliquots (130 gram-hour-equivalents (GHE)) of Porapak Q volatile extracts from diseased elm wood. Experiment 2 tested the beetles' response to a synthetic blend (SB) (130 GHE) of four candidate semiochemicals [(−)-β-pinene, (−)-α-cubebene, (+)-spiroaxa-5,7-diene, (+)-δ-cadinene] at a blend ratio of 20:1.5:1.25 :175, as found in natural volatiles from diseased elm wood. Experiments 3-6 explored the role of individual components in the 4-component synthetic blend (4CSB) by testing the beetles response to SB's (130 GHE) lacking (+)-spiroaxa-5,7-diene (experiment 3), (−)-β-pinene (experiment 4), (−)-α-cubebene (experiment 5), or (+)-δ-cadinene (experiment 6). Experiment 7 explored whether a 10-fold lower concentration (13 GHE) of SB would still be attractive, whereas experiments 8 and 9 explored the importance of blend ratio by testing SB at the nonnatural ratio of 1:1:1:1 at 1 GHE (experiment 8) and 7 GHE (experiment 9)

Numbers of beetles responding to stimuli in olfactometer bioassays were analyzed with the χ^2 goodness of fit test (Zar, 1999) using JMP 5.1 statistical package.

2.2.4 Analysis of Volatiles by GC-EAD, GC-MS, HPLC and NMR

Aliquots of Porapak volatile extracts were analyzed by coupled gas chromatographic-electroantennographic detection (GC-EAD) (Arn et al., 1975; Gries, 1995; Gries et al., 2002). For GC-EAD recordings[1], an insect antenna was removed and the base inserted into the tip of a glass capillary filled with a saline solution (Staddon &

[1] GC-EAD analyses were conducted by Regine Gries

Everton, 1980). The club of the antenna was pierced with a sharply-pointed open tip of a second capillary also filled with saline. Volatiles that elicited responses from male or female antennae were analysed by GC-mass spectrometry (MS), employing a Varian Saturn 2000 Ion Trap GC-MS fitted with a DB-5 column (30 m x 0.32 mm ID; J&W Scientific, Folsom, Ca., USA).

Nuclear Magnetic Resonance (NMR) spectra of volatiles were taken with a Varian AS 500 (at 499.77MHz) for ^{1}H spectra. ^{1}H shifts are reported as parts per million [ppm relative to TMS (0.00ppm)].

High performance liquid chromatography (HPLC) of samples employed a Waters LC626 and a Waters 486 variable wavelength UV/visible detector set to 210 nm, HP Chemstation software (Rev.A.07.01), and a reverse phase Nova-Pak C18 column (60 Å, 4 μm, 3.9 x 300 mm).

2.2.5 Acquisition of Candidate Semiochemicals

(–)-α-Cubebene and (–)-β-pinene were purchased (Fluka Chemika-Biochemika, Buchs, Switzerland CH-9470; Sigman-Alrdich Oakville Ont L6H 2J8). (+)-Spiroaxa-5,7-diene (Polovinka, 2000)was formed as a minor product by palladium-catalyzed rearrangement of (–)-α-cubebene during hydrogenation. Reduced palladium (5% on barium sulfate, 200 mg) was added to a solution of (–)-α-cubebene (30 mg) in 10 mL of pentane. While stirring, hydrogen was bubbled through the suspension. The reaction was monitored by GC analysis of aliquots and terminated after 3-6 min when the yield of (+)-spiroaxa-5,7-diene reached its maximum (approximately 3%). (+)-Spiroaxa-5,7-diene was isolated from the mixture by high-performance liquid chromatography (HPLC) on a

reverse-phase Nova Pak® C_{18} column (3.9 x 300 mm) eluted with acetonitrile (1 mL/min). Elution with 88% aq. acetonitrile afforded (+)-δ-cadinene (von Reuβ et al. 2004) which was produced by heating a solution of (–)-α-cubebene (20 mg) in 1,4-dioxane (1 mL) in the presence of 0.2 mL 0.1 M HCl (50°C / 2-4 hrs) and extraction with pentane (Ohta, et al. 1968).

To determine the molecular structure and absolute configuration of (+)-spiroaxa-5,7-diene, the dextro- and levorotatory enantiomers were synthesized[2] by TiO_2/SO_4^{2-}–catalyzed rearrangement (Polovinka, 2000) of (–)-*ent*-aromadendrene [a sesquiterpene derived from (–)-*ent*-bicyclogermacrene which was isolated from the liverwort *Mylia taylorii* (von Reuβ et al. 2004)] and (+)-aromadendrene, respectively, and analyzed by enantioselective GC on an octakis-(2,6-di-*O*-methyl-3-*O*-pentyl)-β-cyclodextrin column which separated them with complete baseline resolution. The synthetic (+)-(1*S*,2*R*)-spiroaxa-5,7-diene had the same retention time as (+)-spiroaxa-5,7-diene of the elm wood volatiles, and elicited in GC-EAD analyses stronger antennal responses than its antipode (data not shown), thereby substantiating the (1*S*,2*R*)-configuration of (+)-spiroaxa-5,7-diene.

2.2.6 Field Testing of Candidate Semiochemicals

Adhesive cardboard traps (45 x 67 cm) (Phero Tech Inc., Delta, BC, V4G 1E9, Canada) were suspended between poles at a height of ~2 m and spacings of 20-25 m in randomized complete blocks separated by 2-5 km. Trap baits consisted of a piece of

[2] Synthesis was conducted by Stephan von Reuβ

dental cotton roll (10 x 15 mm) (Richmond Dental, Charlotte, NC 28234, USA) that was impregnated with (–)-α-cubebene, (+)-spiroaxa-5,7-diene and (+)-δ-cadinene, and affixed to a 400-μL polyethylene microcentrifuge tube containing a 5-μL capillary tube filled with (–)-β-pinene (Sigma-Aldrich Canada Ltd., Oakville Ont. L6H 2J8). Release rates of (–)-β-pinene, (–)-α-pinene, (+)–spiroaxa-5,7-diene and (+)–δ-cadinene were, respectively, 25, 2, 2 and 184 μg/24 h, approximating the ratio found in diseased elm wood. Numbers of *H. rufipes* captured in traps were recorded 24 h after trap placement. Lures were replaced, and traps and their locations were re-randomized within blocks.

2.3 Results

2.3.1 Identification of Semiochemicals in Bioactive Elm Volatile Extracts

Porapak Q volatile extracts of diseased elm wood tissue attracted male and female *H. rufipes* in Y-tube olfactometer (Figure 2, Experiment 1).

GC-EAD analyses of aliquots of this bioactive sample revealed four components that strongly and consistently elicited responses from male and female antennae (Figure 3). GC-mass spectra and retention indices of compounds **1** and **2** (Figures 4, 5; Table 1) suggested and GC-MS of synthetic standards confirmed that they were β-pinene and α-cubebene. The mass spectrum and retention index of compound **4** (Figure 6, Table 1) resembled those of γ- or δ-cadinene. NMR data of HPLC-isolated compound **4** (100 μg) [^{1}H (500MHz, C_6D_6): δ = 0.85 (d, 3 H), 0.99 (d, 3 H), 1.20 – 1.36 (m, 3 H) 1.59-1.65 (m, 2 H), 1.70 (s, 3 H), 1.72 (s, 3 H), 1.92-2.16 (m, 4 H), 2.69 (s [broad], 1 H), 2.75 (m, 1 H), 5.64 (s, 1 H)] were consistent only with those reported for δ-cadinene. The mass spectrum and retention index of trace compound **3** (Figure 7, Table 1) were indicative of

another sesquiterpene. To find a source containing compound **3** in quantities sufficient for isolation, 165 essential oils were screened by GC-MS. Compound **3** was found in cubeb oil (Liberty Natural Products Inc, Portland, OR 97215), and isolated by HPLC to obtain its NMR spectrum [^{1}H (500 MHz, C_6D_6): δ = 0.89 (d, 3 H), 0.99 (d, 6 H), 1.40 (m, 1 H), 1.56 (m, 2 H), 1.61 (s, 3 H), 1.80 (m, 1 H, [d of sept.?]), 1.88 (t, 2 H), 1.91 (m, 2 H), 2.10-2.30 (m, 2 H), 5.11 (d, 1 H), 5.32 (s, 1 H, [broad])]. These NMR data were consistent with those of spiroaxa-5,7-diene (Polovinka, 2000).

Figure 2. Number of male and female *Hylurgopinus rufipes* responding in Y-tube olfactometers to Porapak Q extract of volatiles from ground Dutch elm disease-infested elm wood at 130 gram hour equivalents (GHE) (Experiment 1), or to a synthetic blend (SB) of (–)-β-pinene, (–)-α-cubebene, (+)-spiroaxa-5,7-dinene, and (+)-δ-cadinene at 130 GHE (Experiment 2). Number of insects responding to each stimulus given in bars, number of insects tested given in parenthesis. For each experiment an asterisks (*) indicates a significant preference for a particular treatment; χ^2 test with Yates correction for continuity, treatment versus control; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

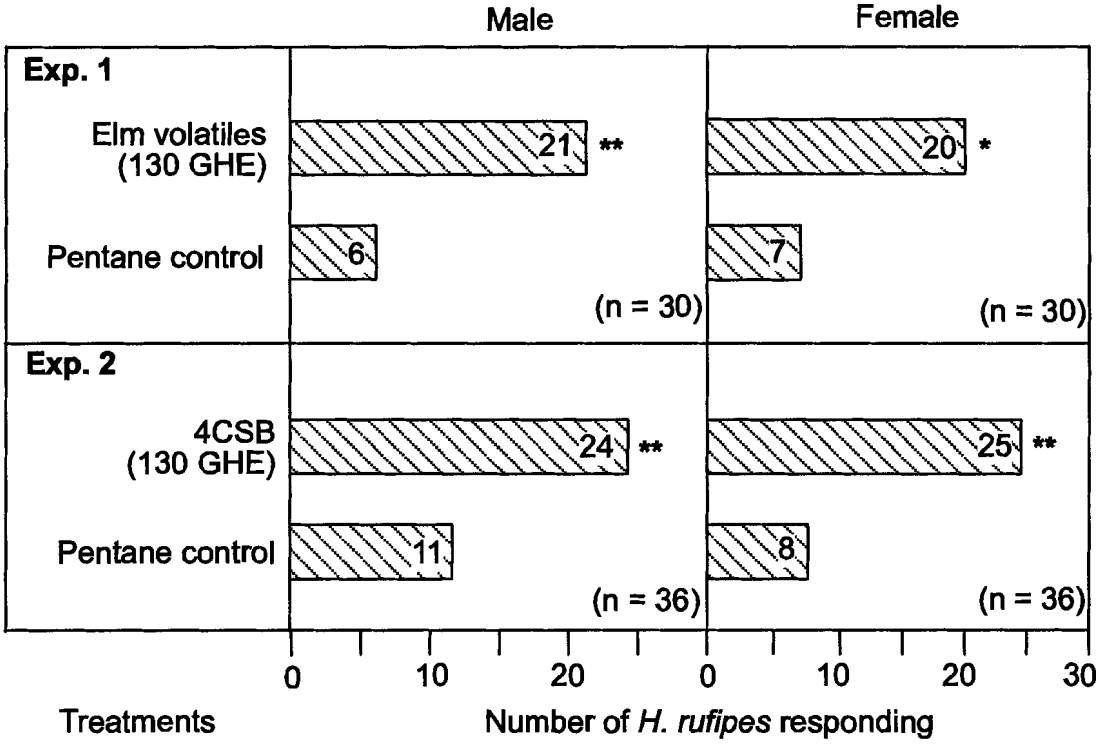

Male
Female
Exp. 1
Elm volatiles (130 GHE)
21
**
20
*
Pentane control
6
7
(n = 30)
(n = 30)
Exp. 2
4CSB (130 GHE)
24
**
25
**
Pentane control
11
8
(n = 36)
(n = 36)
0
10
20
0
10
20
30
Treatments
Number of H. rufipes responding

Figure 3. Gas chromatograms of volatiles (desorbed from Porapak Q) emanating from ground elm wood infected with the fungal pathogen *Ophiostoma novo-ulmi*. Hewlett Packard 5890A gas chromatograph with DB-5 column (30 m x 0.32 mm ID; J&W Scientific, Folsom, CA 95630) and flame ionization (FID) or electroantennographic detector (EAD: male and female *Hylurgopinus rufipes* antenna); splitless injection; temperature program: 50°C (2 min), then 10°C per min to 280°C.

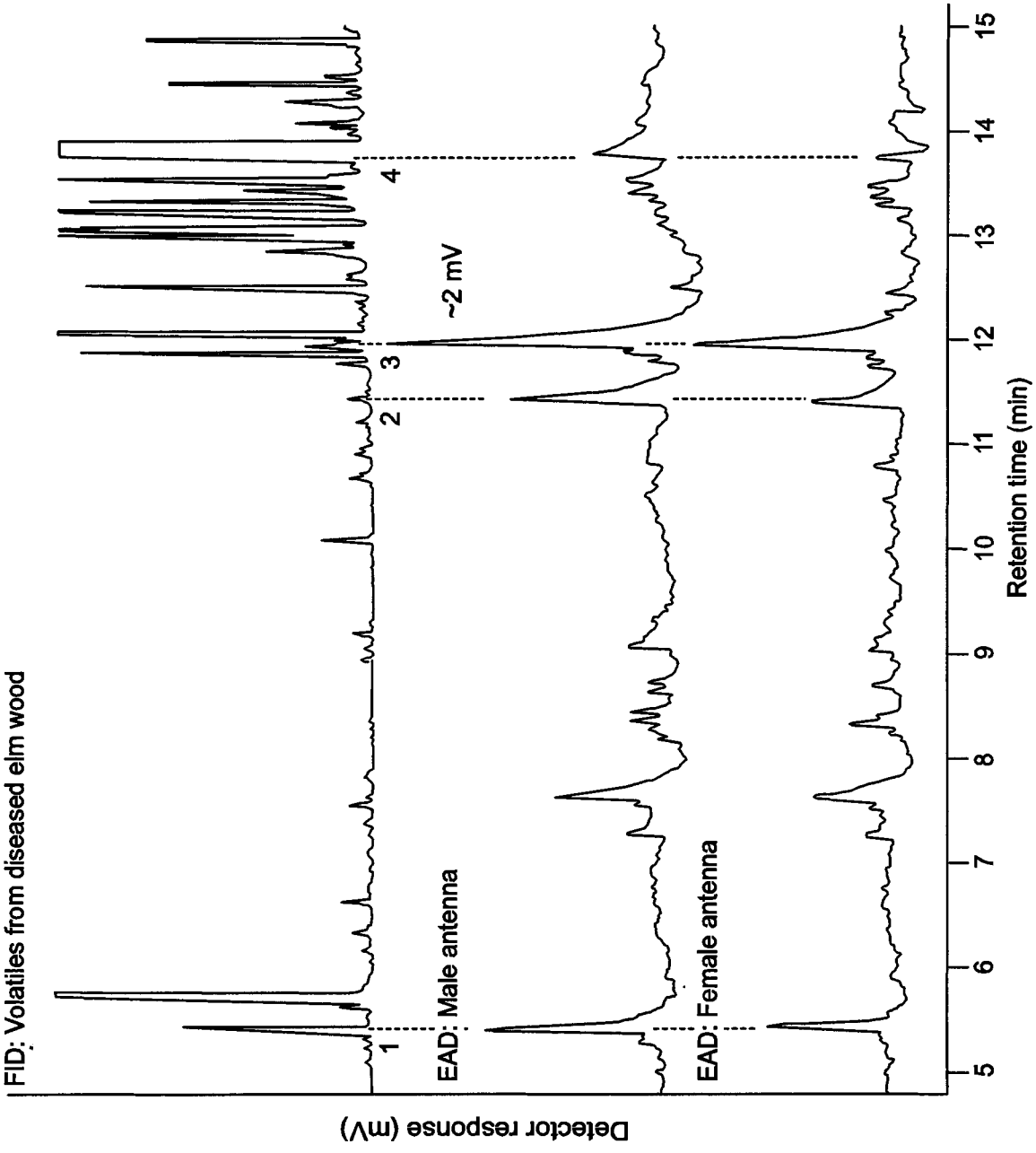

FID: Volatiles from diseased elm wood
EAD: Male antenna
EAD: Female antenna
~2 mV
1
2
3
4
Detector response (mV)
Retention time (min)
5
6
7
8
9
10
11
12
13
14
15

Figure 4. Mass spectrum of EAD-active compound **1** in figure 3 (top) and authentic (–)-β-pinene (bottom). Varian Saturn 200 ion trap GC-mass spectrometer.

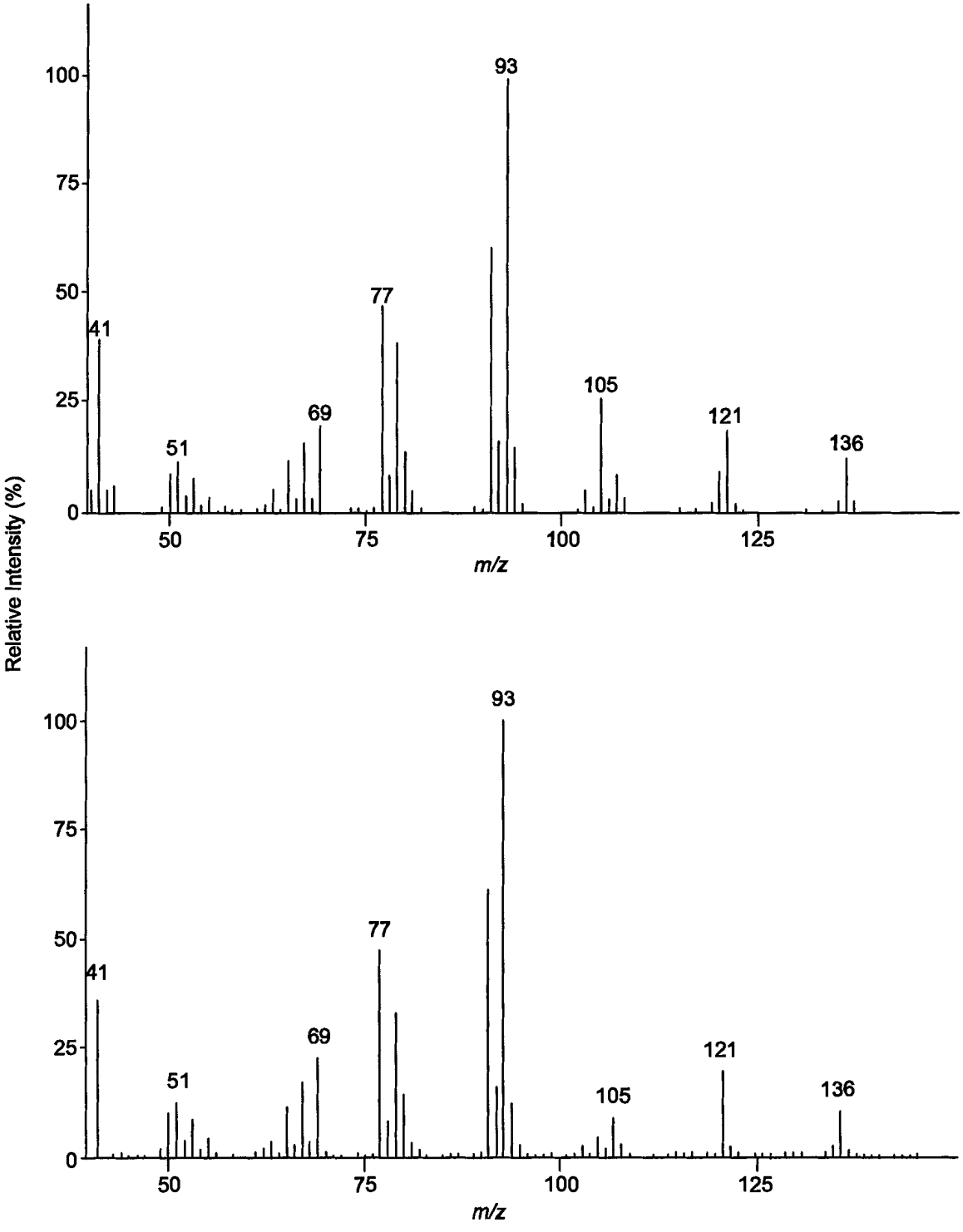
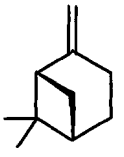
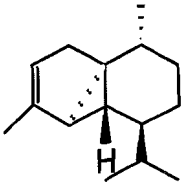
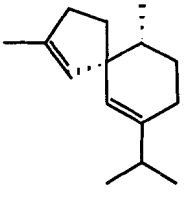
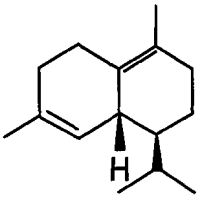

Relative Intensity (%)
100
75
50
25
0
93
77
41
105
69
121
136
51
50
75
100
125
m/z
100
75
50
25
0
93
77
41
69
121
51
105
136
50
75
100
125
m/z

Table 1. Name, structure, retention index purity and source of chemicals identified in volatiles of diseased elm tissue eliciting antennal responses by female and male *H. rufipes*.

Compound	# in Figure 3	Structure	Retention Index (DB-5)	Chemical purity	Source[a]
(−)-β-pinene	1		980	99%	Sigma-Aldrich
(−)-α-cubebene	2		1346	> 97%	Fluka
(+)-spiroaxa-5,7-diene	3		1384	> 95%	Isolated by R. Gries, SFU
(+)-δ-cadinene	4		1570	99%	Isolated by R. Gries, SFU

[a] Sigma-Aldrich Canada Ltd., 2149 Winston Park Drive, Oakville Ont. L6H 2J8
Fluka Chemika-Biochemika, CH-9470 Buchs, Switzerland

Figure 5. Mass spectrum of EAD-active compound **2** in Figure 3 (top) and of authentic (–)-α-cubebene (bottom). Varian Saturn 2000 ion trap GC-mass spectrometer.

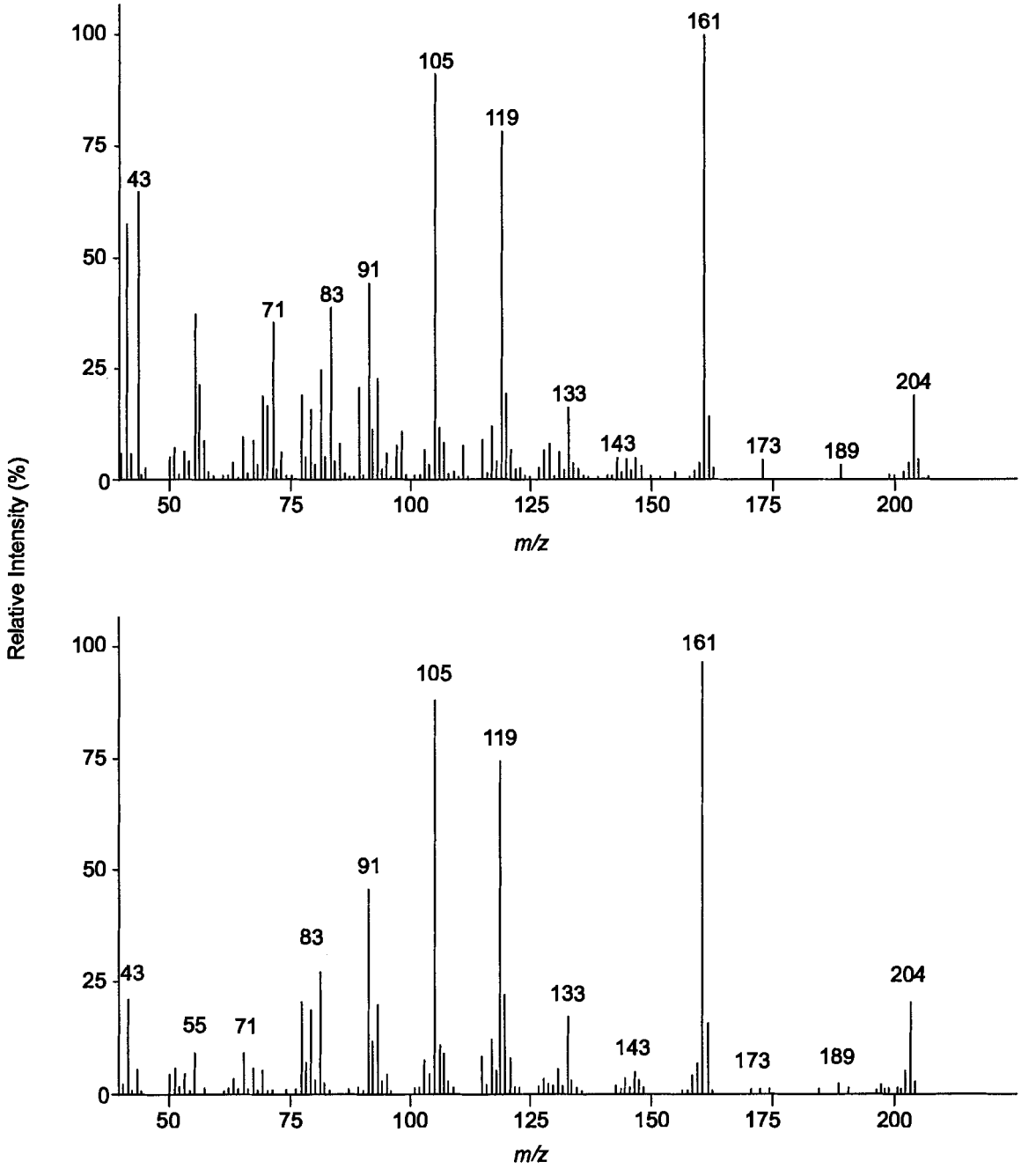

Relative Intensity (%)
100
75
50
25
0
161
105
119
43
91
83
71
133
204
143
173
189
50
75
100
125
150
175
200
m/z
100
75
50
25
0
161
105
119
91
83
43
204
133
55
71
143
173
189
50
75
100
125
150
175
200
m/z

Figure 6. Mass spectrum of EAD-active compound **4** in Figure 3 (top) and of authentic (+)-δ-cadinene (bottom). Varian Saturn 2000 ion trap GC-mass spectrometer.

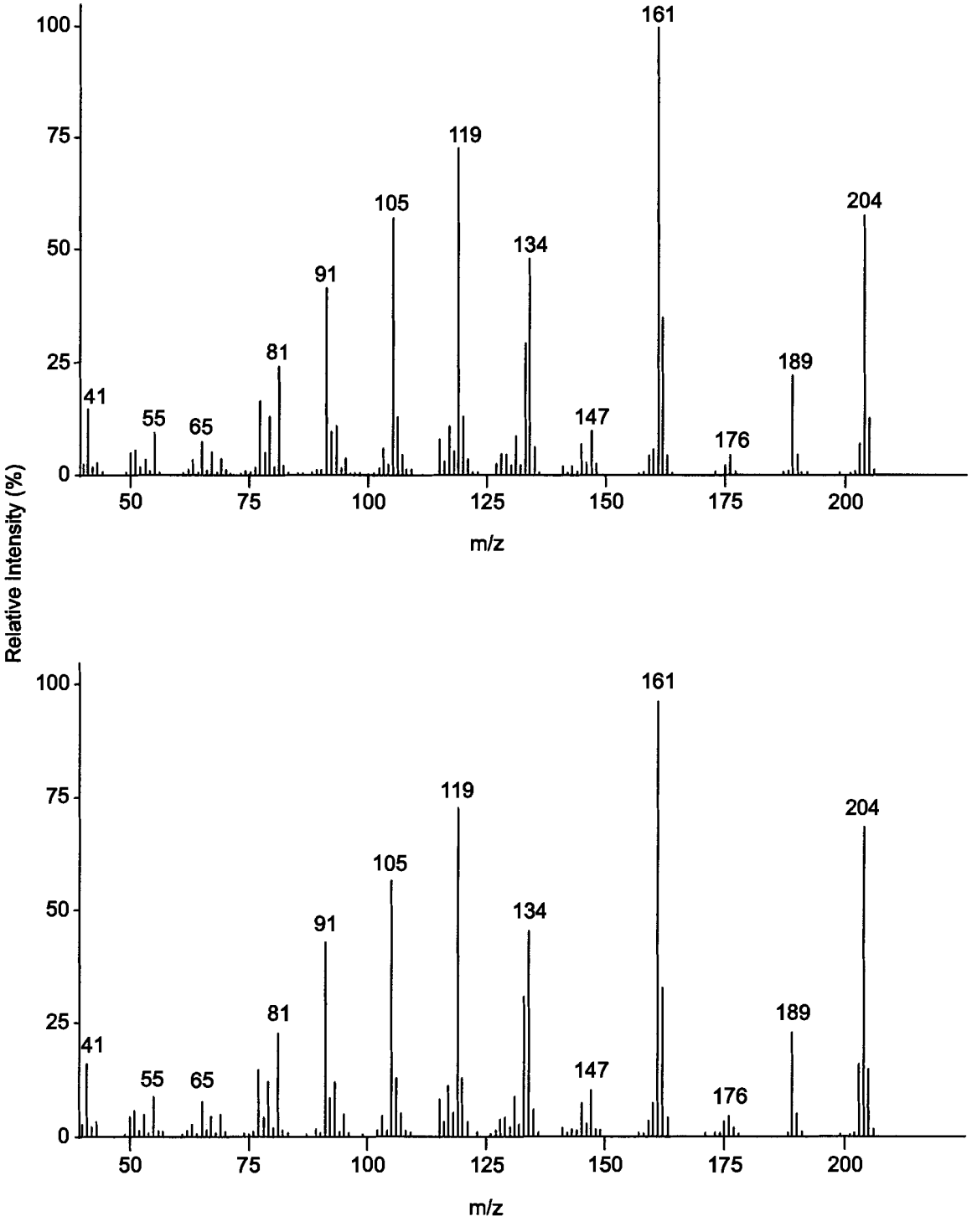
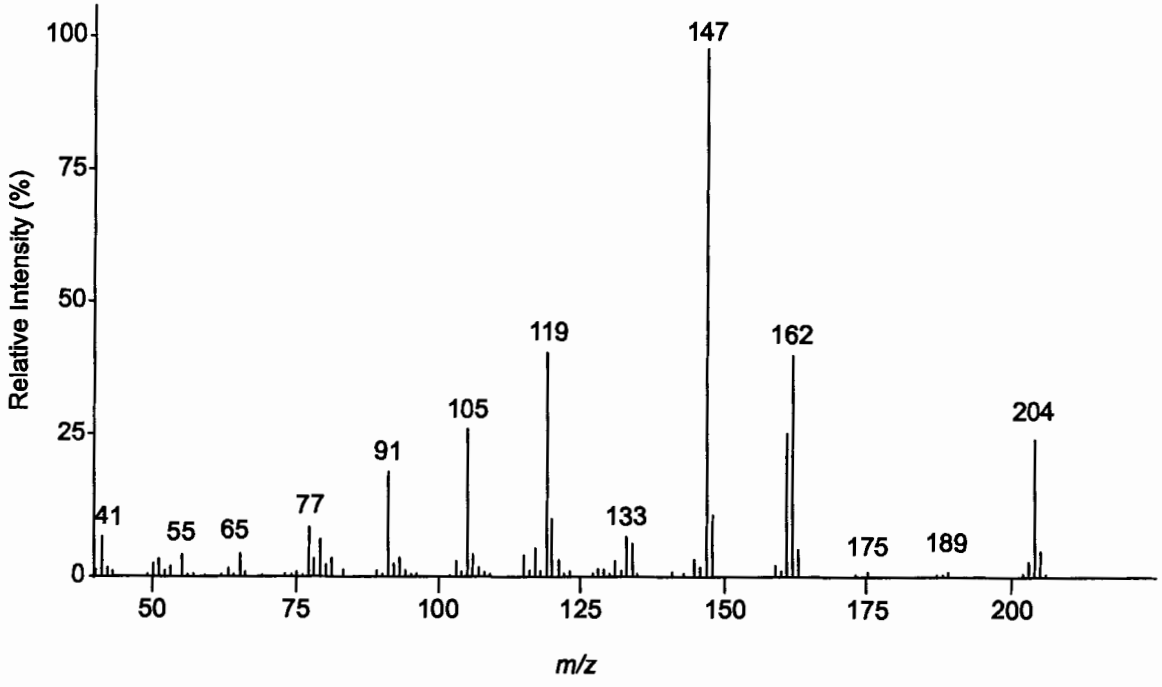

Relative Intensity (%)
100
75
50
25
0
161
119
105
204
134
91
81
189
41
55
65
147
176
50
75
100
125
150
175
200
m/z
100
75
50
25
0
161
119
204
105
134
91
81
189
41
55
65
147
176
50
75
100
125
150
175
200
m/z

Figure 7. Mass spectrum of EAD-active compound **3** in Figure 3 (top). Varian Saturn 2000 ion trap GC- mass spectrometer.


100
75
50
25
0
Relative Intensity (%)
147
119
162
105
204
91
77
41
55
65
133
175
189
50
75
100
125
150
175
200
m/z

2.3.2 Laboratory and Field Experiments with Candidate Semiochemicals

In laboratory experiment 1 elm volatiles (130 GHE) and in experiment 2 the 4-component synthetic blend (SB; 130 GHE) of (–)-β-pinene, (–)-α-cubebene, (+)-spiroaxa-5,7-diene and (+)-δ-cadinene attracted significantly more males and females than did a pentane control stimulus (Figure 2). In experiments 3-6, SB lacking any one of the four components were as unattractive to males as the pentane control stimulus (Figure 8). Similar results were obtained with females except that SB lacking spiroaxa-5,7-diene still attracted females. In experiment 7 (Figure 9), SB at the natural blend ratio but 10-fold lower dose (13 GHE) than in experiment 2 still attracted males and females more strongly than did the pentane control. In experiment 8 and 9, however, SB at an unnatural (1:1:1:1) blend ratio failed to attract beetles, irrespective of the dose (1 and 7 GHE) that was tested (Figure 9).

In experiment 10 conducted in elm forests, traps baited with SB captured 10 times more males and females than did unbaited control traps (Figure 10). In field experiment 11, traps baited with SB again captured 10 times more males and females than unbaited control traps, but all lures lacking any one of the four components were as unattractive as unbaited control traps (Figure 10).

Figure 8. Number of male and female *Hylurgopinus rufipes* responding in Y-tube olfactometers to synthetic blends (SB) (130 gram-hour equivalents) of (–)-β-pinene, (–)-α cubebene, (+)-spiroaxa-5,7-diene, and (+)-δ-cadinene lacking one of the four components. Number of insects responding to each stimulus given in bars, number of insects tested given in parenthesis. For each experiment an asterisks (*) indicates a significant preference for a particular treatment; χ^2 test with Yates correction for continuity, treatment *versus* control; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

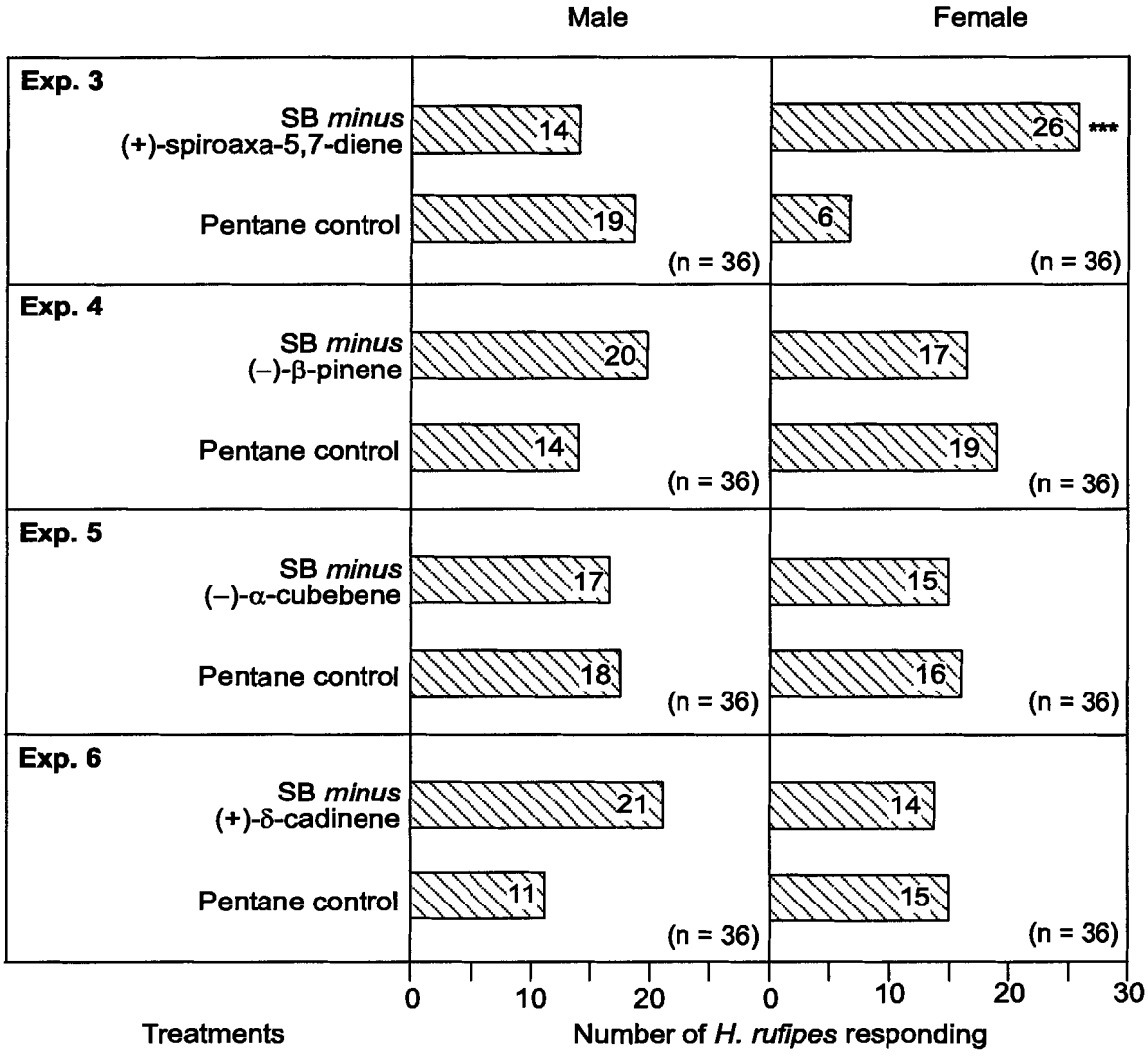

Male
Female
Exp. 3
SB minus (+)-spiroaxa-5,7-diene
14
26
***
Pentane control
19
6
(n = 36)
(n = 36)
Exp. 4
SB minus (–)-β-pinene
20
17
Pentane control
14
19
(n = 36)
(n = 36)
Exp. 5
SB minus (–)-α-cubebene
17
15
Pentane control
18
16
(n = 36)
(n = 36)
Exp. 6
SB minus (+)-δ-cadinene
21
14
Pentane control
11
15
(n = 36)
(n = 36)
0
10
20
0
10
20
30
Treatments
Number of H. rufipes responding

Figure 9. Number of male and female *Hylurgopinus rufipes* responding in Y-tube olfactometers to a synthetic blend (SB) of (–)-β-pinene, (–)-α cubebene, (+)-spiroaxa-5,7-diene, and (+)-δ-cadinene at a natural ratio of components (20:1.5:1.25:175) (Experiment 7), or at an equal ratio of components (1:1:1:1) (Experiments 8, 9). Number of insects responding to each stimulus given in bars, number of insects tested given in parenthesis. For each experiment an asterisks (*) indicates a significant preference for a particular treatment; χ^2 test with Yates correction for continuity, treatment *versus* control; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

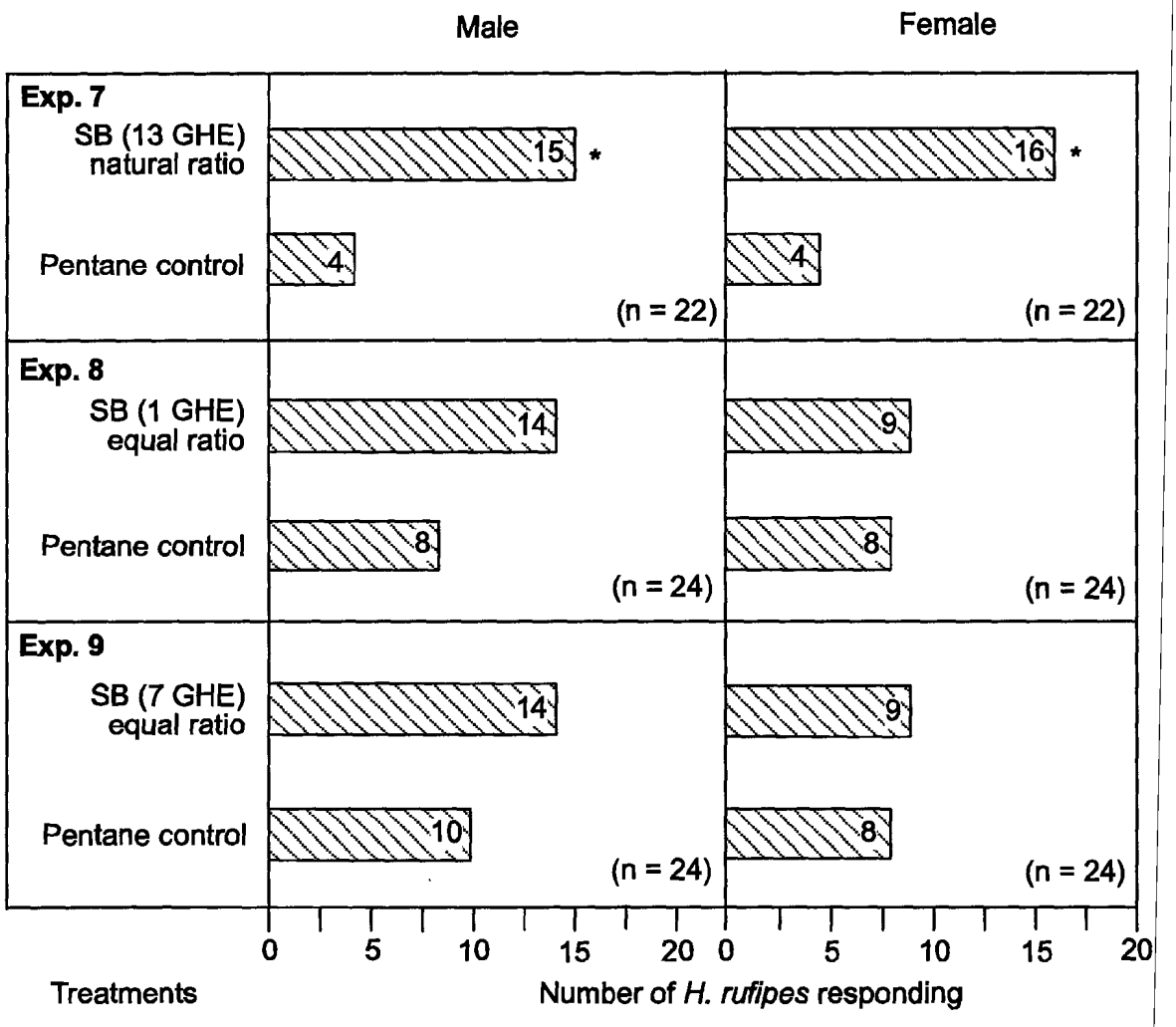
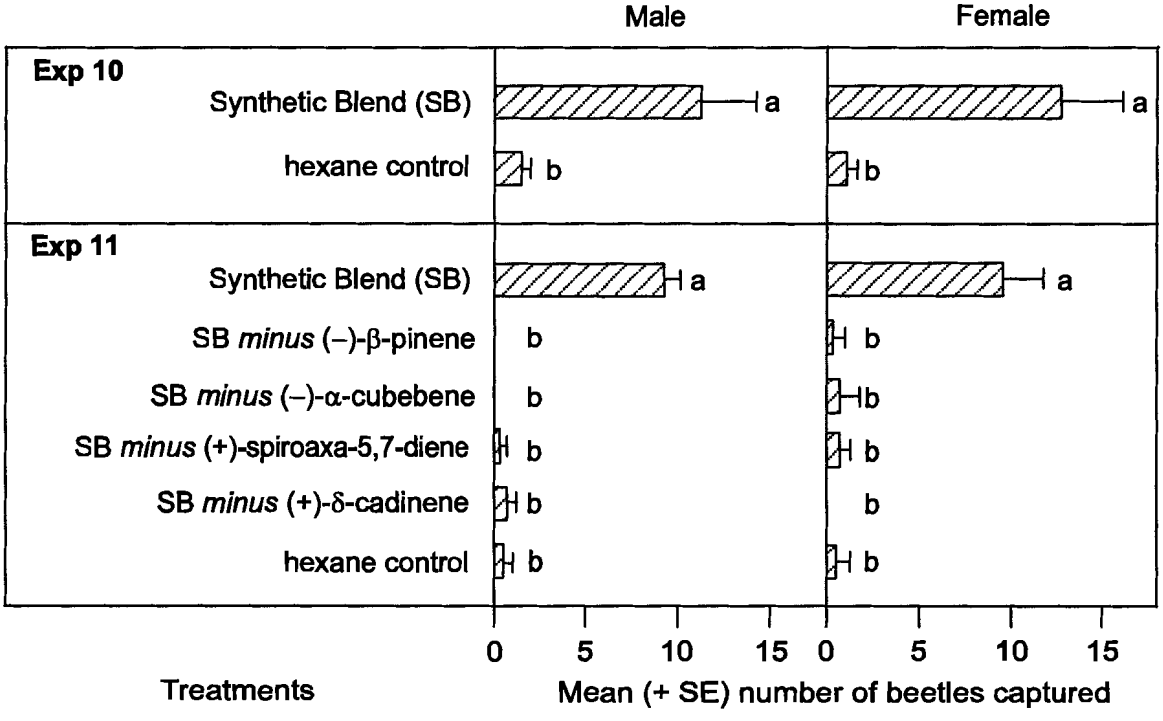

Male
Female
Exp. 7
SB (13 GHE) natural ratio
Pentane control
15
*
4
(n = 22)
16
*
4
(n = 22)
Exp. 8
SB (1 GHE) equal ratio
Pentane control
14
8
(n = 24)
9
8
(n = 24)
Exp. 9
SB (7 GHE) equal ratio
Pentane control
14
10
(n = 24)
9
8
(n = 24)
0 5 10 15 20 0 5 10 15 20
Treatments
Number of H. rufipes responding

Figure 10. Mean (+ SE) number of male and female *Hylurgopinus rufipes* captured in field experiment 10 (2-5 June 2004; 24 replicates) on sticky cardboard traps baited with the synthetic blend (SB) of (–)-β-pinene, (–)-α cubebene, (+)-spiroaxa-5,7-diene, and (+)-δ-cadinene (at release rates of 25, 2, 2, and 184 μg per 24 hr, respectively), or baited with SBs lacking one of the four components. Elm forests near Lumsden, Saskatchewan. In each experiment, bars with different letters are significantly different. ANOVA followed by Tukey-Kramer HSD comparison of means; JMP statistical software; $P < 0.05$.


Male
Female
Exp 10
Synthetic Blend (SB)
hexane control
Exp 11
Synthetic Blend (SB)
SB minus (–)-β-pinene
SB minus (–)-α-cubebene
SB minus (+)-spiroaxa-5,7-diene
SB minus (+)-δ-cadinene
hexane control
a
b
0
5
10
15
Treatments
Mean (+ SE) number of beetles captured

2.4 Discussion

My data are consistant with previous conclusions (Millar et al., 1986) that elm trees infested with *O. novo-ulmi* are strongly attractive to *H. rufipes*. My study also reveals the semiochemicals that are essential for the attraction of beetles. In both laboratory and field experiments, the 4-component blend of (–)-β-pinene, (–)-α-cubebene, (+)-spiroaxa-5,7-diene, and (+)-δ-cadinene strongly attracted males and females (Figure 10). The attractiveness of this semiochemical blend required both the presence and near-natural ratio of all four components. Blends lacking any one of the four components [(–)-β-pinene, (–)-α-cubebene, (+)-spiroaxa-5,7-diene, and (+)-δ-cadinene], or containing them at an unnatural ratio (1:1:1:1), failed to attract beetles in laboratory or field experiments. The fact that females did show significant attraction to blends lacking spiroaxa-5,7-diene in laboratory experiment 3 may be explained by trace amounts of the spiroaxa-5,7-diene that could have been present in synthetic δ-cadinene or α-cubebene. If so, this would suggest that females are more sensitive to this semiochemical than males, and that females are more likely than males to locate host trees.

Primary attractiveness of host trees to foraging bark beetles has been demonstrated in various studies (e.g. Raffa et al, 1993), but the semiochemicals that mediate the primary attractiveness have rarely been identified. A synthetic blend of ethanol and α-pinene attracts several species of ambrosia beetles, including *Trypodendron lineatum*, *Xyleborus ferrungineus*, and *Gnathotrichus sulcatus*, (Moeck, 1970; Kelsey, 1997). Moreover, a blend of α-pinene and pinene oxide attracts the

European pine shoot beetle, *Tomicus piniperda*. Finally, fir engraver beetles, *Scolytus ventralis*, respond to a semiochemical blend of α-pinene, camphene, β-pinene, myrcene, limonene, β-phellandrene, α-terpinoline, α-cubebene, longifolene, bornyl acetate, (-)-borneol, nerolidol, and methyl-isoeugenol (Macias-Samano, 1998).

The 4-semiochemical lure identified in my study shows promise for deployment in programs that monitor *H. rufipes* population densities, and the incidence of DED in the Prairie Provinces. Assuming that the semiochemicals can be commercially produced and formulated, lures may even be deployed in programs aimed at mass trapping *H. rufipes*. Such programs may help sustain elm shelterbelts that protect farm houses from harsh winds and may intercept beetles that might use shelterbelts as corridors to urban centers. The semiochemicals identified in my study may also enhance the attractiveness of trap lures for European elm bark beetles, *Scolytus multistriatus*, and for the exotic banded elm bark beetle, *Scolytus schevyrewi*. Currently α-cubebene, is the only elm-derived semiochemical in commercial lures developed for attraction of *S. multistriatus*. New containing both synthetic *S. multistriatus* pheromone, and the 4-component semiochemical blend identified in my study, may prove more attractive to *S. multistriatus* than the lure concurrently used for monitoring or (mass) trapping *S. multistriatus* populations.

III
RESPONSE TO INOCULATION WITH *Ophiostoma novo-ulmi*

3.1 Introduction

Non-motile parasites that complete one or more stages of their life cycle in intermediate or definitive hosts can manipulate these hosts to optimize transportation to new hosts (Poulin, 2002). For example, protozoan parasites, *Toxoplasma gondii*, cause their intermediate rat hosts to approach and be eaten by cats, the definitive host (Berdoy, 2000). Likewise, the fungal pathogen *Ophiostoma ulmi* kills elm trees (Hubbes, 1999; Brasier, 2001) and then requires transportation to new elms (Agrios, 1988). Since its introduction into the United States in the 1930s, it has ravaged forest and urban American elms across the northeastern United States and Canada. With the appearance in the 1960's - 1970's of the more virulent strain *O. novo-ulmi,* elms have been severely decimated across all geographic locations. In North America, *O. novo-ulmi* relies on *H. rufipes,* or the smaller European elm bark beetle, *Scolytus multistriatus,* to be transported to new host elms (Hubbes, 1999; Agrios, 1988; Millar, 1986). In the prairie regions of North America, *H. rufipes* can withstand cold winter temperatures (Agrios, 1988), and is the primary vector of Dutch elm disease (DED).

Plants are known to synthesize and emit semiochemicals in response to invading or damaging organisms (Turlings, 1990) in order to recruit natural enemies of those organisms. Tobacco, cotton and maize plants each produce distinct semiochemical blends in response to damage by caterpillars of two closely related herbivore species. The specialist parasitic wasp *Cardiochiles nigriceps* exploits these differences to

distinguish infestation by its host *Heliocoverpa virescens* from that by the non-host *H. zea* (Demoraes, 1998). Volicitin, N-(17-hydroxylinolenoyl)-L-glutamine, in the oral secretion of beet armyworms triggers the release of plant semiochemicals which attract natural enemies of the caterpillar (Alborn et al., 1997; Paré et al., 1998). Insects that feed by sucking plant sap also induce changes in plants' semiochemicals to attract parasitic wasps (Powell et al., 1998; Guerrieri, 1993; Du et al, 1996). *cis*-Jasmone has been found to attract an insect predator and parasitoid of aphids (Birkett et al., 2000); it may even serve as a phyto-pheromone in plant-plant communications (Powell et al, 2003). Trees under attack by bark beetles that carry symbiotic fungi respond by forming necrotic lesions around the infection, and by increasing the concentration of allelochemicals with fungistatic properties within the lesions (Raffa, 1988). In all these cases, the plants' response helps alleviate the impact of the damage caused by insects or fungi.

Like other fungi, *O. novo-ulmi* obtains its energy from monosaccharide sugars such as glucose, mannose and galactose (Kendrick, 1992). Enzymatically, it is well equipped to break down cellulose from host trees (Kendrick, 1992). However, dead and dying host trees discontinue cellulose production and become a finite energy resource for *O. novo-ulmi*. Acquiring a vector relationship with a bark beetle that shares a similar niche and is able to disperse to new hosts seems advantageous. Attracting the beetle vector by semiochemicals, rather than relying on chance encounters, would be an optimal strategy for *O. novo-ulmi*. In this chapter I will test the hypothesis that *O. novo-ulmi* emits the four semiochemicals that are attractive to *H. rufipes* (see chapter II), or that it makes host trees emit them.

3.2 Methodology

3.2.1 Culturing of *Ophiostoma novo-ulmi* and Preparation of Inoculum

Ophiostoma novo-ulmi cultures were obtained from the Government of Saskatchewan Crop Protection Laboratory (Regina, SK. S4P 3V7, Canada). Cultures devoid of contamination were sub-cultured onto potato-dextrose agar (PDA) in sterilized Petri plates (15 x 85 mm). Cultures were incubated in the dark for approximately four weeks at 23°C. Those cultures that were randomly selected for inoculation of trees were flooded with 10 mL of distilled water, and the surface rubbed with a glass rod to suspend the condiospores. The solution was then filtered twice through eight layers of cheesecloth to remove any hyphal fragments that may have become dislodged during the spore suspension process. Spore concentrations were estimated using a compound microscope and a Fuchs Rosenthal Ultra Plane counting chamber.

3.2.2 Inoculation of American Elm Saplings with *Ophiostoma novo-ulmi*

American elm (*Ulmus americana*) saplings (bare root; 2 m tall, 2 cm in diam at a height of 24 cm) were obtained from Bylands Nursery, Kelowna, BC, V1Z 1H6. Trees were transported to the greenhouse of the Global Forest quarantine facility at SFU, and were potted in 20 L containers using unsterilized potting soil.

To determine whether *O. novo-ulmi* emits the four semiochemicals that attract *H. rufipes* or induces host trees to emit them, I conducted the following inoculation experiment. I flooded culture plates of *O. novo-ulmi* grown on potato dextrose agar with distilled water and diluted the suspended conidia to 6×10^7 mL^{-1}. Using a sterile probe, I then punched ten holes similar in diameter to those bored by *H. rufipes* into the stem and

twig crotches of the healthy elm saplings (n = 3) maintained in a quarantine greenhouse. Into each hole, I pipetted 10 μL of *O. novo-ulmi* spore suspension. Control saplings (n = 3) with the same number and distribution of holes received equivalent volumes of distilled water, and additional control saplings (n = 3) received no treatment. After 12 weeks, when treatment saplings exhibited disease symptoms, I ground wood tissue from all nine saplings into separate samples of fine sawdust, weighed them, and collected volatiles on Porapak Q (see above: collection of semiochemicals from elm wood).

All treatment and control saplings were monitored regularly for DED symptoms including wilting of leaf tissue. The presence of DED in each of the inoculated elms that exhibited DED symptoms was confirmed through sterile transfer of wood tissue slivers to separate potato dextrose agar plates. Plates were incubated as described above and monitored daily for the presence of developing hyphae and mycelium.

3.2.3 Quantification of Semiochemicals derived from *Ophiostoma novo-ulmi*-Inoculated Elms

Following 12 weeks of inoculation, each sapling (total n = 9) was separately ground into sawdust with a table top electric grinder. To avoid any cross contamination, control trees were ground up first, and the grinder was thoroughly cleaned and sterilized with 95% ethanol after grinding of each sapling. Samples were adjusted to ensure that they were of similar weight before their volatiles were captured on Porapak Q as described above (2.2.1). An internal standard (7-methyl-heptadecane) was added to Porapak Q extracts to quantify compounds **1**,**2**,**3** and **4**.

3.3 Results and Discussion

At approximately 4 weeks, saplings inoculated with a suspension of *O. novo-ulmi* were observed to be symptomatic of Dutch elm disease. Leaves persisted on saplings but were severely wilted compared with leaves of control saplings. At nine weeks, when the bark was peeled away from stem tissue, inoculated saplings exhibited the characteristic streaking of xylem tissue caused by *O. novo-ulmi*.

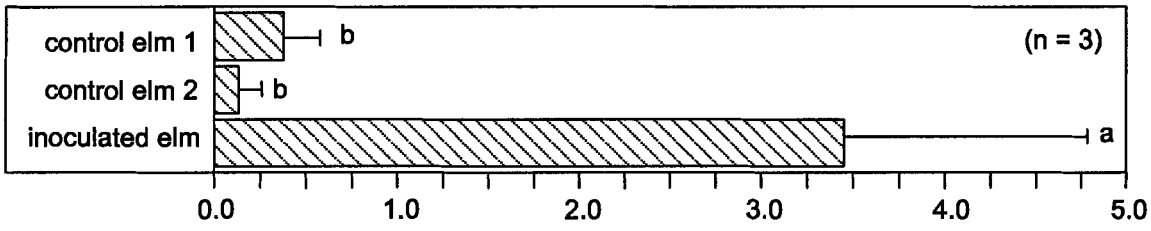
The presence of *O. novo-ulmi* in inoculated saplings was confirmed by plating slivers of wood tissue on potato dextrose agar revealing the white fibrous hyphae and mycelium that are characteristic of *O. novo-ulmi* (Seifert, 1993).

Quantitative GC-MS analyses of each Porapak Q extract revealed that spiroaxa-5,7-diene, α-cubebene, and δ-cadinene were significantly more abundant in diseased than healthy elm saplings (Figure 11, Exp. 12). β-Pinene is typically produced by plants in response to mechanical injury (Trapp, 2001); punching 10 holes with a probe may not have inflicted a sufficiently severe injury to induce and maintain its production. None of the four semiochemicals was present in Porapak Q volatile extracts of *O. novo-ulmi* grown on potato dextrose, indicating that *O. ulmi* itself does not produce these semiochemicals. However, it is conceivable that a modified diet including host material would lend itself to produce these semiochemicals. However, data (data not shown) indicates that the presence of host material in media does not do this.

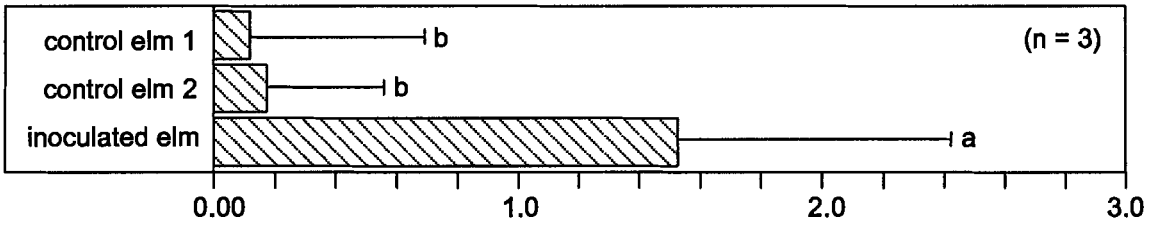
Figure 11. Results of an inoculation experiment comparing quantities of (–)-α-cubebene, (+)-spiroaxa-5,7-diene, and (+)-δ-cadinene in Porapak Q volatile extracts of wood tissue from potted elm saplings (n = 3) 12 weeks after inoculation with an aqueous spore suspension of *O. novo-ulmi* (inoculated elm; n = 3), distilled water (control elm 1; n = 3), or left untreated (control elm 2; n = 3). (–)-β-Pinene occurred in amounts too low for analysis. Bars with different letters are significantly different (ANOVA followed by Tukey-Kramer HSD comparison of means; JMP statistical software): $P < 0.05$.

Exp 12

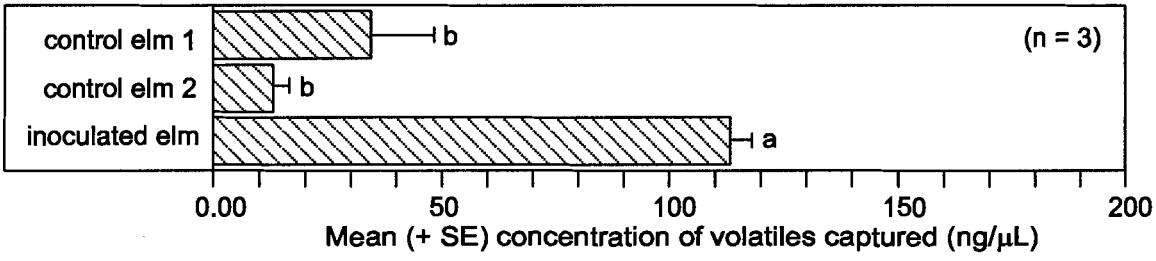
(–)-α-cubebene



(+)-spiroaxa-5,7-diene



(+)-δ-cadinene



My data provide strong evidence that the four elm-derived semiochemicals that attract *H. rufipes* occur at low quantities in healthy elms. By an as yet unknown mechanism, *O. novo-ulmi* up-regulates the production of these semiochemicals, thus enhancing the apparency of host trees to foraging beetles, and increasing the probability of transportation of the pathogen to new hosts. It would now be intriguing to investigate (i) whether European elms, which unlike North American elms have coevolved with the fungal pathogen, show an analogous semiochemical response upon *O. ulmi* invasion; and (ii) whether the virulence of the fungus is expressed, in part, by its ability to provoke the trees semiochemical response that attracts the bark beetle vector.

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