THE CHEMICAL ECOLOGY OF HOST FORAGING, AGGREGATION, AND PROPHYLACTIC MICROBIAL DEFENSE IN THE WESTERN BOXELDER BUG, *BOISEA RUBROLINEATA* (BARBER) (HETEROPTERA: RHOPALIDAE)

by

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ABSTRACT

The boxelder bug (BEB), *Boisea rubrolineata* (Heteroptera: Rhopalidae), is a specialist herbivore of boxelder trees, *Acer negundo*. BEBs form spectacular aggregations on (i) pistillate *A. negundo* when seeds are maturing, (ii) in and around shelters in fall and winter, and (iii) in response to warm sunlight. When sunbathing, BEBs release from their posterior dorsal abdominal gland an odorous blend of monoterpenes with heretofore unknown biological function. We reveal that (1) BEBs exploit semiochemicals (phenylacetonitrile, 2-phenethyl acetate) from host trees to locate them during foraging and colonization, (2) aggregation and sexual communication of BEBs are mediated by a complex pheromone system, and (3) BEBs in warm sunlight, but not in shade, exude and spread copious amounts of monoterpenes onto their cuticle. These monoterpenes do not serve as a pheromone, but rather a means of sanitation that interferes with spore germination and growth of the pathogenic fungus *Beauvaria bassiana.*

Keywords: Boxelder bugs, *Boisea rubrolineata,* Heteroptera, Rhopalidae, Serinethinae, boxelder trees, *Acer negundo*, semiochemicals, phenylacetonitrile, host foraging, plant defense, seed predation, aposematism, feces, aggregation pheromone, ventral abdominal gland, sex-pheromone, 2-phenylethanol, posterior dorsal abdominal gland, monoterpenes, microbial defense, fungal pathogen, *Beauveria bassiana*, sunbathing, secretion-grooming

DEDICATIONS

To my mother, who taught me to follow my heart and to reach for my dreams…I love you.

To my father, whose kind offering focused me on the intimate world of nature, filling me with a sense of self and wonder.

Thank you for the camera, it literally changed my view of the world.

To Chip, whose friendship, guidance and encouragement brought hope beyond the pale.

Unclouded by the enchanting beauty

of the imminent dawn, I embrace with sanity

the challenges that await the new day

— Joseph J. Schwarz

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1 INTRODUCTION

In my first chapter, I shall review the taxonomy of hemipterous insects (1.1) and scentless plant bugs (1.2) of which the boxelder bug, *Boisea rubrolineata,* is one representative species. Focusing on *B. rubrolineata,* I then shall describe its distribution and morphology (1.3), exocrine system (1.4), life history (1.5), preferred host tree (1.6), and research opportunities and objectives (1.7) that present themselves based on the current status of knowledge.

1.1 Hemiptera

Hemipterous insects possess piercing-sucking mouthparts in which the mandibles and maxillae have evolved into a flexible feeding tube (proboscis) containing both a feeding channel and a salivary channel, sheathed within a modified labium, forming the "beak" or rostrum capable of piercing plant tissue and insect cuticle (Triplehorn and Johnson, 2005). The forewings of Hemiptera ("Hemiptera" meaning "half wing") are termed hemelytra. They are hardened at the basil end (corium), but membranous at the apical end. However, this is true only for the Heteroptera, contrasting with (i) the completely membranous or slightly thickened wings of species of the two suborders Auchenorrhyncha and Sternorrhyncha, and (ii) the completely hardened elytra of Coleoptera (beetles) (Triplehorn and Johnson, 2005). Other external features, such as position of the wings and rostrum, separate Heteroptera from both Auchenorrhyncha and Sternorrhyncha. For example, the wings of Heteroptera lie flat over the abdomen at

rest and overlap at the membrane, whereas in Auchenorrhyncha and Sternorrhyncha the wings at rest are held roof-like over the body (Triplehorn and Johnson, 2005). The Heteroptera as one suborder of the Hemiptera are referred to as true bugs of which *B. rubrolineata* is one representative species. Monophyletic characters of the Heteroptera include the possession of functional abdominal scent glands in nymphs and meta-thoracic scent glands in adults (Schaefer and Chopra, 1982; Schuh and Slater, 1995).

Heteropterans undergo simple or gradual metamorphosis. Terrestrial bugs, such as *B. rubrolineata*, undergo paurometabolous development. Their offspring are referred to as nymphs that greatly resemble adults and that occupy the same habitat (Elzinga, 2004; Triplehorn and Johnson, 2005). Paurometabolous insects differ from hemimetabolous insects (commonly used to generalize Hemiptera; see Elzinga, 2004) in that in the latter group the nymphs are aquatic and gill-breathing, but the adults are terrestrial, such as in dragonflies (Odonata) and stoneflies (Plecoptera) (Triplehorn and Johnson, 2005). In true bugs, such as *B. rubrolineata*, the final molt from the fifth-instar to the adult involves little more than the development of functional wings and genitalia. Taxonomically, *B. rubrolineata* belongs to the scentless plant bugs (Rhopalidae).

1.2 Scentless plant bugs

Scentless plant bugs (Rhopalidae) are split into 2 subfamilies. The Rhopalinae comprise 18 genera and the Serinethinae 3 genera (Schuh and Slater, 1995; Göllner-Scheiding, 1980). They have worldwide distribution (Chopra, 1967; Schaefer, 1975; Schuh and Slater, 1995) and feed primarily on woody plants in the subclasses Rosidae

and Asteridae, within which they prefer the orders Sapindales, Asterales, Malvales, Cyperales, and Rosales (Schaefer and Chopra, 1982; Schaefer and Mitchell, 1983). Based on apomorphic characters, Serinethinae are considered more advanced than Rhopalinae (Chopra, 1967; Cobben, 1978; Schaefer and Chopra, 1982; Li and Zheng, 1994; Davidova-Vilimova et al., 2000). For example, Serinethines entirely lack, or have severely reduced paired metathoracic glands coupled with the absence of gland openings and associated structures, while Rhopalinae possess the meta-thoracic scent gland (Schaefer and Chopra, 1982; Aldrich, 1988). The implication that the metathoracic scent glands are non-functional has earned them the status of "scentless" true bugs (Aldrich et al., 1990b; Davidova-Vilimova et al., 2000). This loss or severe reduction of the metathoracic gland is consistent with the retention and function of the nymphal dorsal abdominal gland as a co-evolutionary response to host specialization and the sequestering of host metabolites for secondary defense (Aldrich et al., 1979, 1990a, b).

There are 3 genera in the Serinethinae. *Leptocoris* Hahn includes 30 Old World tropical species, *Jadera* Stål 17 New World tropical and sub-tropical species, and *Boisea* (formerly *Leptocoris*) Kirkaldy two Nearctic, one Central and West African, and one South Indian species (Göllner-Scheiding, 1980; Schuh and Slater, 1995; Schaefer and Chopra, 1983). Serinethines are seed predators of sapindaceous plants (Schaefer and Chopra, 1982; Carroll and Loye, 1987; Carroll, 1988) from which they sequester secondary plant metabolites for defense against predators (Braekman et al, 1982; Ribeiro, 1989; Aldrich et al, 1990b).

1.3 Distribution and morphology of *B. rubrolineata*

In North America, *B. rubrolineata* (Barber) and *B. trivittata* (Say) are considered the "Pacific" species (west of 115°) and "Eastern" species (east of 115°), respectively. Both occur in sympatry in the south western United States (Barber, 1956; Schaefer, 1975). There is evidence, however, questioning their separate species status (Schaefer, 1975; Aldrich et al, 1990a). For example, their genitalia are nearly identical and differences in morphology and color are subtle and inconsistent (Barber, 1956; Schaefer, 1975).

Boisea rubrolineata is aposematically colored, conspicuous, and highly gregarious. Adults have a distinctive notch along the lateral margins of the pronotum behind the collar (Schuh and Slater, 1995) which separates the Rhopalinae from Serinethinae (see above). The body shape is elliptical and slate black with contrasting orange coloration. The spade-shaped head is broader than long and bears red compound eyes and ocelli. The antennae are elbowed and four-segmented. The piercing-sucking mouthparts fold longitudinally into a ventral median groove along the thorax between the coxae, and the legs are black with orange coxae. The dorsal abdominal surface is orange in color and expands onto the ventral surface. Adults retain the functional posterior dorsal abdominal scent gland of the nymph stage. The wings (hemelytra) are black with orange veins on the corium (Barber, 1956; Schaefer, 1975). Unlike *B. rubrolineata,* heterospecific *B. trivittata* often lack the corium-vein coloration, a morphological feature that may help separate the two alleged species (Barber, 1956; Schaefer, 1975). When the wings of *B. rubrolineata* are folded over the body, they form an orange V pointed towards the head. The trapezoidal pronotum has three parallel

orange stripes, one central and two at the outer margins running from the collar to the mesoscutum bordering the scutellum. When exposed to the sun, their orange coloration "ignites" with brilliancy, becoming highly conspicuous (JJS observations).

Adult females lay an average of 10 eggs per mass, which hatch in ~ 10-14 days (Smith and Shepherd, 1937; Yoder and Robinson, 1990). In the trees' canopy, egg masses are typically oviposited on the underside of leaves or on seedpods (samaras). Freshly laid eggs are light orange in color and turn dark red just prior to hatching. There are five nymphal instars as is the general rule for hemipterans (Bouldrey and Grimnes, 1995), which take ~ 60 days to reach adulthood (Smith and Shepherd, 1937). Upon hatching from the egg, first-instar nymphs are bright red in color from antennae to legs, and remain clustered about the egg casings for some time. The head, legs, antennae, and thoracic region of the first instar soon turn a deep maroon-burgundy color, whereas the abdomen remains deep bright red. Second instars resemble first instars, but are mobile and larger in all aspects as are successive instars (Bouldrey and Grimnes, 1995). Third instars have small dark wing pads protruding from the thorax under the cuticle (Smith and Shepherd, 1937; Bouldrey and Grimnes, 1995), and they are not as brightly red as earlier instars. Fourth instars have wing pads that appear dark brown to black in color spanning the first tergite and being free at the tips. Fifth instars are the largest, with black-colored wing pads spanning the third tergite and sometimes part of the fourth. Their abdomen is dark orange-red with a conspicuous pale orange patch (also noticeable in fourth instars and to a lesser degree in third instars) at the convergence of tergites 4-6, marking the area of the openings to both the anterior (tergites 4-5) and posterior (tergites 5-6) dorsal abdominal glands.

1.4 Exocrine system of *B. rubrolineata*

Meta-thoracic scent glands are common in the Heteroptera but are vestigial in *Boisea* spp. (Aldrich et al., 1990b). Nymphs have an anterior and posterior dorsal abdominal gland located between the 4th and 5th (anterior) and the 5th and 6th (posterior) tergites. The anterior dorsal abdominal gland (ADAG) of *B. trivittata* nymphs contains a mixture of (*E*)-2-hexenal, (*E*)-2-octenal, 4-oxo-(*E*)-2-hexenal, and 4-oxo-(*E*)-2-octenal (Aldrich et al., 1990b). The ADAG of adult *Boisea* spp. is flaccid, but the posterior dorsal abdominal gland (PDAG) remains functional, and is identical between *Boisea* spp. and between nymphs and adults (Aldrich et al., 1990b). The PDAG contains a mixture of 3-carene, sabinene, β-pinene, myrcene, limonene, and *trans*-ocimene (Aldrich et al., 1990b). The male-specific ventral abdominal gland (VAG) of *Boisea* spp. contains 2-phenylethanol (Aldrich et al., 1990b).

ADAG constituents do not evoke alarm responses by conspecific *Jadera* nymphs. Alkenal and keto-alkenal ADAG constituents of *Jadera* spp. and *Boisea* spp. may instead serve in arthropod predator defense (Aldrich et al., 1990b). Species-specificity of monoterpenes in the PDAG of *Jadera* spp. suggests a pheromonal role of these compounds, but their chemical nature also implies a function in interspecific defense and aposematic odor signaling (Aldrich et al., 1990b). This interpretation may also be applicable to *B. rubrolineata*. VAG gland constituents are believed to serve in mate marking or to prevent male-male homosexual encounters (Aldrich et al., 1990b), but they could also be pheromone components. That constituents of both the PDAG and

VAG of adult *B. rubrolineata* and *B. trivittata* are identical (Aldrich et al., 1990b) corroborates doubt about their distinct species status (Schaefer, 1975).

1.5 Life history of *B. rubrolineata*

When the weather warms in the spring, boxelder bug adults emerging from diapause feed on fallen seeds and insects in the soil and on developing and flowering buds in the canopy of trees, where they deposit their eggs. First-generation nymphs reach adulthood in about 60 days (Smith and Shepherd 1937) by early to mid-summer, feeding heavily on maturing seeds of pistillate trees, which they colonize as a rendezvous site for mating (Long 1928; Tinker 1952; Yoder and Robinson 1990). During the summer, a second overlapping generation may emerge. Late summer into the fall, surviving non-mating adults cease feeding, depart from their host trees, and form large aggregations in protective shelters for overwintering diapause. In their search for overwintering shelters, large numbers of bugs may enter houses and buildings unintentionally where their feces stain textiles, making them an unsavory urban pest.

1.6 Characteristics of boxelder maple, *Acer negundo*

Boxelder maple, *Acer negundo* Linnaeus (Sapindales) is one of the most widespread of the maples that range from the east to the west coast of North America and from Canada to Guatemala (Peattie, 1953; Elias, 1980; Overton, 1990). It is also referred to as ashleaf maple, Manitoba maple, sugar ash, California boxelder, eastern boxelder, and western boxelder. The genus *Acer* is cladistically assigned to the family Aceraceae. However, recent phylogenetic analysis supports taxonomic placement of the

Aceraceae into the Sapindaceae (Harrington et al., 2005), which are known to contain secondary metabolites that are sequestered by specialist rhopalid seed predators for secondary defense against predation (see Braekman et al., 1982; Ribeiro, 1989; Aldrich et al., 1990b).

Acer negundo is most common on moist soils of water basins and bottomland hardwoods but they can grow in a variety of climates (Elias, 1980; Overton, 1990). Companion trees include cottonwood, sycamore, sweetgum, elm, silver maple, and willow. The bark of *A. negundo* is pale gray to light brown with deep furrows and broad rounded ridges. Pinnate-compound leaves bear 3-7 leaflets that are opposite, deciduous, and coarsely serrate along the margin, light green and smooth above, and paler with hair beneath. Petioles are long and slender. Flowers are imperfect (petals absent) and dioecious, occurring on pistillate (seed-bearing) and staminate (pollen-bearing) trees. Staminate flowers are fascicled, and pistillate flowers are drooping racemes appearing before leaves in early spring. Fruit is two-winged (one wing = samara = flattened, fibrous tissue developed from the ovary wall encasing the ovule or seed, allowing for wind dispersal), ~ 3.5-4.5 cm long and 5-10 mm wide with an angle of spread < 45° (Preston, 1976; Elias, 1980). Seeds that ripen from August to October are wind-distributed throughout winter into the following spring, allowing for prolific reproduction of *A. negundo* (Overton, 1990).

Acer negundo is an excellent shade tree able to endure extreme heat, drought, cold, and wind. It grows very rapidly in the first 15-20 years, reaching heights of 21 m with an even broader crown (Peattie, 1953, 1966; Elias, 1980). It was therefore, extensively planted in sprawling Mid-West communities. However, *A. negundo* is

considered of the poorest of quality among the maples, lacking profitable wood and autumn display (Peattie, 1953, 1966; Overton, 1990). Despite rapid growth, *A. negundo* is short-lived (i.e., 60 years on average). With a shallow root system and weakening wood when aging, they readily succumb to heavy storms (Peattie, 1953, 1966; Overton, 1990). Native North Americans tapped its sap for sugar and used its wood to make charcoal for ceremonial painting and tattooing (Peattie, 1953).

1.7 Research opportunities and objectives

Boisea rubrolineata is an herbivore specialist and prefers to feed on seeds of *A. negundo*. The high quality of the ephemeral seed resources may explain why *B. rubrolineata* populations "explode" when host samaras mature and seeds therein reach an optimal stage for feeding. If *B. rubrolineata* forage in response to the presence of ephemeral seed resources, and gear their reproductive ecology accordingly, then one could argue that *B. rubrolineata* should be capable of exploiting semiochemical cues that might be associated with these profitable resources. My objective in **Chapter 2** is to elucidate the semiochemicals that mediate host foraging by *B. rubrolineata*.

Boxelder bugs, *Boisea* spp., are well known to form spectacular aggregations numbering thousands of specimens (Aldrich *et al.*, 1990b; Schowalter, 1986). Aggregations form on pistillate *A. negundo* with maturing seeds (Long, 1928; Tinker, 1952; Yoder and Robinson, 1990; Aldrich et al., 1990a; Robinson, 1996) and in or near shelters suitable for overwintering (Smith and Shepherd, 1937; Knowlton, 1944; Tinker, 1952; Schowalter, 1986; Yoder and Robinson, 1990; Aldrich et al., 1990a; Robinson, 1996). Aggregations apart from host tree sources suggest that they are mediated by

pheromone (Schowalter, 1986). Yet, aggregation or sex pheromones are unknown. My objective in **Chapter 3** is to investigate intraspecific communication signals in *Boisea rubrolineata.*

As excessive thermoregulators, boxelder bugs break overwintering dormancy to sunbathe gregariously (Smith & Shepherd, 1937; Knowlton, 1944; Schowalter, 1986). Sunbathing boxelder bugs have a higher thoracic temperature (Tinker, 1952; Yoder & Robinson, 1990; Robinson, 1996; JJS & ST, unpublished data), metabolism and activity than their conspecifics in the shade (Tinker, 1952). They also release an odorous blend of monoterpenes (JJS, unpublished), which are known to have anti-microbial properties (Cole & Blum 1975; Okamoto et al., 1978; Berryman, 1988; Chen et al., 2003; Alma et al., 2004; Grujic-Jovanovic et al., 2004; Sonboli et al., 2005). Phytophagous insects, such as *B. rubrolineata*, that are exposed to and risk infection by pathogens thriving on leaf surfaces (Wertheim et al., 2005) or in overwintering shelters should have evolved antimicrobial defense strategies. My objective in **Chapter 4** is to test whether *B. rubrolineata* sunbath as a prophylactic defense against pathogens.

Without further ado, we will now turn our attention to *B. rubrolineata* to better understand and appreciate some of the communication dynamics veined not only in its host, *A. negundo*, but in its physiology as well.

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2 PHENOLOGY OF SEMIOCHEMICAL-MEDIATED HOST FORAGING BY THE WESTERN BOXELDER BUG, *BOISEA RUBROLINEATA*, AN APOSEMATIC SEED PREDATOR

2.1 Abstract

The western boxelder bug (BEB), *Boisea rubrolineata* (Heteroptera: Rhopalidae), is a specialist herbivore of boxelder trees, *Acer negundo*. We tested the hypothesis that BEBs exploit semiochemicals from host trees to locate them. Headspace volatiles from trees bearing staminate inflorescences ("staminate trees") and from trees bearing pistillate inflorescences ("pistillate trees") were separately collected throughout the season, and bioassayed in Y-tube olfactometer experiments. Headspace volatile extracts of early-season, pollen-bearing staminate trees and mid-season pistillate trees with mature samaras (seed pods) attracted female and male BEBs. To determine the key semiochemical(s), we analyzed extracts by coupled gas chromatographic-electroantennographic detection (GC-EAD) and GC-mass spectrometry (MS). Considering antennal stimulatory components and their relative abundance in headspace volatile extracts of trees with immature, mature and senescing samaras, we prepared a 5-component synthetic blend of candidate semiochemicals (hexanol, pentyl acetate, phenylacetonitrile, 2-phenethyl acetate, *trans*-nerolidol) that attracted females, males, and 5^{th}-instar nymphs. Phenylacetonitrile as a single component was as attractive as the 5-component blend to both adults and nymphs. By responding to phenylacetonitrile emitted by pollen-bearing staminate trees and pistillate trees with maturing seeds, BEBs

appear to track and exploit the availability of nutrient-rich food sources, suggesting that their reproductive ecology is synchronized to the phenology of their host boxelder tree.

2.2 Introduction

In well-studied relationships between phytophagous insects and their host plants, there is consensus that the synchrony between host plant phenology and herbivore ecology is governed by ephemeral resources (Wolda and Tanaka 1987; Carroll 1988; Braman and Besheasr 1994; Yukawa 2000; Carroll et al. 1998, 2005b; Grimm and Führer 1998; Kurota and Shimada 2002; Ivashov et al. 2002; Kohno and Bui Thi 2005; Yukawa and Akimoto 2006), suggesting that insects maximize reproductive fitness or survivorship in response to host phenology. The boxelder bugs' adaptations in response to its hosts' ephemeral resources (pollen and seeds) appears diverse and may include recognition of plant semiochemicals (message-bearing chemicals) and timing of the breeding season according to resource availability. Indeed, the evolutionary trend in heteropteran true bugs from generalist predators to phytophagous specialists may be driven by interactions with host plants (Cobben 1978), leading to synchrony of the plants' and insects' reproductive phenologies (Aldrich et al., 1979; Bowers 1990).

The basic biology of the western boxelder bug (BEB), *Boisea* (formerly *Leptocoris*) *rubrolineata* (Heteroptera: Rhopalidae), is still poorly understood. BEBs belong to the "scentless" true bugs (Aldrich et al, 1990a) and feed primarily on seeds of boxelder maple trees, *Acer negundo* (Schuh and Slater, 1995; Robinson 1996), and to a lesser extent on seeds of silver maple, *A. saccharinum* (JJS observations) and sugar maple, *A. saccharum* (Robinson, 1996). When the weather warms in the spring, adults

emerging from diapause feed on fallen seeds (Tinker, 1952; Yoder and Robinson, 1990) and insects in the soil (JJS observations) and on flowering buds in the canopy of pistillate trees as well as on anthers and associated tissue of staminate trees (JJS observations). Females deposit eggs on or near pistillate trees. First-generation nymphs roam the ground litter in search of nutrition, feeding on fallen seeds (Tinker, 1952) and insects (JJS observations), and later moving into host trees to feed on maturing seeds. Nymphs reach adulthood in about 60 days (Smith and Shepherd 1937) by early to mid-summer, feeding heavily on maturing seeds of pistillate trees, which they colonize as a rendezvous site for mating (Long 1928; Tinker 1952; Yoder and Robinson 1990). During the summer, a second overlapping generation may emerge. Late summer into the fall, surviving non-mating adults cease feeding, depart from senescing host trees (Tinker, 1952), and form large aggregations in protective shelters for overwintering diapause

The closely related and well studied soapberry bugs, *Jadera haematoloma*, assemble on seed-bearing specimens of their golden rain host tree, *Koelreuteria elegans* (Carroll et al. 1998). Feeding by *J. haematoloma* (Carrol 1988) or *J. obscura* (Tanaka and Wolda 1988) on such trees attracts male and/or female conspecifics, likely mediated by semiochemicals, which originate from the bugs, the plant, or both. Similarly, the tracking of hosts by *Leptocoris* spp. as related to host phenology and fruit dehiscence (Carroll et al., 2005a) suggests plant volatiles. Emission of herbivore- or wound-induced semiochemicals, or semiochemicals associated with a particular stage of the hosts' phenology, may also attract BEBs to boxelder host trees. If mate choice is tied to host choice (Feder et al. 1994; Carroll et al. 1998; Linn et al. 2003, 2004), then

an assembly cue may facilitate feeding and mating during host colonization, thus driving host-specific foraging by BEBs.

Acer negundo, like others plants, may have defense lines against seed predation. One of the plants' many possible defenses against herbivory is to become inedible by staging a secondary chemical defense (Bonner 1970; Dethier 1970; Whittaker and Feeny 1971; Feeny 1975; Rosenthal and Janzen 1979; Duffey and Isman 1981; Rosenthal and Berenbaum 1991) which, in turn, may alter the plants' attractiveness. The host-specific odors that arise from metabolic reactions of secondary plant metabolites (Visser 1986) may not only deter the general herbivore (Pickett et al. 2003), but may also attract the specialist feeder (Dethier 1970; Bernays et al. 1977; Feeny 1975, 1980; Rothschild and Moore 1987; Bowers 1990; Moore et al. 1990; Nottingham et al. 1991; Seigler 1991; Bernays and Chapman 1994a, b) that sequesters secondary plant metabolites for aposematic defense (Rosenthal and Janzen 1979; Bowers 1990; Guilford 1990; Rosenthal and Berenbaum 1991).

The nitrogen content of food-source protein affects the growth, development and performance of many phytophagous insects (Strong et al. 1984), and their response to nitrogenous secondary plant metabolites may drive host selection (Bernays and Chapman 1994a, b). The plants' reproductive parts, such as pollen, flowers and seeds, contain high levels of protein, and secondary metabolites for herbivore defense. Seeds store non-protein amino acids and defensive amines, alkaloids or cyanogenic glycosides in high concentrations (Bernays and Chapman 1994a), and thus are potentially great resources for aposematic seed predators such as BEBs. Furthermore, herbivore-induced stress may upregulate the production of free amino acids and nitrogenous constituents in

plant tissues, thus modifying host quality (Rhoades 1985; Bernays and Chapman 1994a).

True bugs sequester host-plant secondary metabolites (Duffey and Scudder 1972; Scudder and Duffey 1972; Braekman et al. 1982; Aldrich 1988; Aldrich et al. 1990; Aliabadi et al. 2002) for aposematic defense (Riberio 1989; Berenbaum and Milickzy 1984; Aldrich et al. 1990; Aliabadi et al. 2002). Conceivably, aposematic and gregarious BEBs may obtain secondary defense, as well as nutrition, when feeding on pollen-producing tissue or maturing seeds of boxelder trees.

The high quality of ephemeral seed resources may explain why BEB populations "explode" when host samaras (indehiscent winged fruits or pods) mature and seeds therein reach an optimal stage for feeding. Reproductive synchrony between the golden rain trees' phenology and their *Jadera* seed predators (Carroll and Loye 1987; Carroll 1988; Carroll et al. 1998) and between *Leptocoris mitellatus* and seeding *Atalaya oleifolius* (Carroll et al., 2005a) may have been driven by the ephemeral state and quality of host plant resources (Carroll et al. 1998, 2003a, b; 2005a).

Therefore, if BEBs indeed forage in response to the presence of ephemeral pollen and seed resources, and gear their reproductive ecology accordingly, then one could argue that BEBs should be capable of exploiting semiochemical cues that might be associated with these profitable resources. We tested the hypotheses that (1) BEBs are attracted to volatiles of early-season, pollen-bearing staminate trees, but not to those of pistillate trees; (2) volatile profiles of pistillate trees change as samaras mature and senesce; (3) BEBs discern between changing volatile blends and are attracted to only those associated with mature samaras (encasing maturing seeds); and (4) that specific

semiochemicals, perhaps linked to the plants' herbivory defense, mediate attraction of BEBs to boxelder trees with pollen or seed resources.

2.3 Methods and Materials

2.3.1 Collection and Maintenance of Experimental Insects

Late-instar nymph and adult BEBs were field-collected weekly on or near pistillate trees of *A. negundo* or *A. saccharinum* in Kelowna, Westbank, and Princeton, British Columbia (Canada), during June to September 2005, 2006 and 2007 (see Figure 2.1 for seasonal phenology). BEBs were maintained at 22-30 C°, 30-70% relative humidity and a natural photoperiod, and were provisioned with water and new-growth shoots of *A. negundo* with intact leaves and seeds that were clipped weekly and transported from field sites to SFU in 19-L buckets of water. Post-diapause adult BEBs of unknown age were field-collected in March/April, separated by sex, maintained on water in separate cages, and used in bioassays as needed. Newly-molted sexually immature adult females or males were kept separate and bioassayed within 14 days, before they reach sexual maturity (Smith and Shepherd 1937; Yoder and Robinson 1990; Miller et al. 1991). In 2007, newly-molted adults were bioassayed when they were 4-6 days old, an age at which long-lived seed predatory bugs feed heavily before they reach sexual maturity (Caldwell and Rankin 1974). Reproductive adults (as evidenced by mating activities) were > 1 month old when they were used in bioassays.

2.3.2 Collection of *A. negundo* volatiles

Clipped branches (4-6 ramates) (*i*) with intact leaves and reproductive parts devoid of apparent insect or mechanical damage, (*ii*) with BEBs feeding on them, or (*iii*) with mechanical damage were placed in separate water beakers (200 mL) to allow for transpiration, and were enclosed in separate cylindrical Pyrex® glass chambers (each 14 × 37.5 cm). The above treatments were chosen to help discern between effects caused by mechanical damage or BEB herbivory. Ramates were provided from four different sources: (1) early-spring pollen-bearing staminate trees, or pistillate trees, with swollen and opening buds, unfurling leaves, and inflorescences; (2) mid-spring pistillate trees with developing samaras (containing ovules) and unfurled leaves; (3) mid-summer pistillate trees with maturing seeds (Tinker, 1952); and (4) late-summer pistillate trees with senescing foliage and ripening seeds (see Figure 2.1 for seasonal phenology).

We simulated BEB-produced mechanical damage by puncturing with a 000-pin leaf veins, petioles, and samara ovaries. Each leaf received 3-4 vein punctures, each petiole 2-3 punctures, and each samara 2 punctures. We studied the effects of herbivory by starving 25 male and 25 female for 3 days before we placed them into a cylindrical Pyrex® glass aeration chamber (14 × 37.5 cm) to feed on intact branch clippings. Charcoal-filtered air was drawn for 3 days at ~ 0.5 L per min through the chamber and a Pyrex® glass tube (14 ×1.3 cm OD) containing 500 mg of Porapak-Q (50-80 mesh, Waters Associates, Inc., Milford, MA). Volatiles were eluted from Porapak-Q with 2 mL of redistilled pentane and extracts were concentrated as needed under a stream of nitrogen. The dry weight of ramates with attached foliage was obtained to determine Gram-Hour-Equivalents [GHE; 1 GHE = volatiles released from 1 g of ramate biomass during 1 h] as a quantitative descriptor of stimuli to be tested in bioassays. The

procedures for obtaining control stimuli were identical to those described above except that aeration chambers did not contain any insects or plant material.

2.3.3 Analyses of Extracts

Aliquots of headspace (air space above sample) volatile extracts were analyzed by coupled gas chromatographic-electroantennographic detection (GC-EAD; Arn et al. 1975; Gries et al. 2002), employing a Hewlett Packard 5890A gas chromatograph equipped with a GC column (30 m × 0.25 ID) coated with DB-5 (J&W Scientific, Folsom, CA). Helium was used as carrier gas (35cm/sec); the temperature program was: 50°C (2 min) 10°C per min to 280°C (5 min). GC-mass spectrometry (MS) of compounds that (*i*) elicited responses from antennae of male or female BEBs, and (*ii*) varied in abundance as the season progressed (Figure 2.4) employed a Saturn 2000 Ion Trap GC-MS (Varian) fitted with the DB-5 column referred to above. Compounds were identified by comparing their retention indices (Van den Dool and Kratz 1963) and mass spectra with those reported in the literature [(*Z*)-3-hexenol, hexanol, nonane, pentyl acetate, 6-methyl-5-hepten-2-one, *trans*-ocimene, linalool (enantiomeric composition not known), nonanal, methyl salicylate, decanal, 2-phenethyl acetate, tridecane, *cis*-jasmone, (*E*)-β-farnesene, α-farnesene, *trans*-nerolidol, hexadecane, eicosane, heneicosane, tricosane (Adams 1989); (*E*)-4,8-dimethyl-1,3,7-nonatriene, phenylacetonitrile, *trans*-caryophyllene, tetradecanal, pentadecanal, methyl hexadecanoate (McLafferty 1994-98); (*Z*)-3-hexenyl acetate, (*Z*)-3-hexenyl butyrate, undecanal (Jennings and Shibamoto 1980); *iso*-caryophyllene (Joulain and König 1998] and of authentic standards (Table 2.1).

2.3.4 General Bioassay Procedures

Anemotactic (upwind) responses of walking 3-d starved adults or 3- to 4-d starved 5th instar nymphs to 10 GHE aliquots of headspace volatile extracts (a dose found suitable in preliminary experiments) or to synthetic chemicals (see Table 2.2 for specific test stimiuli) were bioassayed at 25-28°C in a horizontal Y-shaped Pyrex® glass olfactometer (stem 20 cm × 2.5 cm ID; side arms at 120°; 18 cm long) illuminated from above by two horizontal fluorescent mercury lamps (GE™, Ecolux; 5000 K, 32 W). For each replicate, treatment and control stimuli were randomly assigned (coin toss) to and pipetted onto Whatman® 1.5-cm filter paper positioned near the orifice of side arms. In all experiments, a water aspirator drew humidified air at 0.5 L/min through the Y-tube carrying volatiles from the treated filter paper towards the insect that was released from a holding tube at the base of the Y-tube. For each replicate, a clean Y-tube and a new insect were employed. An insect was scored as a responder if within 5 min it approached a test stimulus within 2.5 cm. Non-responding insects were excluded from statistical analyses. Olfactometers were washed in warm water with Sparklene™ detergent, rinsed with cold tap and distilled water, and oven-dried at ca. 125°C for at least 1 h.

I am predicting that females, males and nymphs respond similarly to host semiochemicals, but have tested them separately not to miss any gender- or stage-specific host foraging behavior. Similarly, we had kept track of the insects' sexual maturity and reproductive state in the unlikely event that they modulate the insects' response to host semiochemicals

2.3.5 Specific Experiments (Table 2.2)

Experiments 1-8 were designed to test whether the attractiveness of pistillate and staminate trees change as the growing season progresses. Specifically, experiments 1-4 (Figure 2.2) tested the response of post-diapause adult females and males to headspace volatile extracts from early-season (flowering) staminate or pistillate trees. Experiments 5-8 (Figure 2.2) tested the response of new-generation adult female and males to headspace volatile extracts of pistillate trees with immature samaras (experiments 5, 6) and mature samaras (experiments 7, 8).

Experiments 9-14 were designed to fast-track the identification of semiochemicals that mediated the attraction of BEBs to volatiles of pistillate trees with mature samaras (Figure 2.2). I considered the 22 volatiles that elicited an antennal response (Figure 2.3; Table 2.1) and their relative abundance in headspace analyses of pistillate trees with immature, mature and senescing samaras (Figure 2.4). Because 1-hexanol, pentyl acetate, phenylacetonitrile, 2-phenethyl acetate and *trans*-nerolidol appear when seeds are maturing on trees and decline thereafter, we therefore prepared a 5-component synthetic blend and tested the response of females, males and 5^{th}-instar nymphs to it (experiments 9-11) and to a 22-component synthetic blend that contained all EAD-active candidate semiochemicals (experiments 12-14) as a "positive control". Expecting attraction of insects to treatment stimuli in experiments 9-14, I anticipated no such responses in experiments 15 and 16, which were to volatiles from pistillate trees with senescing foliage and samaras (Figure 2.5).

With significant insect responses to the 5-component synthetic blend obtained in experiments 9-11 (Figure 2.5), experiments 17-28 (Figure 2.6) were designed to explore whether attractive volatile blends remain attractive to insects irrespective of time of

season, or the insects' life history stage or reproductive status. Specifically, experiments 17-20 and 21-24, respectively, tested the response of post-diapause and new-generation adults to the 5-component synthetic blend and to headspace volatiles from early-season staminate trees. Moreover, experiments 25-28 tested the response of sexually immature adults and reproductively active adults to the 5-component blend.

The block of experiments 29-42 (Figure 2.8) then aimed at determining the key components(s) in the 5-component blend. In experiments 29 and 30, we tested the 2-component blend of phenylacetonitrile and 2-phenethyl acetate because both compounds also occur in the volatile blend of early-season staminate trees, which are attractive to insects. Taking into account the attractiveness of the 2-component blend, follow-up experiments 31-34 tested it in comparison with the 5-component blend. To determine whether a single component may suffice for attraction of adults, experiments 35-36 tested 2-phenethyl acetate, and parallel-run experiments 37-39 compared the attractiveness of 2-phenethyl acetate (experiment 37), phenylacetonitrile (experiment 38) and the 5-component synthetic blend (experiment 39). Experiment 40 tested the response of males to phenylacetonitrile, and final experiments 41 and 42 compared the response of 5^{th}-instar nymphs to phenylacetonitrile (experiment 41) and 2-phenethyl acetate (experiment 42).

2.3.6 Statistical Analyses

Data of all experiments were analyzed with the χ^2 goodness-of-fit test, using Yates correction for continuity ($\alpha = 0.05$) (Zar, 1999). Logistic Regression analyses (Table 2.3) were used to test for effects of insect gender, developmental stage and reproductive state, host type, season, and synthetic chemical blend, and interactions thereof ($\alpha = 0.05$)

Fishers Exact test was used to test for differences in response by 5th-instar nymphs to synthetic-chemical blends ($\alpha = 0.05$). All analyses employed JMP statistical software.

2.4 Results

Headspace volatiles of early-season staminate trees, but not of pistillate trees, attracted both male and female post-diapause BEBs (Figure 2.2; experiments 1-4). Host type had a significant effect on the insects' response, but their gender did not (Table 2.3). Volatiles of branches with immature samaras (Table 2.1) did not attract females or males (Figure 2.2; experiments 5-6), whereas those of branches with mature samaras did (Figure 2.2; experiments 7-8). Host season had a significant effect on the insects' response, but their gender did not (Table 2.3).

In GC-EAD recordings of attractive extracts in experiments 7 and 8, 22 volatile components (Table 2.1) elicited antennal responses, most of which are shown in figure 2.3. To rapidly determine the key semiochemicals, we considered the components that elicited an antennal response (Figure 2.3) and varied in relative abundance in volatile profiles of pistillate trees throughout the season (Figure 2.4). Based on these criteria, five components [1-hexanol, pentyl acetate phenylacetonitrile, 2-phenethyl acetate and *trans*-nerolidol] were selected and bioassayed at quantities and ratios equivalent to those found in 10 GHE of pistillate trees with mature samaras (Table 2.1).

In experiments 9-14, both the 5-component synthetic blend and the 22-component synthetic blend of all antennal stimulatory components (Table 2.1) attracted females (Figure 2.5, experiments 9, 12), males (Figure 2.5, experiments 10, 13) and 5th-instar nymphs (Figure 2.5, experiments 11, 14). These results suggested that the key

semiochemicals were present in the 5-component blend. In experiments 9-14, insect gender and developmental stage as well as synthetic semiochemical blend had no effect on the insects' response (Table 2.3).

Considering that the five components were present at much reduced quantities in headspace volatile extracts of trees with senescing samaras (Figure 2.4), we predicted — and the results of experiments 15 and 16 confirmed (Figure 2.5) — that such extracts are not attractive to BEBs. Expectedly then, host season had a significant effect on the insects' response but their gender did not (Table 2.3).

Time of season, the insects' life history stage, and their gender did not significantly modulate their positive response to attractive volatile blends (Figure 2.6, experiments 17-24; Table 2.3), but the insects' reproductive state did (Figure 2.6, experiments. 25-28; Table 2.3). The 5-component blend attracted post-diapause and new-generation females and males (Figure 2.6, experiments 17, 18, 21, 22), strongly attracted sexually immature females and males (Figure 2.6, experiments 25, 26), and moderately attracted reproductively active males, but not females (Figure 2.6, experiment 27, 28). Attraction of post-diapause and new-generation females and males to the volatile blend of early-season staminate trees (Figure 2.6, experiments 19, 20, 23, 24) suggested — and GC-MS analyses confirmed (Figure 2.7) — that it contained constituents [phenylacetonitrile, 2-phenethyl acetate (trace)] of the 5-component blend.

In experiments 29 and 30, the synthetic blend of phenylacetonitrile and 2-phenethyl acetate equally attracted females and males (Figure 2.8; Table 2.3). In follow-up and parallel-run experiment 31-34, this 2-component blend was as effective as the 5-component blend in attracting females and males (Figure 2.8; Table 2.3). In

experiments 35 and 36, synthetic 2-phenethyl acetate as a single component attracted both females and males (Figure 2.8). In parallel-run experiments 37-39, 2-phenethyl acetate and phenylacetonitrile as single components were as effective as the 5-component blend in attracting females (Figure 2.8; Table 2.3). Similarly, in experiment 40, synthetic phenylacetonitrile strongly attracted males. In experiments 29-40, neither gender nor synthetic blend affected the insects' response (Table 2.3). In parallel-run and final experiments 41-42, phenylacetonitrile, but not 2-phenethyl acetate, strongly attracted 5th-instar nymphs (Figure 2.8).

2.5 Discussion

My data support the hypotheses that (1) BEBs are attracted to volatiles of early-season staminate trees bearing pollen; (2) volatile profiles of pistillate trees change as the season progresses; (3) pistillate trees with mature samaras are strongly attractive to BEBs; and (4) that the semiochemical phenylacetonitrile, mediates attraction of BEBs.

Insects emerging from overwintering diapause seek food sources that may increase their reproductive potential prior to mating (Tanaka et al. 1987; Tanaka and Wolda 1987; Kalberer et al. 2001). Post-diapause female and male BEBs are strongly attracted to volatile blends of early-season pollen-bearing staminate boxelder trees (Figure 2.2, experiments 1, 2; Table 2.3), but can be observed feeding on new-growth tissue of both staminate and pistillate trees.

The protein/nitrogen content of plants affects the growth, development and performance of many phytophagous insects (Strong et al. 1984), and its content in pollen is proportionally similar to that of insects (Strong et al. 1984), and likely

supplements the BEBs diet they eat as facultative cannibals and insect scavengers (JJS observations; see Brown and Norris 2004). Similarly, western conifer seed bugs, *Leptoglossus occidentalis*, and plum curculio beetles, *Conotrachelus nunuphar,* supplement their diets by feeding on staminate flowers in the spring (Blatt 1997; Leskey and Prokopy 2000).

Attraction of BEBs to pistillate trees (Figure 2.2, experiments 7, 8; Table 2.3) is correlated with the appearance of essential semiochemicals (Figures 2.3, 2.4) which, in turn, is linked to the presence of mature samaras. BEBs appear to synchronize their reproductive ecology with that of their host trees by responding to, and reproducing on, trees at the time of seed maturation when food energy and possibly metabolites for aposematic defense are abundant. As suggested for the heteropteran seed predators *Jadera* (Carroll and Loye 1987) and *Pachycoris* (Grimm and Führer 1998) and their respective hosts, the boxelder trees' tactics to escape seed predation may be to (*i*) mature seeds late in the season with little time for BEB populations to build and predation to take place, and (*ii*) synchronize fruiting so that not all fruits among trees are exploitable. Such compensatory plant-escape mechanisms against herbivory (Järemo et al. 1999; Agrawal 2000) are typically not under selection for subtropical trees that do not senesce and are fed on year-round by *Jadera haematoloma* bugs (Carroll, 1988; see Carroll and Loye, 1987 for exceptions). Here instead, trees and soapberry bugs appear to have entered an arms race, respectively evolving adaptive features that prevent seed predation, and mouthparts capable of breaching that defense (Carroll and Loye 1987; Carroll et al., 1998). Similarly, relationships between the timing of host colonization of *Leptocoris* spp. and bugs' beak length and seed

dehiscence are reported (Carroll et al., 2005a, b). Moreover, with seeds of host trees in temperate regions available only during two months per year (Carroll, 1988), time is of the essence for *J. haematoloma* populations that exploit these ephemeral resources. Accelerated growth and reproduction of temperate *J. haematoloma* populations likely evolved in response to host tree phenology (Carroll 1988; Carroll et al. 1998), and may be adaptive traits to save time and to most effectively utilize these fleeting resources. Locating them rapidly is another means of saving time and would explain why both *Jadera* spp. (Aldrich et al. 1990) and BEBs respond to semiochemicals indicative of seed availability (Figure 2.2, experiments 7, 8; Table 2.3).

Although volatile blends of early- and late-season pistillate trees were not attractive in bioassays (Figure 2.2, experiments 5, 6; Figure 2.5, experiments 15, 16; Table 2.3), field observations indicate that pistillate trees in spring and early summer suffer feeding damage by BEBs and other herbivores (i.e., aphids and thrips). Clipped branches of pistillate trees with BEBs feeding or with mechanical damage were most attractive to BEBs, and released higher levels of the key semiochemicals phenylacetonitrile and 2-phenethyl acetate than clipped branches with foliage devoid of insect and mechanical damage (data not shown). These data suggest that herbivory may promote the apparency of trees that approach seed maturation, thus likely affecting successional colonization of trees by BEBs.

Insects with piercing/sucking mouthparts, such as BEBs, are known to induce emissions of plant volatiles (Walling 2000; Kessler and Baldwin 2001; Rodriguez-Saona et al. 2002; Blackmer et al. 2004; Colazza et al. 2004; Williams III et al. 2005; Delphia et al. 2007), which could be exploited by herbivores as foraging cues (Walling

2000). Indeed, many of the antennal stimulatory compounds identified in our study (Table 1) have been identified in plants in response to herbivory or mechanical damage (Paré and Tumlinson 1996, 1997, 1999; Rose et al. 1996; Walling 2000; Pickett et al., 2003).

Moreover, early-season herbivory may activate dormant seed-producing meristems, thus enhancing the trees' reproductive output, and ultimately the food source for seed predators (Agrawal 1999, 2000; Järemo et al. 1999; Wise and Cummins 2006). Herbivory may even manipulate the seasonal ontogeny of a host tree to an optimal feeding stage for herbivores by "induced susceptibility" (Karban and Agrawal 2002). This may explain why trees colonized with BEBs senesce, and likely mature their seeds, earlier in the season than their uninfested counterparts (JJS, personal observation).

Availability and quality of food resources can affect insects' decisions regarding when to stay and feed and when to leave (Dingle 1974). When boxelder seeds enter the stage of dormancy (Figure 2.1) and BEBs cease to reproduce, phenylacetonitrile and 2-phenethyl acetate decline to trace quantities (Figure 2.4), conveying both unattractiveness of such volatile sources to BEBs (Figure 2.5, experiments 15, 16; Table 2.3), and possibly a low quality food resource. That reproductively diapausing, pre-overwintering BEBs in late-season laboratory bioassays were still attracted to synthetic blends containing phenylacetonitrile and 2-phenethyl acetate (Figure 2.5, experiments 9-11, 12-14; Table 2.3) suggests that these two semiochemicals are indicative of high-quality food sources. Provisioned with a low-quality diet, female milkweed bugs, *Oncopeltus fasciatus*, delayed reproduction and undertook long flights similar to those of diapausing conspecifics (Rankin and Riddiford 1977, 1978). Reproductively

diapausing, pre-overwintering BEBs feeding on senescing hosts (JJS, personal observation) were not attracted to them (Figure 2.5, experiments 15, 16) but remained there to attain the "last-minute" nutrients they need to complete development as adults and to build nutritional reserves for winter survival. Small pre-overwintering adults (JJS, personal observation) may have traded off size for time to complete development as adults and to attain sufficient nutritional reserves before seeking overwintering sites. That food resources or nutritional reserves of insects trigger behavior associated with pre-overwintering and winter survival is well reflected in ladybird beetles, *Coccinella septempunctata.* Late-season foraging *C. septempunctata* with sufficient nutritional reserves changed behavior from feeding to overwintering aggregation, and experienced significantly lower overwintering mortality than their malnourished counterparts (Barron and Wilson, 1998).

The BEBs' reproductive state considerably modulated their positive response to host semiochemicals (Figure 2.6, experiments 25-28; Table 2.3). During the first week post molting, sexually immature adults feed heavily (JJS personal observations), which may explain their relatively stronger response to host semiochemicals (Figure 2.6, experiments 25, 26) than that of reproductive adults (Figure 2.6, experiments 27, 28). Heavy feeding of newly molted adults has also been observed in *O. fasciatus* (Caldwell and Rankin, 1974), and been interpreted as an adaptation to a lifestyle of migration and colonization (Dingle 1985). The fact that mated females concerned with oviposition, unlike virgin females and other adults, no longer respond to the attractive 5-component blend (Figure 2.6, experiments 25-28; Table 2.3) is likely linked to their reproductive ecology and physiology. Assuming that such females are already present on resources

they seek for food and oviposition, directional orientation to resource-related semiochemicals may no longer be needed. Moreover, females focused on oviposition may trade off mobility for optimal fecundity. In female cotton stainer seed bugs, *Dysdercus* spp., availability of food (and oviposition sites) induced histolysis of wing muscles and rapid development of eggs (Dingle and Arora 1973). Similarly, well-fed female *O. fasciatus* contain high levels of juvenile hormone, which suppress migratory flight and stimulate ovarian development (Rankin and Riddiford 1977, 1978), mechanisms highly adaptive at ensuring successful oviposition (Caldwell and Rankin 1974). In sub-tropical regions, temporal food availability also induced histolysis of wing muscle in *J. haematoloma* bugs, but this phenomenon correlated with the lack of both migration and overwintering dormancy, and instead selected for traits in both females and males that produced flightless morphs, i.e. stunted wings and no flight muscle, compared to migrating conspecifics (Carroll et al., 2003b). *B. rubrolineata* is a migrating temperate species that enters overwintering dormancy (JJS observations). That reproductively active BEB males, unlike sedentary and gravid females, remain responsive to the 5-component blend (Figure 2.6, experiment 28) may allow them to seek further food sources and ultimately mating opportunities with females.

Phenylacetonitrile, rather than 2-phenethyl acetate, appears to be the key host plant kairomone. Our conclusion is based on the findings (*i*) that only phenylacetonitrile as a single component attracted both adults and nymphs (Figure 2.8, experiment 38, 40, 41; Table 2.3), and (*ii*) that phenylacetonitrile greatly exceeded and paralleled the abundance of 2-phenethyl acetate in headspace volatiles of early-season staminate trees (Figure 2.7) and mid-season pistillate trees, respectively (Figure 2.4).

There is suggestive evidence that phenylacetonitrile is linked to the trees' production of secondary metabolites (i.e., flavonoids, cyanogenic glycosides, glucosinolates) that help plants defend against herbivores. Plants producing phenylacetonitrile are known to store secondary metabolites that specialist herbivores, like BEBs, may sequester for secondary (aposematic) defense. Plants in the Brassicaceae family produce phenylacetonitrile in the biosynthesis of defensive benzylglucosinolates (Van Etten and Tookey 1979; Louda and Mole 1991). In oil seed rape, *Brassica napus* and *B. rapa*, phenylacetonitrile as a glucosinolate metabolite attracts both the specialist cabbage seed weevil, *Ceutorhynchus assimilis* (Bartlet et al. 1997; Smart and Blight 1997) and the cabbage butterfly, *Pieris rapae* (Ômura et al. 1999). All major groups of vascular plants store cyanogenic glycosides for defense against herbivory and biosynthesize them from amino acids such as phenylalanine (Seigler, 1991). Plants also use phenylalanine to biosynthesize flavonoids (Herbert 1989; Petersen et al. 1999), which function as attractive floral colors as well as feeding deterrents against herbivores (Harborne 1991). However, some flavonoids, such as isoquercitrin, stimulate feeding by specialist herbivores, as demonstrated for silkworm larvae, *Bombyx mori*, feeding on foliage of mulberry trees, *Morus alba* (Hamamura et al. 1962). BEBs as specialist herbivores of boxelder trees attain their aposematic coloration by ingesting flavonoids (Palmer and Knight 1924), and may gain secondary defense by ingesting or metabolizing cyanogenic toxins from seeds of their host trees, as other aposematic true bugs do (Aliabadi et al. 2002).

Phenylacetonitrile has been reported also as a pheromone in diverse contexts. It is a male-produced anti-aphrodisiac pheromone in the cabbage butterfly, *Pieris brassicae,*

rendering mated females less attractive to conspecific males, but attractive to the egg-parasitic wasp *Trichogramma brassicae* (Fatouros et al. 2005). Phenylacetonitrile is a male-produced courtship-inhibition pheromone in the desert locust, *Schistocera gregaria*, masking mated females and inhibiting male-male homosexual encounters (Seidelmann et al. 2000; Seidelmann and Ferenz 2002; Seidelmann et al. 2005). It is produced in wings and legs of *S. gregaria* as revealed by incubating them with ^{14}C-phenylalanine resulting in the biosynthesis of ^{14}C-phenylacetonitrile (Seidelmann et al. 2003).

2-Phenethyl acetate is a wide-spread constituent of floral and fruit scents (Knudsen and Tollsten 1993). Emitted from boxelder trees with mature fruit (samaras), 2-phenethyl acetate attracts adult females and males (Fig.7, experiments 35-38), conceivably as a foraging cue indicative of harvestable food sources. 2-Phenethyl acetate is likely a metabolite formed from phenylalanine followed by acetylation of 2-phenylethanol by alcohol acetyltransferases unique to plants (Guterman et al. 2006).

In summary, my data indicate that specific semiochemicals from the boxelder tree attract the specialist boxelder bug herbivore. The semiochemical phenylacetonitrile attracts foraging BEBs to early-season, pollen-bearing staminate trees and to mid-season pistillate trees with maturing seeds. By responding to phenylacetonitrile, BEBs appear to track and exploit nutrient-rich food sources. Considering the ecology of BEBs, there is suggestive evidence that phenylacetonitrile is likely linked to the trees' defense against herbivory. However, it remains to be shown that BEBs sequester secondary metabolites from boxelder trees for secondary defense against predation. If so demonstrated, phenylacetonitrile would constitute a plant kairomone that conveys

information to a specialist herbivore where and when to obtain food and secondary defense.

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TABLE 2.1 List of antennal stimulatory components in GC-EAD analyses (Figure 2.3) in headspace volatile extracts of pistillate boxelder trees, *Acer negundo*, with immature ovules and maturing seeds.

L[A]	RI[B]	Developing Ovules[C]	ng[D]	Maturing Seeds[C, E, F]	ng[D]	Source
a	860	(*Z*)-3-hexenol*	7	(Z)-3-hexenol*	13	Aldrich
b	868			**hexanol**	4.8	Aldrich
c	900	nonane	0.4			Aldrich
d	909			**pentyl acetate**	1.2	SFU[H]
e	981	6-methyl-5-hepten-2-one*	0.2	6-methyl-5-hepten-2-one*	0.6	Aldrich
f	1003	(*Z*)-3-hexenyl acetate*	2.4	(Z)-3-hexenyl acetate*	18	SFU[I]
g	1049	*trans*-ocimene*	17.8	*trans*-ocimene*	10	IFF
h[G]	1099	(+)-linalool*	1.8	(+)-linalool*[G]	1.4	Fluka
i	1105	nonanal	1.6	nonanal	1.2	Aldrich
j	1114	(*E*)-4,8-dimethyl-1,3,7-nonatriene*	22.6	(*E*)-4,8-dimethyl-1,3,7-nonatriene*	25	SFU[J]
k	1140			**phenylacetonitrile**	1	Aldrich
l	1185	(*Z*)-3-hexenyl butyrate*	1.8	(*Z*)-3-hexenyl butyrate*	0.2	Aldrich
m	1193	methyl salicylate*	1.2	methyl salicylate*	2	Aldrich
n	1206	decanal	1	decanal	0.4	Sigma
o	1257			**2-phenethyl acetate**	1.4	SFU[K]
p	1300	tridecane	0.2			Aldrich
q	1308	undecanal	0.2			SFU[L]
r	1393	*cis*-jasmone*	0.5			Aldrich
s	1409	iso-caryophyllene*	0.2	iso-caryophyllene*	0.04	Aldrich
t	1426	*trans*-caryophyllene*	5.6	*trans*-caryophyllene*	0.1	Sigma
u	1449	(*E*)-β-farnesene*	0.3	(*E*)-β-farnesene*	0.5	Fluka
v	1490	unknown sesquiterpene	2.8	unknown sesquiterpene	2	
w	1505	α-farnesene*	42.8	α-farnesene*	94	Treatt[M]
x	1562			***trans*-nerolidol**	5.4	Sigma
y	1600	hexadecane	0.3			SFU[N]
z	1626	tetradecanal	0.8			SFU[O]
aa	1715	pentadecanal	0.2			Aldrich
bb	1926	methyl hexadecanoate	0.5	methyl hexadecanoate	0.4	Sigma
cc	2000	eicosane	0.4			ICN
dd	2100	hencosane	0.3			Aldrich
ee	2300	tricosane	0.2			Aldrich

[A]L = Letters refer to components in figure 3 that elicited antennal responses.
[B]RI = Retention indices (Van den Dool and Kratz 1963) on a DB-5 column (Figure 2.3).
[C]An asterisk (*) indicates a compound that reportedly is released by plants in response to herbivore-induced or mechanical damage.
[D]Amount in nanogram (ng) present in 2 Gram-Hour-Equivalents [GHE; 1 GHE = volatiles released from 1 gram (dry-weight) of ramate biomass during 1 h]; experimental dose = 10 GHE.
[E]All compounds in this column are constituents of the 22-component synthetic blend tested in behavioral experiments (Table 2.2).

tested in behavioral experiments (Table 2.2).

[F]Bold-face compounds were present in headspace volatile extracts only of mature samaras with maturing seeds, and are constituents of the 5-component synthetic blend (Table 2.2)

[G]At times of bioassays, the absolute configuration of linalool in headspace volatiles of boxelder trees was not yet known; we anticipated and bioassayed the (-)-enantiomer (~ 85% ee) but later learned that (+)-linalool is the natural enantiomer.

[H]Acetylated pentanol (Aldrich)

[I]Acetylated (*Z*)-3-hexen-1-ol

[J]Synthesized by Grigori Khaskin in Gries-laboratory

[K]Acetylated 2-phenylethanol (Fluka)

[L]Oxidized undecanol

[M]Treatt USA Inc. (Lakeland, Florida, 33805-7637)

[N]GC standard

[O]Oxidized tetradecanol (Aldrich)

TABLE 2.2 Stimuli and experimental insects tested in Y-tube olfactometer experiments 1-42

Exp.[a,b,c]	Treatment stimuli[d,e]	Bioassay insects	N[f]
1	Volatiles of staminate trees in early spring[g]	Post-diapause ♀[o]	49.
2	Volatiles of staminate trees in early spring[g]	Post-diapause ♂[o]	52.
3	Volatiles of pistillate trees in early spring	Post-diapause ♀[o]	50.
4	Volatiles of pistillate trees in early spring	Post-diapause ♂[o]	50.
5	Volatiles of pistillate trees with immature samaras[h]	New-generation ♀[p]	27.
6	Volatiles of pistillate trees with immature samaras[h]	New-generation ♂[p]	27.
7	Volatiles of pistillate trees with mature samaras[i]	New-generation ♀[p]	27.
8	Volatiles of pistillate trees with mature samaras[i]	New-generation ♂[p]	27.
9	5-Component synthetic blend[j]	Pre-overwintering ♀[p]	32.
10	5-Component synthetic blend[j]	Pre-overwintering ♂[p]	35.
11	5-Component synthetic blend[j]	5th-Instar nymphs	26.
12	22-Component synthetic blend[k]	Pre-overwintering ♀[p]	32.
13	22-Component synthetic blend[k]	Pre-overwintering ♂[p]	24.
14	22-Component synthetic blend[k]	5th-instar nymphs	29.
15	Volatiles of pistillate trees with senescent samaras[l]	Pre-overwintering ♀[p]	29.
16	Volatiles of pistillate trees with senescent samaras[l]	Pre-overwintering ♂[p]	23.
17	5-Component synthetic blend[j]	Post-diapause ♀[o]	25.
18	5-Component synthetic blend[j]	Post-diapause ♂[o]	24.
19	Volatiles of staminate trees in early spring[g]	Post-diapause ♀[o]	25.
20	Volatiles of staminate trees in early spring[g]	Post-diapause ♂[o]	25.
21	5-Component synthetic blend[j]	New-generation ♀[q]	21.
22	5-Component synthetic blend[j]	New-generation ♂[q]	20.
23	Volatiles of staminate trees in early spring[g]	New-generation ♀[q]	21.
24	Volatiles of staminate trees in early spring[g]	New-generation ♂[q]	25
25	5-Component synthetic blend[j]	Immature ♀[q]	23
26	5-Component synthetic blend[j]	Immature ♂[q]	22
27	5-Component synthetic blend[j]	Reproductive ♀[r]	25.
28	5-Component synthetic blend[j]	Reproductive ♂[r]	25
29	Phenylacetonitrile + 2-phenethyl acetate (5 + 7 ng)[m]	New-generation ♀[q]	30
30	Phenylacetonitrile + 2-phenethyl acetate (5 + 7 ng)[m]	New-generation ♂[q]	32
31	5-Component synthetic blend[j]	New-generation ♀[q]	20
32	5-Component synthetic blend[j]	New-generation ♂[q]	20
33	Phenylacetonitrile + 2-phenethyl acetate (5 + 7 ng)[m]	New-generation ♀[q]	20
34	Phenylacetonitrile + 2-phenethyl acetate (5 + 7 ng)[m]	New-generation ♂[q]	20
35	2-Phenethyl acetate (10 ng)	New-generation ♀[q]	18
36	2-Phenethyl acetate (10 ng)	New-generation ♂[q]	18
37	2-Phenethyl acetate (10 ng)	Pre-overwintering ♀[q]	20
38	Phenylacetonitrile (10 ng)[n]	Pre-overwintering ♀[q]	20
39	5-Component synthetic blend[j]	Pre-overwintering ♀[q]	20
40	Phenylacetonitrile (10 ng)[n]	Pre-overwintering ♂[q]	22
41	Phenylacetonitrile (10 ng)[n]	5th-Instar nymphs	20
42	2-Phenethylacetate (10 ng)	5th-Instar nymphs	20

[a]Grouped experiments were run in parallel.
[b]Dose-response experiments (not listed here) determined that 10 Gram-Hour-Equivalents (see footnote of Table 2.1) were a suitable dose.
[c]A control- vs. control-stimulus experiment revealed no side bias of the bioassay set-up.
[d]The control stimulus (pentane) consisted of the same amount of solvent (10 μL) as dispensed with the treatment stimulus.
[e]Headspace volatiles of early-season staminate and pistillate trees in 2006 were tested in parallel at 10 GHE, at quantities of ca. 900 ng and ca. 1000 ng, respectively. In 2007, volatiles of early-season staminate trees, when tested in parallel to either the 5- or 22-component synthetic blends, were tested at ca. 930 ng in 10-μL aliquots (equivalent to the amount of the 22 antennal stimulatory components in pistillate trees with maturing seeds at 10 GHE; see footnote of table 1)
[f]N = number of insects bioassayed
[g]Bearing pollen
[h]Immature samaras contain immature ovules
[i]Mature samaras contain maturing seeds
[j]The blend comprised hexanol (24 ng), pentyl acetate (6 ng), phenylacetonitrile (5 ng), 2-phenethyl acetate (7 ng) and *trans*-nerolidol (27 ng)
[k]For blend constituents and their quantities see columns 5 and 6 of Table 2.1
[l]Senescent samaras contain ripening seeds entering dormancy
[m]The amounts of synthetic test chemicals reflect those of natural constituents in head space volatiles of pistillate trees with maturing seeds at 10 GHE.
[n]The amount of synthetic test chemical reflects that of the natural constituent in head space volatiles of early-season staminate trees at 10 GHE.
[o]adult age unknown
[p]< 14 days old adults; (note: pre-overwintering adults are in reproductive diapause)
[q]4- to 6-day-old adults; (note: pre-overwintering adults are in reproductive diapause)
[r]> 30-day-old adults

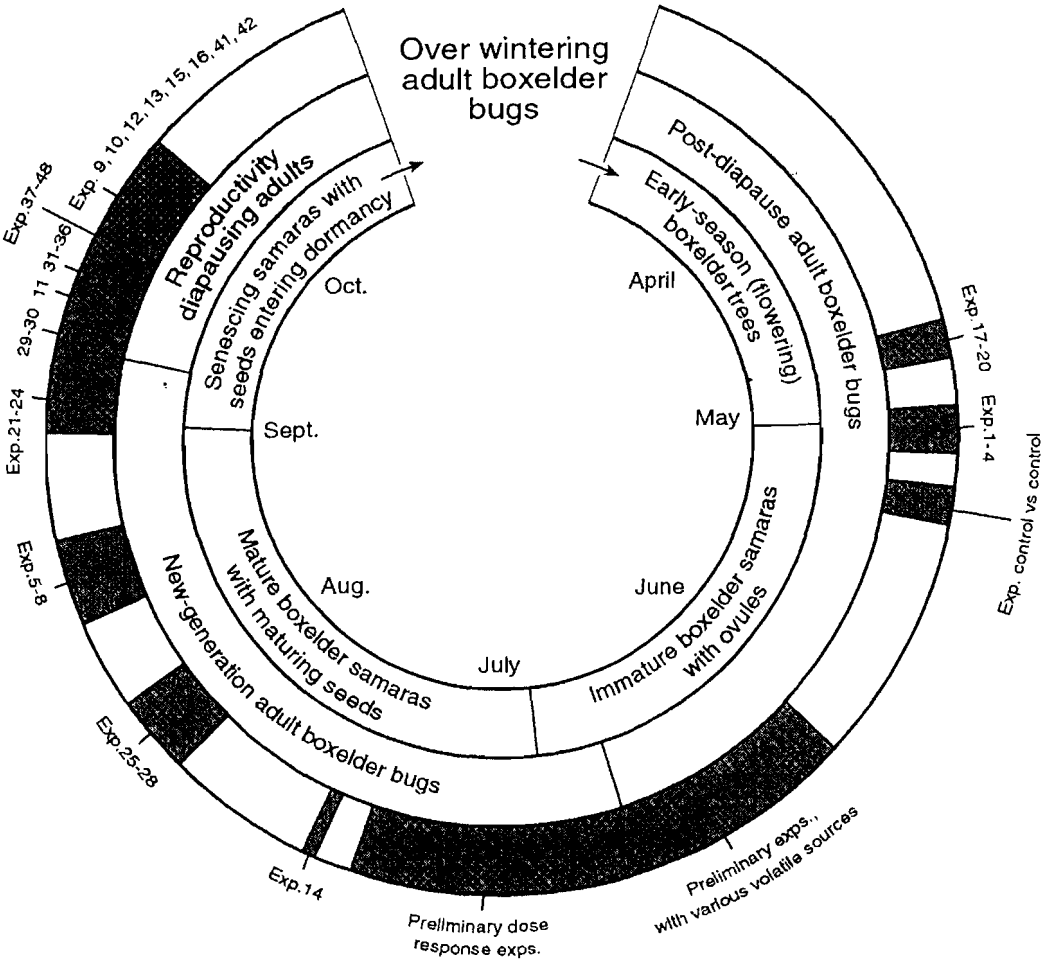
TABLE 2.3 Logistic Regression analyses of the effects of *Boisea rubrolineata* gender, developmental stage, reproductive state, host season, host type, synthetic blends, or interactions (×) between them, tested in experiments 1-40. An asterisk (*) indicates a significant effect (L-R $\chi 2$ test; *P ≤ 0.05, **P ≤ 0.01)

Logistic Regression Analysis					
Experiments	Source(s)	Effects	*df*	$\chi 2$	*P*-value
1-4	(early-season)	insect gender	1	0.163	NS[a]
	staminate tree	host type	1	4.63	**0.03***
	pistillate tree	gender × type	1	0.02	NS
5-8, 15-16	immature ovules	insect gender	1	0.112	NS
	maturing seeds	host season	2	6.965	**0.03***
	senescing tree	gender × season	2	0.147	NS
5-8	immature ovules	insect gender	1	0.004	NS
	maturing seeds	host season	1	5.032	**0.02***
		gender × season	1	0.004	NS
5-6, 15-16	immature ovules	insect gender	1	0.131	NS
	senescing tree	host season	1	0.004	NS
		gender × season	1	0.131	NS
7-8, 15-16	maturing seeds	insect gender	1	0.163	NS
	senescing tree	season	1	5.593	**0.02***
		gender × season	1	0.074	NS
9-10, 15-16	5-SB[b]	insect gender	1	0.342	NS
	senescing tree	season	1	4.319	**0.04***
		gender × season	1	0.031	NS
9-14	5-SB	insect gender[g]	2	2.914	NS
	22-SB[c]	synthetic blends	1	0.24	NS
		gender × blends	2	0.467	NS
		insect stage[h]	1	3.003	NS
		stage × blend	1	0.134	NS
17-24	5-SB	insect gender	1	0.012	NS
	staminate tree	insect stage[i]	1	0.001	NS
	(early-season)	gender × stage	1	0.338	NS
		host blend	1	1.396	NS
		stage × blend	1	0.004	NS
25-28	5-SB	insect gender	1	1.499	NS
		reproductive state	1	7.179	**0.007****
		gender × state	1	0.251	NS
25, 27	5-SB	reproductive state	1	6.335	**0.01****
26, 28	5-SB	reproductive state	1	1.927	NS
31-34	2-SB[d]	insect-gender	1	1.7	NS
	5-SB	synthetic blend	1	0.581	NS
		gender × blend	1	0.014	NS

37-39	5-SB PAN[e] 2-PEOAc[f]	synthetic blend	2	0.784	NS
31-32, 35-36, 38, 40	2-SB PAN 2-PEOAc	insect gender host blend gender × blend	1 2 2	0.35 0.151 0.293	NS NS NS
38, 40, 41	PAN	insect-gender[g]	2	0.105	NS

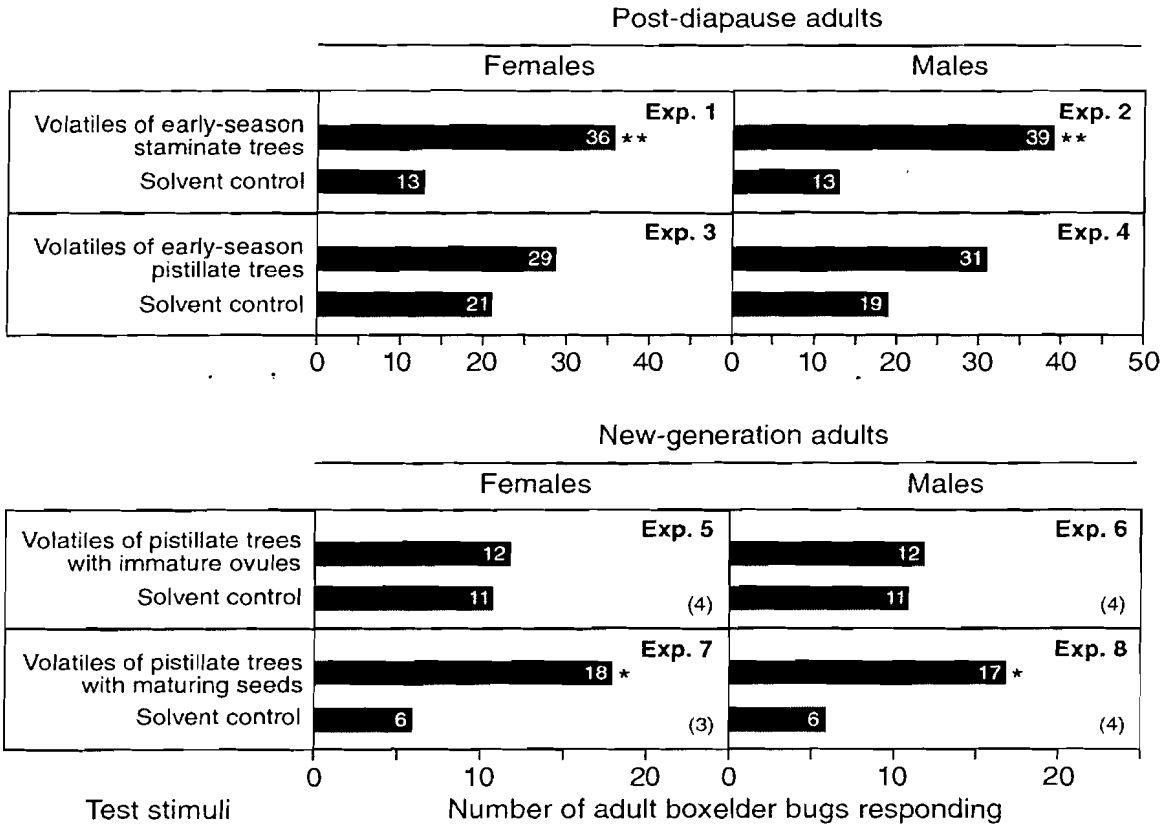
[a]NS = Not significant
[b]5-SB = Synthetic blend of the 5 antennally active volatiles of pistillate trees with maturing seeds, not present in trees with immature ovules (Table 2.1; Figure 2.3)
[c]22-SB = Synthetic blend of all 22 antennally active volatiles of pistillate trees with maturing seeds (Table 2.1; Figure 2.3)
[d]2-SB = A blend of phenylacetonitrile and 2-phenethyl acetate
[e]PAN = Phenylacetonitrile
[f]2-PEOAc = 2-Phenethyl acetate
[g]Insect genders: female, male, and 5th-instar nymph
[h]Insect stages: adults and 5th-instar nymphs
[i]Insect stages: post-diapause adults and new-generation adults

Fig. 2.1 Graphical illustration depicting aspects of the seasonal phenology of boxelder trees, *Acer negundo*, and western boxelder bugs, *Boisea rubrolineata*, and the time of season experiments (shaded portion of outer circle) were conducted.



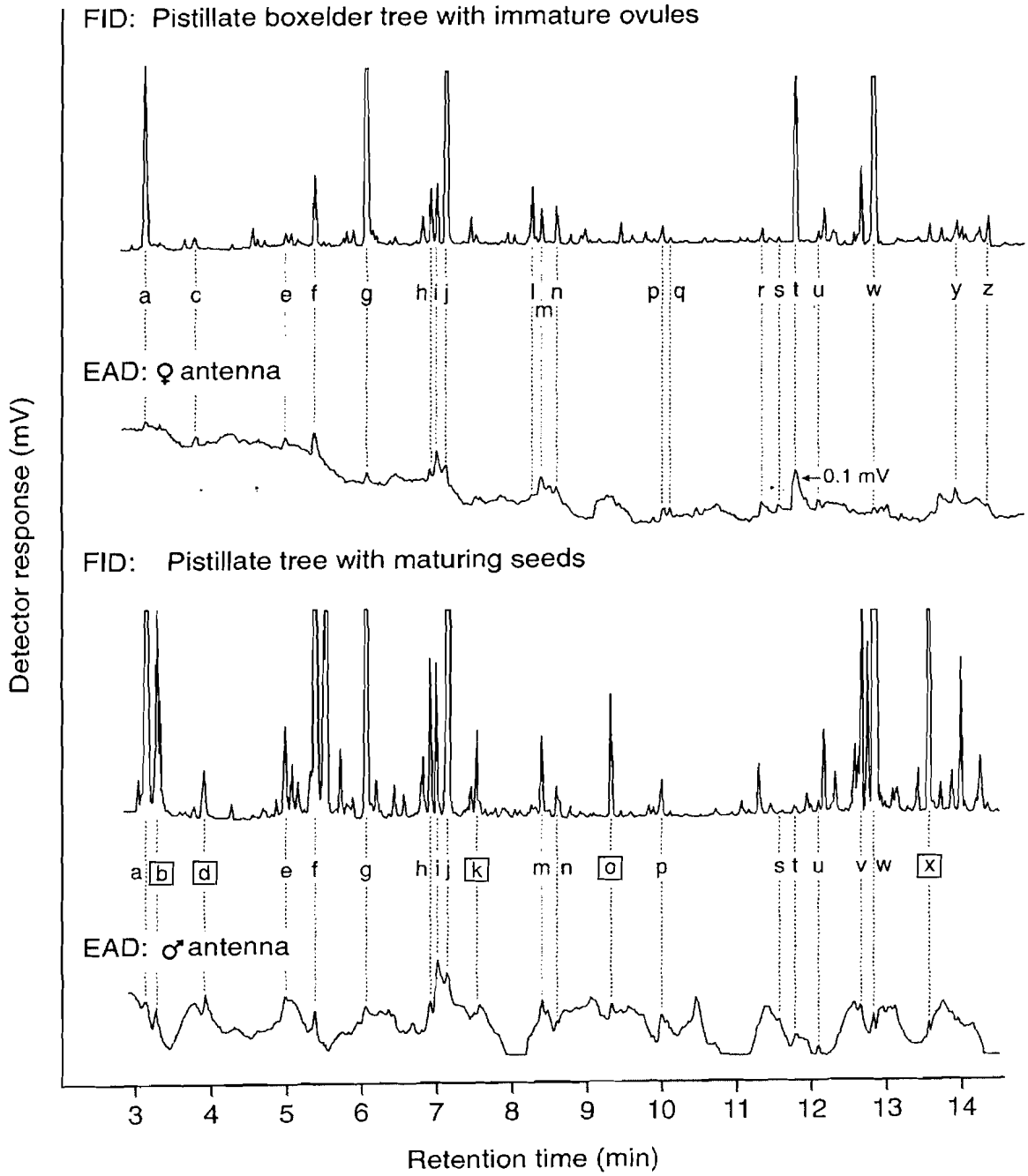
Gries_BEB_Fig1

Fig. 2.2 Response of post-diapause adult female and male western boxelder bugs, *Boisea rubrolineata*, in experiments 1-4 and of new-generation virgin adult females and males in experiments 5-8 to headspace volatile extracts of staminate (♂) and pistillate (♀) host trees, *Acer negundo*, in Y-tube olfactometers. Numbers in parentheses indicate number of non-responding insects. An asterisk (*) indicates a significant preference for a particular test stimulus ($\chi 2$ test; *$P < 0.05$; **$P < 0.01$).



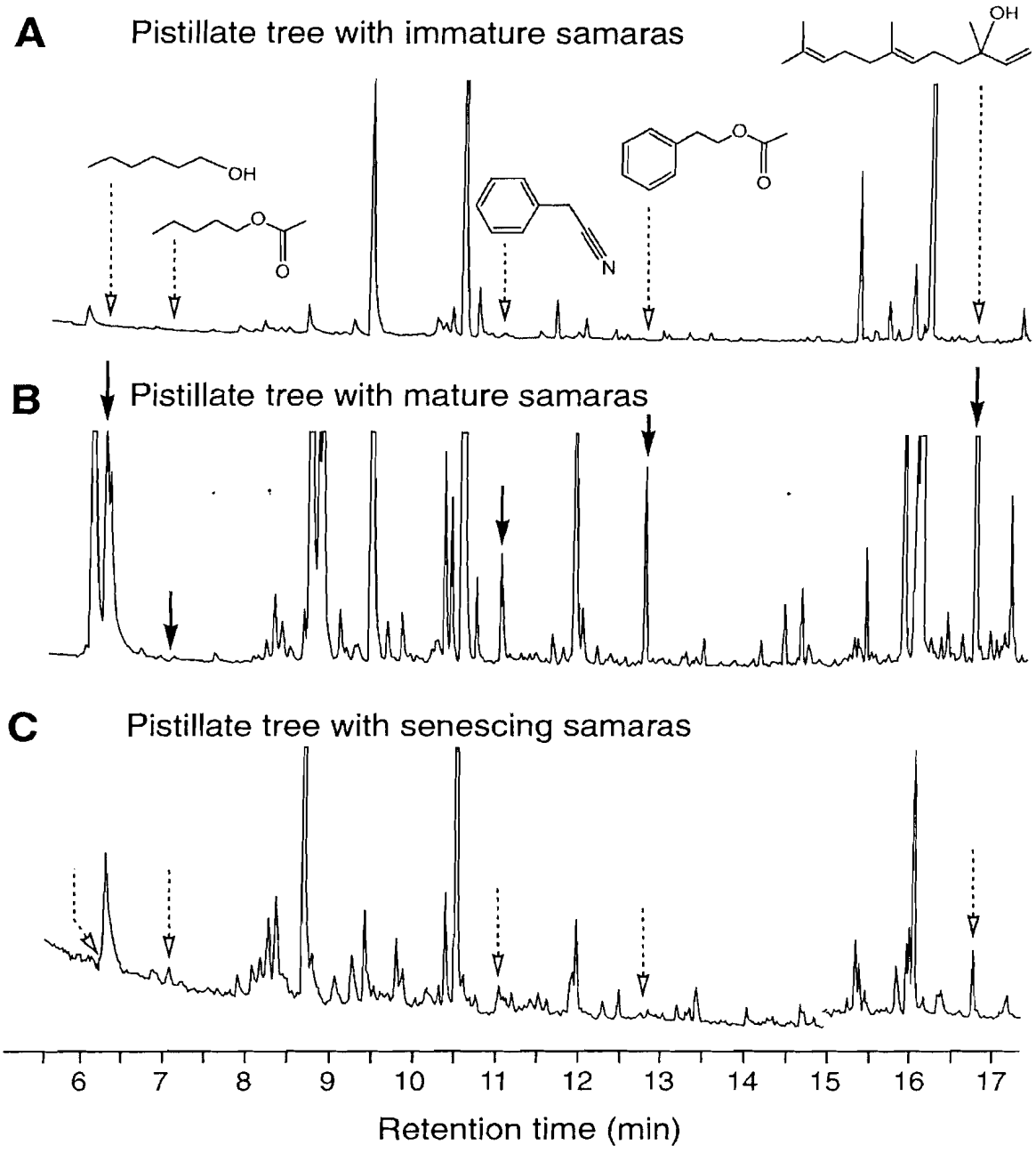
Gries_BEB_Fig2

Fig. 2.3 Representative recordings (N = 3) of flame ionization detector (FID) and electroantennographic detector (EAD: female or male *Boisea rubrolineata* antenna) responses to aliquots of headspace volatile extract from pistillate (♀) trees with immature samaras (top) and mature samaras (bottom). Compounds b, d, k, o and x, respectively, are 1-hexanol, pentyl acetate, phenylacetonitrile, 2-phenethyl acetate and trans-nerolidol. Additional antennal stimulatory components are listed in table 1. Chromatography: DB-5 column; splitless injection; temperature of injection port and FID: 240°C; temperature program: 50°C (2 min), 10°C per min to 280°C (5 min).



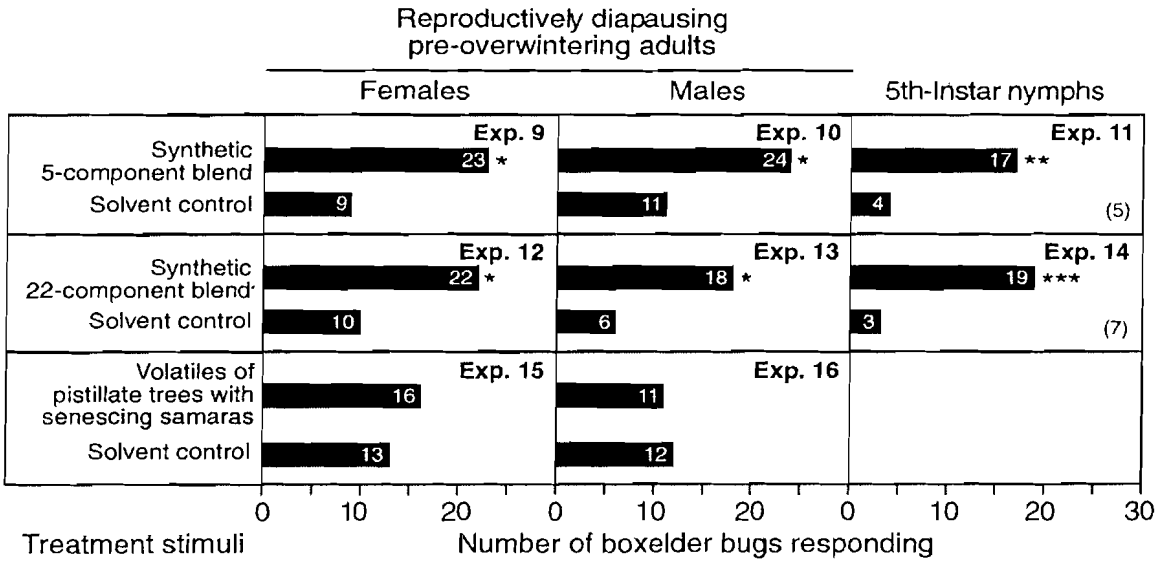
Gries_BEB_Fig3_1

Fig. 2.4 Headspace volatiles of pistillate boxelder trees, *Acer negundo*, with immature samaras (A), mature samaras (B) and senescing samaras (C). Note the absence of 1-hexanol, pentyl acetate phenylacetonitrile, 2-phenethyl acetate, and trans-nerolidol in (A), their prevalence in (B) and decline in (C). Chromatography: DB-5 column; splitless injection; temperature of injection port and FID: 250°C; temperature program: 50°C (2 min) 10°C per min to 280°C (5 min).



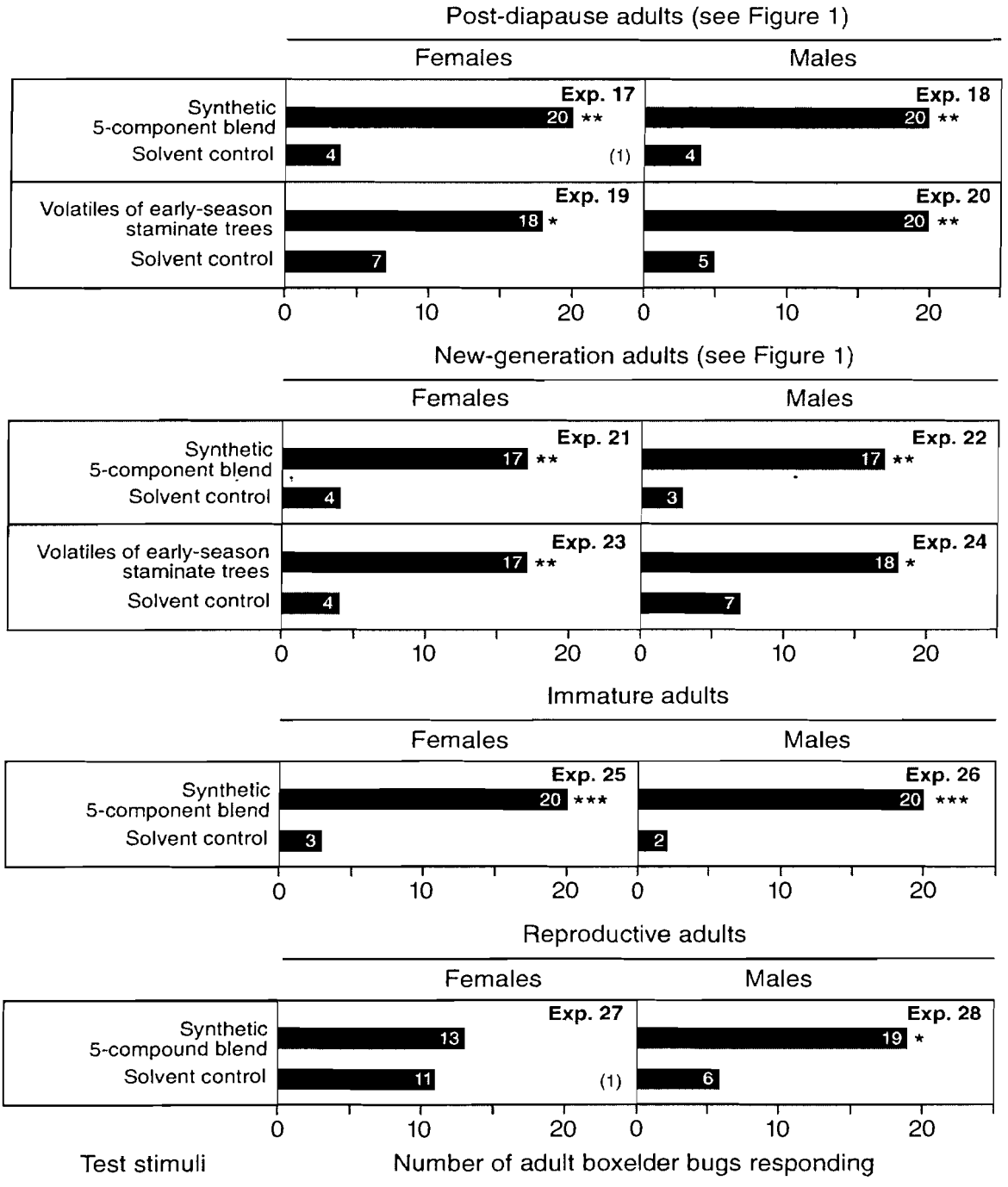
Gries_BEB_Fig4

Fig. 2.5 Response of 5th-instar nymph and virgin adult female and male western boxelder bugs, *Boisea rubrolineata*, in Y-tube olfactometer experiments to 5- or 22-component synthetic blends of candidate semiochemicals and to headspace volatile extract of pistillate (♀) trees with senescing samaras. Constituents of the synthetic blends are listed in Table 1. Numbers in parentheses indicate numbers of non-responding insects. An asterisk (*) indicates a significant preference for a particular test stimulus ($\chi 2$ test; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).



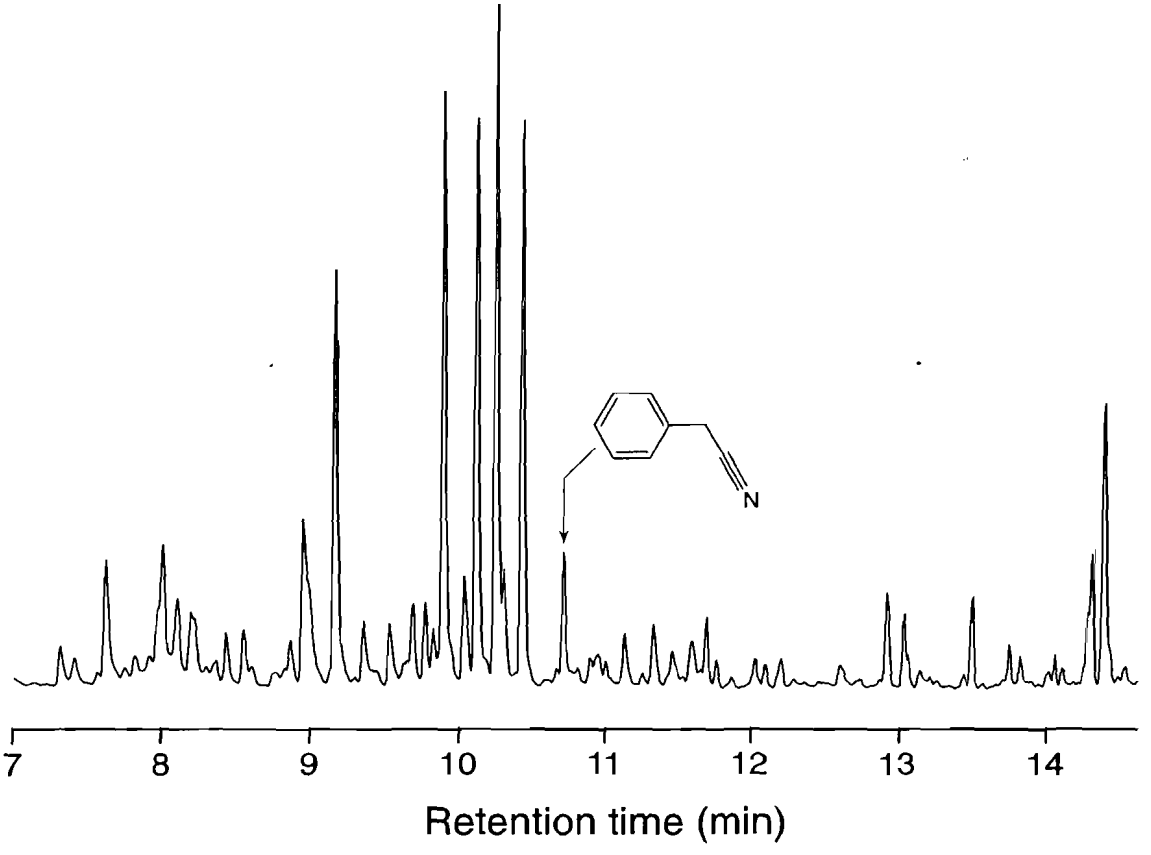
Gries_BEB_Fig5

Fig. 2.6 Response of female and male western boxelder bugs, *Boisea rubrolineata*, of different life history stage and reproductive status to a 5-component synthetic blend (see Table 1) or to headspace volatile extract of early-season staminate (♂) trees. Numbers in parentheses indicate numbers of non-responding insects. An asterisk (*) indicates a significant preference for a particular test stimulus ($\chi 2$ test; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).



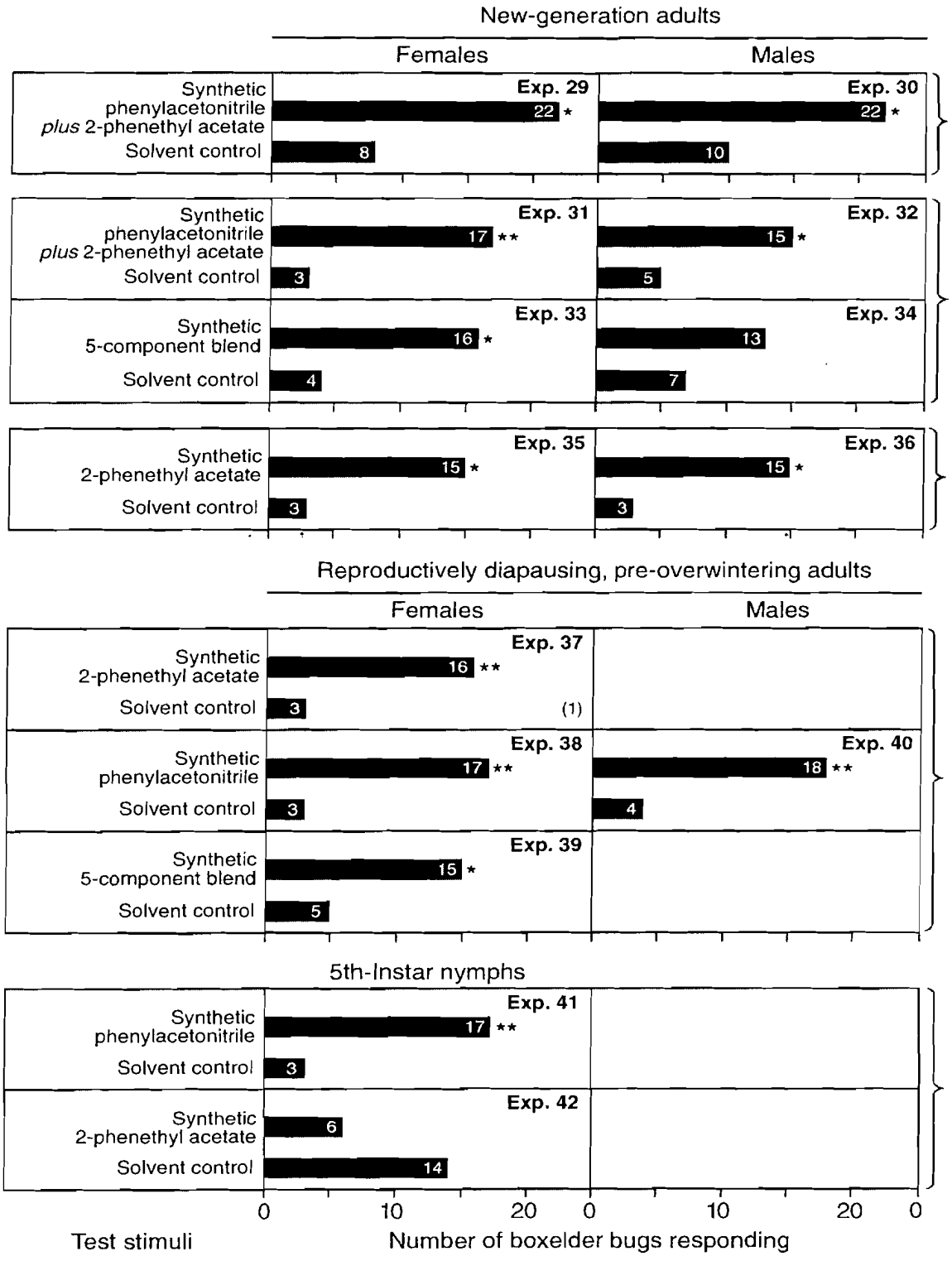
Gries_BEB_Fig6

Fig. 2.7 Headspace volatiles of early-season, pollen-bearing staminate boxelder trees, *Acer negundo*, containing appreciable amounts of phenylacetonitrile. Chromatography: DB-5 column; splitless injection; temperature of injection port and FID: 250°C; temperature program: 50°C (2 min) 10°C per min to 280°C (5 min).



Gries_BEB_Fig7

Fig. 2.8 Response of 5th-instar nymph and sexually immature adult female and male western boxelder bugs, *Boisea rubrolineata*, to various synthetic blends of candidate semiochemicals. Constituents of the 5-component blend are listed in Table 1. Numbers in parentheses indicate numbers of non-responders. An asterisk (*) indicates a significant preference for a particular test stimulus ($\chi 2$ test $^{*}P < 0.05$, $^{**}P < 0.01$). Note: experiment 40 was run not in parallel with experiments 37-39; in experiments 41 and 42, the test stimulus had a significant effect on responding of 5^{th} instar nymphs (Fisher's Exact Test, $\alpha = 0.05$, one-tailed, $df = 1$, $P = 0.0005$).



Gries_BEB_Fig8_1

3 2-PHENYLETHANOL: CONTEXT-SPECIFIC AGGREGATION OR SEX PHEROMONE OF THE WESTERN BOXELDER BUG, *BOISEA RUBROLINEATA*, (HETEROPTERA: RHOPALIDAE) AN APOSEMATIC SEED PREDATOR

3.1 Abstract

Boxelder bugs, *Boisea rubrolineata* (Heteroptera: Rhopalidae), form spectacular aggregations on pistillate boxelder host trees, *Acer negundo*, with mature seeds, in and around shelters in fall and winter, and in response to warm sunlight. We have recently shown that *B. rubrolineata* is attracted and aggregates in response to the host tree semiochemicals phenylacetonitrile and 2-phenethyl acetate. Here we reveal results of pheromone analyses and laboratory bioassays, demonstrating that aggregation and sexual communication in *B. rubrolineata* are also mediated by a complex pheromone system. It appears to include a female-specific sex pheromone that attracts males, a male-specific sex pheromone that attracts females, and a bi-functional pheromone (2-phenylethanol) that serves as an aggregation pheromone in mid-summer for females, males and 5th-instar nymphs, and as a female attractant pheromone in early spring. As an aggregation pheromone, 2-phenylethanol originates from the feces of seed-feeding females and males and/or the ventral abdominal gland of males. As a sex pheromone, 2-phenylethanol originates from the ventral abdominal gland of males that emerge from overwintering diapause. Attractiveness of the aggregation pheromone 2-phenylethanol to females and males in mid-summer, but not in fall, winter and spring, suggests that the aggregations of

B. rubrolineata in mid-summer, and in fall and winter, are mediated by at least two different pheromones.

3.2 Introduction

Boxelder bugs (Heteroptera: Rhopalidae) are well known to form spectacular aggregations numbering thousands of specimens (Aldrich *et al.*, 1990b; Schowalter, 1986). Aggregations form (*i*) on pistillate boxelder host trees, *Acer negundo*, with maturing seeds, (*ii*) in or near shelters suitable for overwintering, or (*iii*) in response to warm sunlight (Long, 1928; Smith and Shepherd, 1937; Knowlton, 1944; Tinker, 1952; Schowalter, 1986; Yoder and Robinson, 1990; Aldrich et al., 1990b; Robinson, 1996). Aggregations may enhance the insects' aposematic coloration that advertises noxiousness to predators (Guilford, 1990). Aggregations of *B. rubrolineata* apart from host tree sources suggest that they are mediated by pheromone (Schowalter, 1986). If so, the aggregation pheromone may concurrently deter potential predators (Pasteels *et al.*, 1983; Blum, 1996). For example, the milkweed bugs *Tropidothorax cruciger* and *Oncopeltus fasciatus* produce hexyl and octyl acetates as aggregation pheromones but emit methoxypyrazines as warning odor during aggregations (Aldrich *et al.*, 1997, 1999). The ladybird beetle *Coccinella septempunctata*, in contrast, produces a pyrazine aggregation pheromone that is also believed to deter predators (Al Abassi *et al.*, 1998).

Feces is one likely source of aggregation pheromone in *B. rubrolineata*. Feces induce aggregations in many orders of insects, including Collembola (Verhof, 1984; Marica *et al.*, 2001), Dermaptera (Walker *et al.*, 1993), Orthoptera (McFarland *et al.*, 1983), Blattodea (Ishii and Kuwahara, 1968; Scherkenbeck *et al.*, 1999), Thysanura

(Woodbury and Gries, 2008) and Heteroptera (Aldrich *et al.*, 1990a; Lorenzo and Lazzari, 1996; Vitta *et al.*, 2002). Kissing bugs, *Triatoma infestans*, defecate outside their harborage, which attracts and assembles conspecifics (Lorenzo and Lazzari, 1996). Similarly, *T. pseudomaculata* aggregates in response to feces-derived pheromone (Vitta *et al.*, 2002). The seed predatory soapberry bugs *Jadera haematoloma* and *J. sanguinolenta* release with their feces the aggregation pheromone 4-methyl-2(5H)-furanone, which attracts conspecifics to profitable seed resources (Aldrich et al, 1990a).

Specific glands are another likely source of pheromone(s) in *B. rubrolineata*. Male *B. rubrolineata* and *B. trivittata* (Aldrich et al., 1990b), and male leaf-footed bugs *Leptoglossus gonagara, Euthochtha galeator* (Aldrich et al, 1979) and *L. clypealis* (Wang and Millar, 2000), possess ventral abdominal glands (VAGs) which may secrete semiochemicals. In *J. haematoloma*, VAG secretions may help discourage male-male encounters (Carroll, 1988) because males are less likely to mount males than to mount females (Carroll, 1991). In male leaf-footed bugs (Aldrich *et al.*, 1976, 1979) and sympatric soapberry bugs (Aldrich et al, 1990b), VAG secretions are species-specific and correlated with the bugs' sexual maturity (Aldrich et al., 1976, 1979). In *L. clypealis*, VAG secretions contain the sex pheromone components benzyl alcohol and guaiacol (Aldrich et al., 1979; Wang and Millar, 2000).

2-Phenylethanol was isolated as a (major) constituent in VAG secretions of *B. rubrolineata, B. trivittata, L. gonagra,* and *E. galeator* (Aldrich et al., 1976, 1979, 1990b), of which *B. rubrolineata* and *B. trivittata* may be the same species (Schaefer, 1975; Aldrich et al, 1990b). 2-Phenylethanol has not yet been assigned an aggregation or sex pheromonal function in *B. rubrolineata*, but is known to be present in VAGs,

even near the end of the overwintering diapause (Aldrich et al., 1990b) when it might play a role in incipient early-spring communications of bugs.

Similar to *Boisea trivittata* (Yoder and Robinson, 1990), *Boisea rubrolineata* has two distinct mating seasons. Overwintered adults of the preceding year mate in spring, and new-generation adults of the current year mate in mid-summer. Aggregations of current-year adults on boxelder host trees, *Acer negundo*, are mediated by the host tree semiochemicals phenylacetonitrile and 2-phenethyl acetate (Chapter 2). Analogous to *Jadera* spp. (Aldrich *et al.* 1990a), aggregation behavior of *B. rubrolineata* may also be mediated by aggregation pheromone released from bugs heavily feeding and defecating on their host plants. If feces were to contain aggregation or sex pheromone(s), it might also mark feces-stained harborage for overwintering aggregations. *Boisea rubrolineata* emerging from overwintering diapause in early spring do not form large aggregations (as they do in mid-summer), and their sex ratio is male-biased (JJS, unpublished data), suggesting that they might engage in sexual rather than resource-based aggregation communication. If so, sexually receptive females in early spring might even guage the fitness of post-diapause males by their ability to retain or produce 2-phenylethanol in the VAG and to "call" (see Aldrich et al., 1990b).

Here I report (1) evidence for feces-derived, yet unknown female- and male-specific sex pheromones; (2) the presence of 2-phenylethanol in both the feces of seed-fed, mid-summer females and males and in the VAG of early-spring, post-diapause males; and (3) the attractiveness of synthetic 2-phenylethanol as an aggregation pheromone for mid-summer females, males, and fifth-instar nymphs, and as a sex attractant pheromone for early-spring females.

3.3 Methods and Materials

3.3.1 Collection and Maintenance of Experimental Insects

Late-instar nymphs and adults were collected weekly on or near pistillate trees of *A. negundo* or *A. saccharinum* in Kelowna, Westbank, Summerland, and Princeton, British Columbia, Canada during June, July, August and September 2004-2007. Insects were maintained at 22-30°C, 30-70% relative humidity, under a natural photoperiod (5000 K daylight spectrum), and were provisioned with water and new-growth shoots of *A. negundo* with intact leaves and seeds that were clipped weekly and transported to SFU in 19-L buckets of water. Newly molted adults were bioassayed at an age of 1-5 days. Newly-molted sexually immature adult females or males were kept separate and bioassayed within 14 days, before they reached sexual maturity (Smith and Shepherd, 1937; Yoder and Robinson 1990; Miller et al, 1991). Sexually mature virgin adults were bioassayed when they were 2-3 weeks old. Reproductive adults (engaging in mating activities) were 4-5 weeks old when they were used in bioassays. Adults emerging from diapause were field collected from late February through April, kept in 76-L glass tanks provisioned with water and last year's seeds of boxelder (supplemented with cracked sunflower seeds), and were used in bioassays as needed. Insects fed freely prior to experiments.

3.3.2 Collection of *B. rubrolineata* feces volatiles

Throughout the summer and into the fall, gender-specific or mixed groups of adults or of nymphs (totaling 700-1000 insects per group) were kept in 76-L rearing tanks lined with Scott® paper towels and allowed to feed throughout the summer on host plant cuttings

with maturing samaras retained in water in Erlenmeyer flasks (150 ml). Paper towels impregnated with feces were (i) bioassayed, or extracted with redistilled pentane, immediately following insect exposure, or (ii) bioassayed after 7-month left behind in rearing tanks, open to the air, at room temperature, simulating an aging volatile source at or near harborage. Extracts were kept in sealed vials cold-stored (4°C) until bioassayed. Paper towels processed the same way but without insect exposure, or paper towel extracts, served as control stimuli. The amount of analyte in one fresh (< 24-h old) fecal spot [= 1 Fecal-Spot-Equivalent (1 FSE)] was quantified by excising and hexane-extracting fecal spots deposited onto Whatman® 90 mm filter paper inside Pyrex® glass Petri dishes (125 mm) by feeding BEBs and by subjecting the analyte to gas chromatography (see below). Dead insects were replaced thrice daily to keep them from contaminating the filter paper.

3.3.3 Collection of Headspace Volatiles from Adults

Headspace volatiles were collected from 60 sexually mature, ~ 14-day old male or female BEBs. BEBs were starved and given only water for 3 days before they were placed into separate cylindrical Pyrex® glass chambers (10 × 23.75 cm) and allowed to feed on samaras and to obtain water. Charcoal-filtered air was drawn at ~ 0.5 L per min through each chamber and through a Pyrex® glass tube (14 × 1.3 cm OD) containing 500 mg of Porapak (50-80 mesh, Waters Associates, Inc., Milford, MA) for trapping headspace volatiles. After the first and second 24-hr aeration period, seeds and insects, respectively, were removed. The volatile trap was replaced at 24, 48 and 72 hr, and eluted with 2 ml of redistilled pentane. Extracts were cold-stored (4°C) in sealed vials prior to analysis.

3.3.4 Extracts of the Ventral Abdominal Gland (VAG)

CO_2-anesthetized males were pinned through the ventral thorax under tap water onto a wax-covered Petri dish (100 mm). Pressure was applied to the abdominal segments enclosing the genital capsule such that it everted, exposing through the thin inter-segmental membrane the VAG which was excised with forceps. The VAG of each male was placed into a separate ampule on dry ice containing 50 µl of hexane, and then macerated with the blunt end of a 10-µl stainless steal syringe plunger. After 10 min of extraction at room temperature, aliquots were analyzed by gas chromatography and mass spectrometry (see below). Remaining analyte was sealed in ampules and cold-stored (4°C).

3.3.5 Design of Y-tube Olfactometer Experiments

Anemotactic responses of walking BEBs to feces volatiles were tested at 24-27°C in a horizontal Y-shaped Pyrex® glass olfactometer (stem 20 cm × 23 mm ID; side arms at 120°; 18 cm long) illuminated from above by two horizontal fluorescent mercury lamps (GE™, Ecolux; 5000 K, 32 W). Insect-exposed paper towels impregnated with their feces (see above), or pentane extracts thereof, were used as test stimuli. For each replicate, treatment and control stimuli were randomly assigned to and positioned near the orifice of side arms. Extracts were pipetted onto Whatman® 1.5 cm filter paper. In all experiments, a water aspirator drew humidified air at 0.5 L min^{-1} through the Y-tube carrying volatiles from the treated sources towards the insect which was released from a

glass holding tube (60 × 22 mm OD) at the base of the Y-tube. For each replicate, a clean Y-tube and a new insect were employed. An insect was scored as a responder if within 5 min it approached a test stimulus within 2.5 cm. Non-responding insects were excluded from statistical analyses. Olfactometers were washed in warm water with Sparklene™ detergent, rinsed with cold tap and distilled water, and oven-dried at ca. 125°C for at least 1 h.

3.3.6 Design of Still-air Olfactometer Experiments

Arrestment responses of males and females to test stimuli were bioassayed at 25-28°C in a horizontal, three-chambered Pyrex® glass still-air olfactometer (Tremblay and Gries, 2003) illuminated from above by fluorescent "wide-spectrum grow light". Olfactometers consisted of a central Pyrex® glass Petri dish connected to two lateral Petri dishes (each dish 9 × 3 cm, and covered with a lid) via Pyrex® glass tubing (2 × 2.5 cm). For each replicate, a treatment and solvent (pentane) control stimulus were randomly assigned to and pipetted onto a Whatman® filter paper disc (42.5 mm) placed into each of the two lateral chambers. The bioassay insect was placed into the central chamber 1 h prior to scotophase and its position was recorded 1, 2, 3 and 15 h later. Insects not responding to either treatment or control stimuli were excluded from statistical analysis. For each replicate, a clean olfactometer and insect were employed. Olfactometers were washed in warm water with Sparklene™ detergent, rinsed with cold tap and distilled water, and were oven-dried at ca. 125°C for at least 1 h.

3.3.7 Specific Experiments

Experiments 1 and 2 tested whether reproductively-diapausing females and males are attracted to volatile extracts of paper towels exposed to and impregnated with feces from adults (Table 3.1). Experiments 3-5 tested whether reproductively-diapausing females (experiment 3), males (experiment 4) and fifth-instar nymphs (experiment 5) are attracted to paper towels exposed to and impregnated with feces from nymphs. Considering the attractiveness of test stimuli in experiments 1 and 2, experiments 6-11 then determined the optimal stimulus dose by testing attraction of virgin females (experiments 6, 8, 10) and virgin males (experiments 7, 9, 11) to extracts of paper towels exposed to and impregnated with feces from adults at 0.1, 1.0 or 10.0 FSE.

Attraction of males, but not females, to extracts of paper towels impregnated with adult feces in experiments 6-11, and attraction of males and females, but not nymphs, to paper towels impregnated with nymph feces in experiments 3-5, suggested the presence of gender- and stage-specific pheromones. Thus, experiments 12-17 tested the response of post-diapause females (experiments 12, 14, 16) and males (experiments 13, 15, 17) to insect-exposed paper towels impregnated with feces from males (experiments 12, 13), females (experiments 14, 15) and nymphs (experiments 16, 17), a time that adults come together to mate.

Taking into account both that headspace volatiles of feces-impregnated paper towels contained 2-phenylethanol (Figure 3.5) and that seed-fed females and males defecated large quantities of 2-phenylethanol (Figure 3.6), experiments 18-25 tested whether synthetic 2-phenylethanol (~ 0.25 insect equivalents; 50 ng) attracts females and males (experiments 18-23) as well as nymphs (experiments 24, 25) during their "explosive" breeding ecology of mid-summer.

Considering that 2-phenylethanol is (*i*) attractive to adults and fifth-instar nymphs in experiments 18-25, (*ii*) present in the males' VAG (Aldrich et al. 1990b), and (*iii*) likely released at various times of year, the final set of experiments 26-33 explored the response of adults to 2-phenylethanol before (experiments 26-27), during (experiments 28-31) and after overwintering diapause (experiments 32-33).

3.3.8 Analyses of Extracts

Aliquots of headspace volatile extracts were analyzed by coupled gas chromatographic-electroantennographic detection (GC-EAD; Arn et al. 1975; Gries et al. 2002), employing a Hewlett Packard 5890A gas chromatograph equipped with a GC column (30 m × 0.25 ID) coated with DB-5 (J&W Scientific, Folsom, CA). Helium was used as carrier gas (35cm/sec); the temperature program is reported in Figure 3. GC-mass spectrometry (MS) of compounds that elicited responses from antennae of male or female BEBs, or that were released from defecating insects in large quantities, employed a Saturn 2000 Ion Trap GC-MS (Varian) fitted with the DB-5 column referred to above. 2-Phenylethanol was identified by comparing the retention index (Van den Dool and Kratz 1963) and mass spectrum with that of an authentic standard.

3.3.9 Statistical Analyses

Data of all experiments were analyzed with the χ^2 goodness-of-fit test, using Yates correction for continuity ($\alpha = 0.05$) (Zar, 1999). Logistic Regression analyses (Table 3.3) were used to test for effects of insect gender, developmental stage, reproductive state,

season, and feces source, and interactions thereof ($\alpha = 0.05$). All data were analyzed using JMP statistical software.

3.4 Results

Reproductively-diapausing and pre-overwintering females and males were attracted to volatile extracts of paper towels exposed to and impregnated with feces from adults, with gender not affecting response (Figure 3.1, experiments. 1-2; Table 3.3). Similarly, reproductively-diapausing and pre-overwintering females and males, but not nymphs, were attracted to paper towels exposed to and impregnated with feces from nymphs (Figure 3.1, experiments. 3-5). The insects' developmental stage, but not their gender or the source of feces, had a significant effect on response (Table 3.3). Volatile extract of paper towels exposed to and impregnated with feces from adults tested at 0.1 FSE did not attract females or males (Figure 3.2, experiments 6, 7). However, the same extract tested at 1 and 10 FSE attracted males, but not females (Figure 3.2, experiments 8-11), which gave the first hint to a sex-specific pheromone. Logistic Regression analyses did not reveal a significant effect of gender (Table 3.3), but may do so with a larger sample size that would then corroborate with the results of the One-way Chi-square analysis in Figure 3.2.

Post-diapause females, but not males, were attracted to paper towels exposed to and impregnated with feces from males (Figure 3.2, experiments. 12, 13). Conversely, post-diapause males, but not females, were attracted to paper towels exposed to and impregnated with feces from females (Figure 3.2, experiments. 14, 15). Unlike females, post-diapause males were attracted to paper towels exposed to and impregnated with

feces from nymphs (Figure 3.2, experiments. 16, 17). Source of feces had no effect on the insects' response (Table 3.3). Gender affected the response to female and nymph feces, but not to male feces (Table 3.3). The interaction between gender × adult feces (Table 3.3), was near statistical significance, suggesting that a larger sample size may reveal that feces source affects the response of females and males, corroborating data analyses by One-way Chi-square test in Figure 3.2 (experiments 12-15). The aspect regarding sex-specific pheromones in *B. rubrolineata* clearly needs a more thorough exploration.

Sixty males separate from 60 females that were fed mature samaras during the height of their summer breeding season, defecated large quantities (183 ng/female; 175 ng/male) of 2-phenylethanol as the single most abundant component (Figure 3.5). In GC-EAD analyses of volatile extracts of paper towels exposed to and impregnated with feces from adults, nearly 60 components—including 2-phenylethanol—elicited responses from female and male antennae (Figure 3.6).

Newly molted (4- to 6-day old) females and males, sexually mature (12- to 15-day old) virgin females and males, reproductive (> 1 month-old) males, and fifth-instar nymphs all were attracted to synthetic 2-phenylethanol (Figure 3.3, experiments 19-24), but gravid and ovipositing reproductive females, and 4th-instar nymphs were not (Figure 3.3, experiments 18, 25). Unlike gender, reproductive state significantly affected the response of adult insects (Table 3.3).

Pre-overwintering females and males in reproductive diapause were not attracted to 2-phenylethanol (Figure 3.4, experiments 26, 27), with gender not affecting their response (Table 3.3). Likewise, females and males removed from overwintering

dormancy were not attracted to, or arrested by, 2-phenylethanol (Figure 3.4, experiments 28-31), with neither gender nor bioassay design affecting the insects' response (Table 3.3). However, comparing the response of pre-overwintering adults to adult feces (Figure 3.1, experiments 1, 2), nymph feces (Figure 3.1, experiments 3, 4), or 2-phenylethanol (Figure 3.4, experiments 26, 27), there is a significant effect of test stimulus (Table 3.3). Analyses of the interaction between gender of pre-overwintering adults × test stimulus (nymph feces, 2-phenylethanol) indicated that test stimulus affected the response of females (Table 3.3).

Finally, unlike post-diapause males, sexually mature virgin females (as evident by copulating conspecifics separately kept) carrying an egg load (as evident by their swollen abdomen) were attracted to 2-phenylethanol (Figure 3.4, experiments 32, 33). Gender of post-diapause insects had a significant effect on response, and there was a significant interaction between the insects' seasonal state × gender (Table 3.3). The females' response to 2-phenylethanol coincides with its presence in the males' VAG (Table 3.2), post-diapause mating, and a male-biased sex ratio of 3:2 in early-spring populations.

3.5 Discussion

My data reveal that pheromonal communication of *B. rubrolineata* is complex. It appears to include a female-specific sex pheromone that attracts males, a male-specific sex pheromone that attracts females, and a bi-functional pheromone (2-phenylethanol) that serves as an aggregation pheromone in mid-summer and as a sex attractant pheromone in early spring.

Multi-functional pheromones with identical molecular structure but a context-specific behavioral response are rare (Pasteels et al., 1983; Blum, 1996; Oldham and Boland, 1996; Borden, 1997; Norin, 2001; Hemptinne et al., 2000a,b; Robert et al., 2004; Fatouros et al., 2005; Miller et al., 2005a,b; Turillazzi, 2006; Yoder et al., 2006). This may be attributed to the fact that often candidate pheromones were identified without testing their potential role as communication signals (Aldrich, 1988; Millar, 2005). Multi-functional pheromones are known to occur in aggressive, tree-killing bark beetles. Consistent with the "optimal-attack-density" concept (Raffa and Berryman, 1983), these pheromones elicit dose-dependent, contrasting behavioral responses (Borden, 1997; and references cited therein). For example, at low concentration the aggregation pheromone *cis*- and *trans*-verbenol attracts mountain pine beetles, *Dendroctonus ponderosae*, but repels them at high concentration (Miller et al., 2005b). Similarly, the pheromone blend of ipsdienol plus lanierone is attractive to the pine engraver beetle, *Ips pini*, at low concentration but antagonistic at high concentration (Miller et al., 2005a). In the termite *Ancistrotermes pakistanicus* (3Z,6Z)-dodecadien-1-ol functions as a trail pheromone at very low concentration (0.1-10 pg/cm) and as a sex pheromone at high concentration (1 ng) (Robert et al., 2004).

The bi-functional pheromone 2-phenylethanol of *B. rubrolineata* differs from all others reported in the literature in three ways. First, context-dependent, it functions as an aggregation pheromone or as a sex pheromone. Second, attractiveness and function of the pheromone are in sink with both the seasonal phenology of the host tree and the reproductive ecology of *B. rubrolineata* (see below). Third, 2-phenylethanol as an aggregation and sex pheromone likely originates from different sources, the feces of

females and males in mid-summer and the ventral abdominal gland of males in early spring.

Seeds of trees in temperate regions are fleeting resources (Tinker, 1952; Carroll and Loye, 1987; Carroll, 1988; Kohno and Bui Thi 2005). Thus, time is of the essence for predatory seed bugs that exploit them. Accelerated growth and reproduction of seed predatory soapberry bugs, *Jadera haematoloma,* in temperate regions (Carroll, 1988) have evolved in response to this ephemeral resource (Carroll et al., 1998). As well, attraction of *B. rubrolineata* to host tree semiochemicals associated with mature seeds (Chapter 2) is likely an adaptive trait to save both foraging and developmental times, and to effectively utilize the fleeting resource. Release of aggregation pheromone 4-methyl-2(5H)-furanone by *Jadera* spp. when feeding on seeds of their golden rain host tree (Aldrich et al., 1990a), and release of 2-phenylethanol by female and male *B. rubrolineata* when feeding on seeds of *A. negundo* (Figure 3.5), both seem to signal the availability of high-quality yet ephemeral food. In concert with the host semiochemicals phenylacetonitrile and 2-phenethyl acetate (Chapter 2), 2-phenylethanol may then parsimoniously assemble conspecifics to boxelder trees and enhance the probability of mate encounter. This would explain why sexually mature female and male *B. rubrolineata* produce the aggregation pheromone (Figure 3.5) and respond to it during their "explosive" breeding ecology in mid-summer (Figure 3.3, experiments 19-23; Table 3.3).

The aggregation pheromone 2-phenylethanol in fecal secretions of 60 female or 60 male *B. rubrolineata* was present in large quantities, respectively ~ 11 μg and ~ 10.5 μg (Figure 3.5). The pheromone response threshold in *B. rubrolineata*, however, is

remarkably low. As little as 50 ng of 2-phenylethanol were sufficient to attract individual females, males and fifth-instar nymphs (Figure 3.3, experiments 19-24; Table 3.3). The group-response threshold for *Jadera* spp. to 4-methyl-2(5H)-furanone, by comparison, was estimated to be 50-100 μg for *J. haematoloma,* and 500 μg for *J. sanguinolenta* (Aldrich et al., 1990a). The differences in pheromone response threshold between *Boisea* and *Jadera* may be attributed to contrasting amounts of pheromone released for communication by both groups. 2-Phenylethanol released by female *B. rubrolineata* must have originated entirely from their feces because females lack the ventral abdominal gland, whereas males may have released 2-phenylethanol with their feces and/or from the ventral abdominal gland.

Plants and yeasts biosynthesize 2-phenylethanol from L-phenylalanine *via* the Ehrlich pathway (Albertazzi et al, 1994; Wittmann et al., 2002; Vuralhan et al., 2003). However, the biosynthesis of 2-phenylethanol by *B. rubrolineata* is yet to be studied. Flowering pistillate boxelder trees release trace amounts of 2-phenylethanol in spring, but in mid-summer, when they are heavily colonized and fed on by *B. rubrolineata*, they produce the corresponding acetate instead (Chapter 2). Unique plant acetyltransferases can acetylate 2-phenylethanol (Guterman et al, 2006), and appear do so also in *A. negundo*. These observations coupled with the large quantities of 2-phenylethanol in feces of seed-fed females and males (Figure 3.5) imply that *B. rubrolineata* itself biosynthesizes 2-phenylethanol, perhaps from host phenylalanine. The capability of two strains of yeasts, *Hansenula anomala* and *Kloeckera saturnus*, to respectively produce 2-phenylethanol and 2-phenethyl acetate from L-phenylalanine (Albertazzi et al, 1994) provides evidence for pertinent biosynthetic capacity outside

plants, and supports our hypothesis that *B. rubrolineata* may produce 2-phenylethanol from plant-derived L-phenylalanine.

The attraction of fifth-instar nymphs to 2-phenylethanol (Figure 3.3, experiment 24) may be controlled by their juvenile hormone (JH) titer which, in turn, is affected by "token" stimuli (e.g., photoperiod, food quality, temperature) and thus may induce reproductive behavior or migratory flight in adults. In *O. fasciatus,* a long photophase triggers a rise in JH-titer, which in turn, stimulates ovarian development (Rankin and Riddiford, 1978), whereas low-quality food or food deprivation causes JH-titers to drop, thus suppressing reproduction, inhibiting oviposition (Rankin and Riddiford, 1977), and stimulating migratory flight (Rankin and Riddiford, 1978). Perhaps the bugs' JH-titer regulates their decision whether to stay on the host and reproduce or to allocate energy to flight muscles and either seek alternative food, or enter reproductive diapause and seek overwintering shelter. In *O. fasciatus,* fifth-instars and newly molted adults are most sensitive to the critical effect of photoperiod (Dingle, 1974b). If early-instars were as sensitive as fifth-instars, then as adults they might enter diapause under reproductively favorable conditions (Dingle, 1974b). Similarly, if only adults were sensitive, then they might reproduce when conditions have become unfavorable (Dingle, 1974b). Response of fifth-instar, but not fourth-instar, nymph *B. rubrolineata* to 2-phenylethanol (Figure 3.3; experiments 24, 25; Table 3.3) suggests that only the former are sufficiently sensitive to the token stimuli and thus gear up physiologically for reproduction or diapause.

Attraction of fifth-instars to 2-phenylethanol is consistent with our observations that only fifth-instars form tight clusters within an aggregating colony while feeding on

or "resting" beneath host trees, and near harborages. However, these phenomena may also result from particular molting behavior of nymphs (Carroll, 1988; Ribeiro, 1989). First instars *B. rubrolineata* cluster around egg casings, analogous to first instars of *Nezara viridula* responding to the stage-specific pheromone 4-oxo-(*E*)-2-decenal (Fucarino et al., 2004). Second-, third-, and fourth-instar nymph *B. rubrolineata* are more likely to roam and appear not to form sizable aggregations, akin to behavior of *N. viridula* nymphs, which instead exhibit alarm, defense or perhaps dispersal behavior in response to (*E*)-2-decenal (Pavis et al., 1994; Fucarino et al., 2004). Fifth- instar nymph *B. rubrolineata* are responsive to the host tree semiochemical phenylacetonitrile (Chapter 2), and sometime after fourth-instar nymphs have molted to the fifth-instar stage, they become responsive to the aggregation pheromone 2-phenylethanol (Figure 3.3, experiment 25). As a result of this behavioral reversal from indifference to attraction to semiochemicals, fifth instars remain (*i*) near the highly nutritional seeds they require to complete development to adults and (*ii*) near prospective mates as they become adults and sexually mature. Hence, as newly-molted adults, they can then gear their physiology in preparation for either reproduction or diapause.

The reproductive state of *B. rubrolineata* modulates the response to 2-phenylethanol (Figure 3.3, experiments 18, 19; Table 3.3). Unlike all other females, mated and ovipositioning females neither respond to host semiochemicals (Chapter 2) nor to aggregation pheromone (Figure 3.3, experiment 18), suggesting that their physiological status constrains orientation to resource-related semiochemicals. Females focused on oviposition may trade off mobility for successful oviposition (Caldwell and Rankin 1974). For example, female cotton stainer seed bugs, *Dysdercus* spp., histolyze

flight muscles and rapidly develop eggs in response to food availability (Dingle and Arora 1973). Similarly, well-fed female *O. fasciatus* with high JH-levels mature ovaries and suppress migratory flight mechanisms (Rankin and Riddiford 1977, 1978). That reproductive males (unlike females) remain responsive to both host semiochemicals (Chapter 2) and aggregation pheromone (Figure 3.3, experiment 19) implies that males continue to seek rendezvous sites and mates. This interpretation is also consistent with observations of a second flight period of males when the females' flight has declined and oviposition commenced (Dingle, 1965; Caldwell, 1974).

Pheromonal communication of *B. rubrolineata* is mediated not only by aggregation pheromone but possibly also by gender-specific sex pheromones. Responses of pre-diapause, non-reproductive females and males to extracts of adult feces (Figure 3.1, experiment 1, 2; Table 3.3), response of post-diapause females to extract of male feces, and response of post-diapause males to extract of female feces (Figure 3.2, experiments 12-15) all support the conclusion of gender-specific sex pheromones (Table 3.3). That post diapause-reproductive females, unlike males, did not respond to nymph feces extract (Figure 3.2, experiment 16, 17; Table 3.3) may be adaptive behavior that reduces cannibalism of eggs by nymphs, which likely exists (Tinker 1952; Carroll, 1991). This interpretation is supported by findings that non-reproductive pre-overwintering females, which are not concerned with oviposition, do respond to volatiles from nymph feces, just as males do (Figure 3.1, experiment 3; Table 3.3).

The presence of pheromones other than 2-phenylethanol became also clear when feces extract of adults and of nymphs attracted pre-overwintering females and males

(Figure 3.1, experiments 1-4) but 2-phenylethanol did not (Figure 3.4; experiments 26, 27; Table 3.3). These responses to feces extract coincide with the end of the reproductive season when non-mating *B. rubrolineata* leave their host trees and seek overwintering shelters. Similarly, the aggregation pheromone of male spined citrus stink bugs, *Biprorulus bibax*, does not attract diapausing females in the fall, suggesting that other pheromones mediate fall aggregations (James et al, 1994). Furthermore, overwintering *B. rubrolineata* awakened from the state of dormancy for bioassays were neither attracted to, nor arrested by 2-phenylethanol (Figure 3.4, experiments 28-31; Table 3.3). In contrast, post overwintering diapause, virgin but sexually-receptive, female *B. rubrolineata* with a full complement of eggs were attracted to 2-phenylethanol (Figure 3.4, experiments 32, 33; Table 3.3). Females likely respond to post-diapause males that contain in their ventral abdominal gland 2-phenylethanol (Table 3.2) and use it as a sex pheromone that attracts receptive females in a male-biased spring population. Males with a large amount 2-phenylethanol in their gland may accrue a reproductive advantage compared to males with little or no 2-phenylethanol.

Feces-derived pheromone components attractive to post-diapause adults were persistent for > 7 months (Figure 3.2, experiments 12-17), and thus are conducive for long-term marking of suitable overwintering harborage. They may also guide bugs to the safety of their harborage during harsh spring weather. Their exact chemical identity remains unknown, but analyses of feces extract by GC-EAD (Figure 3.6) and by GC-MS revealed many antennal stimulatory straight-chain and methylated hydrocarbons, which in other species are known to mediate species or sex recognition (Hemeptinne et al., 1998; Schlamp et al., 2005; Ginzel et al., 2006; Eliyahu et al., 2008; Mullen et al.,

2008). Similar to 2-phenylethanol, the apparent sex-specific pheromones of *B. rubrolineata* may be released with feces because bioassay test stimuli consisted of paper towels exposed to, and impregnated with, the bugs' feces. These pheromones, however, could also have derived from the bugs' cuticle.

2-Phenylethanol has been reported both as a pheromone and plant-derived semiochemical in diverse ecological contexts. It is released from hair pencils of courting males in six species of noctuid moths (Aplin and Birch, 1970; Birtch et al., 1976; Jacobson et al., 1976; Bestmann et al., 1977) and one species of pyralid moths (Kuwahara, 1980). As a plant semiochemical, it contributes to the attractiveness of plants to their respective herbivores, including the American palm weevil, *Rhynchophorus palmarum* (Rochat et al., 2000), the pineapple beetle, *Carpophilus humeralis* (Zilkowski et al., 1999), the twelve-spotted lady beetle, *Coleomegilla maculate* (Zhu et al., 1999), the sap beetle *Carpophilus mutilatus* (Bartelt et al., 1993), and the peach tree borer, *Synanthedon exitiosa* (Derksen et al., 2007). In a tri-trophic context, potato plants, *Solanum tuberosum*, under attack by the Colorado potato beetle, *Leptinotarsa decemlineata*, emit 2-phenylethanol that attracts the predatory stink bug *Perillus binculatus* (Weissbecker et al., 1999). As a floral semiochemical of oilseed rape, *Brassica napus*, 2-phenylethanol elicits proboscis extension in honey bees, *Apis mellifera* (Pham-Delegue et al., 1993). As a floral volatile of alfalfa, 2-phenylethanol is attractive to green lacewings, *Chrysoperla carnae* (Zhu et al, 1999), and is correlated with heavy infestations of pea aphids, *Acyrthosiphon pisum*, a source of honeydew on which these lacewings feed (Zhu et al, 2005). As a scent constituent of flower-mimic pseudoflowers that the crucifer *Arabis drummondii* produces in response to the fungus

Puccinia monoica, 2-phenylethanol attracts halictic bees, which then transfer target-specifically both pollen and fungal spermatia (Roy and Ragusa, 1997). Finally, 2-phenylethanol as part of a semiochemical blend attracts the weevil *Oryzaephilus surinamensis* to stored food products (Pierce et al., 1991).

In conclusion, *B. rubrolineata* appears to have a rather unusual and most complex pheromone communication system. Production of, and response to, the aggregation pheromone 2-phenylethanol coincides with both the peak of their mating season and the presence of maturing seeds on boxelder host trees. 2-Phenylethanol is released with the feces of seed-feeding females and males, or from the ventral abdominal gland of males, and would appear to assemble prospective mates to profitable food sources. The aggregation pheromone seems to have a mate-assembling function because fourth-instar nymphs do not respond, and mated females no longer respond, to 2-phenylethanol. Attraction of *B. rubrolineata* both to the host tree semiochemicals phenylacetonitrile and 2-phenethyl acetate (Schwarz et al., 2008), and the aggregation pheromone 2-phenylethanol (this study), appears to be a fail-safe mechanism that would parsimoniously facilitate rapid assemblage of prospective mates near ephemeral seeds against time. Males emerging from overwintering diapause contain in their ventral abdominal gland appreciable amounts of 2-phenylethanol which in early spring attracts only females, thus strongly suggestive of a sex attractant pheromone. Aggregation of pre- and post-overwintering adults around suitable harborages, and possibly close-range sex recognition, appears to be mediated by sex-specific pheromones that attract only the opposite sex. While both the molecular structure and origin of these pheromones

remain unknown, their persistence on surfaces implies low volatility and high molecular weight, characteristics suitable for long-term marking of harborage.

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TABLE 3.1 Stimuli and experimental *Boisea rubrolineata* tested in Y-tube olfactometer experiments 1-33

Exp[a, b]	Treatment stimuli[c]	Bioassay insects	N[d]
1	Extract volatiles of paper towels exposed to and impregnated with feces from adults	Reproductively diapausing pre-overwintering females[g]	44
2	Extract volatiles of paper towels exposed to and impregnated with feces from adults	Reproductively diapausing pre-overwintering males[g]	58
3	Volatiles of paper towels exposed to and impregnated with feces from nymphs	Reproductively diapausing pre-overintering females[g]	32
4	Volatiles of paper towels exposed to and impregnated with feces from nymphs	Reproductively diapauseing pre-overwintering males[g]	35
5	Volatiles of paper towels exposed to and impregnated with feces from nymphs	Fith-instar nymphs	29
6	0.1 Fecal spot equivalent	Virgin females[h]	22
7	0.1 Fecal spot equivalent	Virgin males[h]	30
8	1 Fecal spot equivalent	Virgin females[h]	21
9	1 Fecal spot equivalent	Virgin males[h]	25
10	10 Fecal spot equivalents	Virgin females[h]	28
11	10 Fecal spot equivalents	Virgin males[h]	25
12	Volatiles of paper towels exposed to and impregnated with feces from males	Post-diapause females[g]	26
13	Volatiles of paper towels exposed to and impregnated with feces from males	Post-diapause males[g]	26
14	Volatiles of paper towels exposed to and impregnated with feces from females	Post-diapause females[g]	26
15	Volatiles of paper towels exposed to and impregnated with feces from females	Post-diapause males[g]	27
16	Volatiles of paper towels exposed to and impregnated with feces from nymphs	Post-diapause females[g]	26
17	Volatiles of paper towels exposed to impregnated with feces from nymphs	Post-diapause males[g]	26
18	2-Phenylethanol[f]	Reproductive females[i]	26
19	2-Phenylethanol[f]	Reproductive males[i]	26
20	2-Phenylethanol[f]	Sexually mature females[j]	24
21	2-Phenylethanol[f]	Sexually mature males[j]	24
22	2-Phenylethanol[f]	Sexually immature females[k]	24
23	2-Phenylethanol[f]	Sexually immature males[k]	24

[	24	2-Phenylethanol[f]	Fifth-instar nymphs	26
[	25	2-Phenylethanol[f]	Fourth-instar nymphs	20
[	26	2-Phenylethanol[f]	Reproductively -diapause pre-overwintering females[g]	24
[	27	2-Phenylethanol[f]	Reproductively diapausing pre-overwintering males[g]	24
[	28	2-Phenylethanol[f]	Overwintering females[g]	20
[	29	2-Phenylethanol[f]	Overwintering males[g]	20
[	30[e]	2-Phenylethanol[f]	Overwintering females[g]	26
[	31[e]	2-Phenylethanol[f]	Overwintering males[g]	26
[	32	2-Phenylethanol[f]	Post-diapause females[g]	30
[	33	2-Phenylethanol[f]	Post-diapause males[g]	30

[a] Experiments in brackets were run in parallel.
[b] A control- vs. control-stimulus experiment revealed no side bias of the bioassay set-up;
[c] The control stimulus (pentane) consisted of the same amount of solvent (10 μL) as dispensed with the treatment stimulus;
[d] N = number of insects bioassayed;
[e] Experiments 30 and 31 employed still-air, instead of Y-tube, olfactometers to test arrestment responses of overwintering adults awoken from dormancy;
[f] The amount of synthetic 2-phenylethanol (50 ng) approximated 25% of that released from a bug in its feces after a 24-h feeding period;
[g] Age unknown (note: overwintering adults were 4-5 months old; post-diapause adults were most likely > 6 months old);
[h] < 14 day-old virgin adults;
[i] Four to five week-old adults, which have been engaging in reproductive activities;
[j] Two week-old, sexually mature virgin adults;
[k] Four to six day-old sexually immature adults

TABLE 3.2 Amount of 2-phenylethanol in the ventral abdominal glands (VAG) of 24 post-diapause male *Boisea rubrolineata*, that were excised, extracted and analyzed on March 19, 2008. Mean, standard error, standard deviation and median were 1302.3, 3877.8, 791.5, and 138.0, respectively.

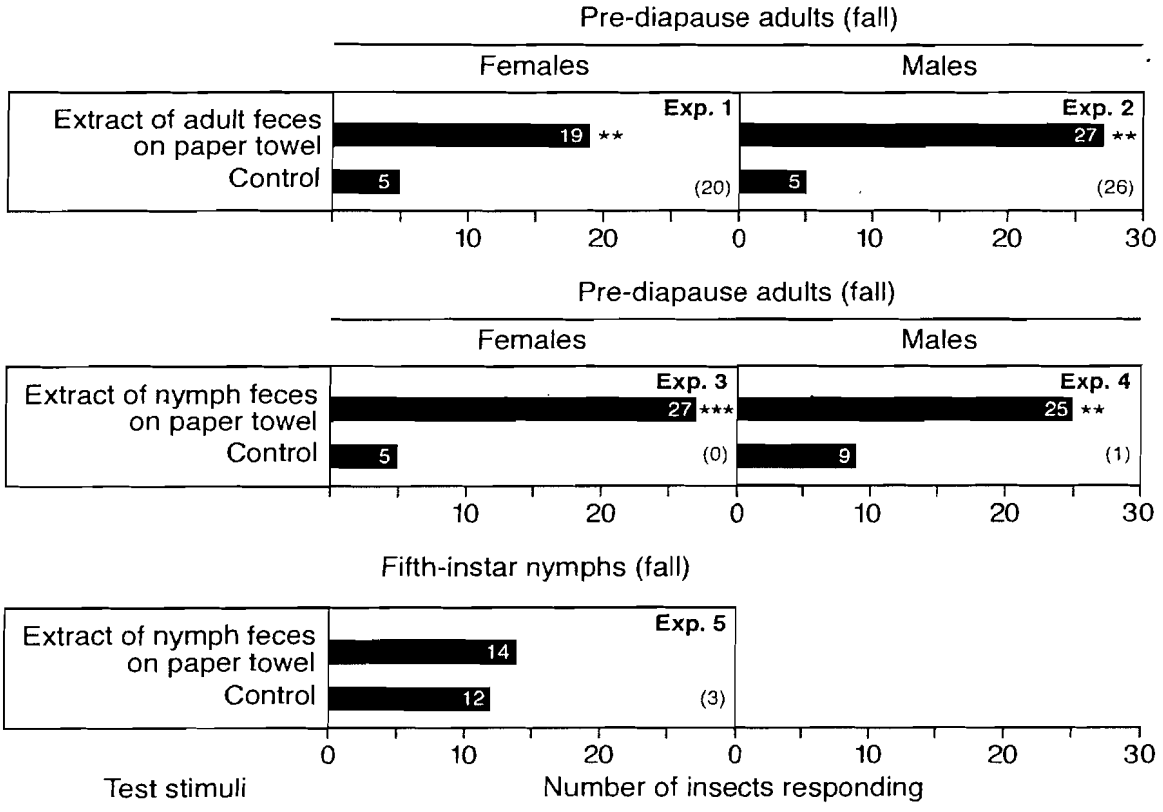
Male #	ng/VAG
1	176.8
2	0
3	16691.0
4	294.0
5	52.6
6	0
7	113.0
8	163.0
9	247.6
10	10283.0
11	394.5
12	10.0
13	30.4
14	10.8
15	7.6
16	24.0
17	0
18	106.4
19	163.4
20	798.0
21	540.0
22	924.5
23	15.6
24	210.5

TABLE 3.3 Logistic regression analyses of the effects of *Boisea rubrolineata* gender, stage, seasonal state, reproductive state, nymph instar, feces source, and bioassay design or interactions (×) between them, tested in experiments 1-33. An asterisk (*) indicates a significant effect (L-R $\chi 2$ test; *P ≤ 0.05, **P ≤ 0.01, *** P ≤ 0.001).

Logistic Regression Analysis					
Experiments	Source(s)	Effects	*df*	$\chi 2$	*P*-value
1, 2, 3, 4	adult feces nymph feces	insect gender feces source gender × source	1 1 1	0.112 0.112 1.184	NS[a] NS NS
3, 4, 5	nymph feces	insect gender[b] insect stage[c]	2 1	6.628 5.453	**0.04*** **0.02***
1, 2, 26, 27	adult feces 2-phenylethanol	insect gender source gender × source	1 1 1	2.344 11.77 0.6	NS **0.0006***** NS
3, 4, 26, 27	nymph feces 2-phenylethanol	insect gender source gender × source	1 1 1	0.215 10.52 4.088	NS **0.001***** **0.04***
3, 26	nymph feces 2-phenylethanol	source	1	13.469	**0.0002*****
4, 27	nymph feces 2-phenylethanol	source	1	0.794	NS
8, 9, 10, 11	adult feces	insect gender	1	3.073	0.08
12, 13, 14, 15	male feces female feces	insect gender feces source gender*source	1 1 1	0.005 0.663 3.17	NS NS 0.08
14, 15, 16, 17	female feces nymph feces	insect gender feces source gender × source	1 1 1	5.581 0.005 0.133	**0.03*** NS NS
18, 19, 20, 21	2-phenylethanol	insect gender reproductive state[d]	1 1	0.435 4	NS **0.05***
20, 21, 22, 23	2-phenylethanol	insect gender reproductive state[e] gender × state	1 1 1	0.305 0.305 0.003	NS NS NS
24, 25	2-phenylethanol	nymph instar	1	6.567	**0.01****
26, 27, 28, 29	2-phenylethanol	insect gender seasonal state[f] gender × state	1 1 1	0.979 0.03 2.106	NS NS NS
28-31	2-phenylethanol	insect gender bioassay design[g] gender × bioassay	1 1 1	0.056 0.944 0.056	NS NS NS
26, 27, 28,29, 32, 33	2-phenylethanol	insect gender seasonal state[f] gender × state	1 2 2	0.155 1.544 8.484	NS NS **0.01****
32, 33	2-phenylethanol	insect gender	1	5.654	**0.02***

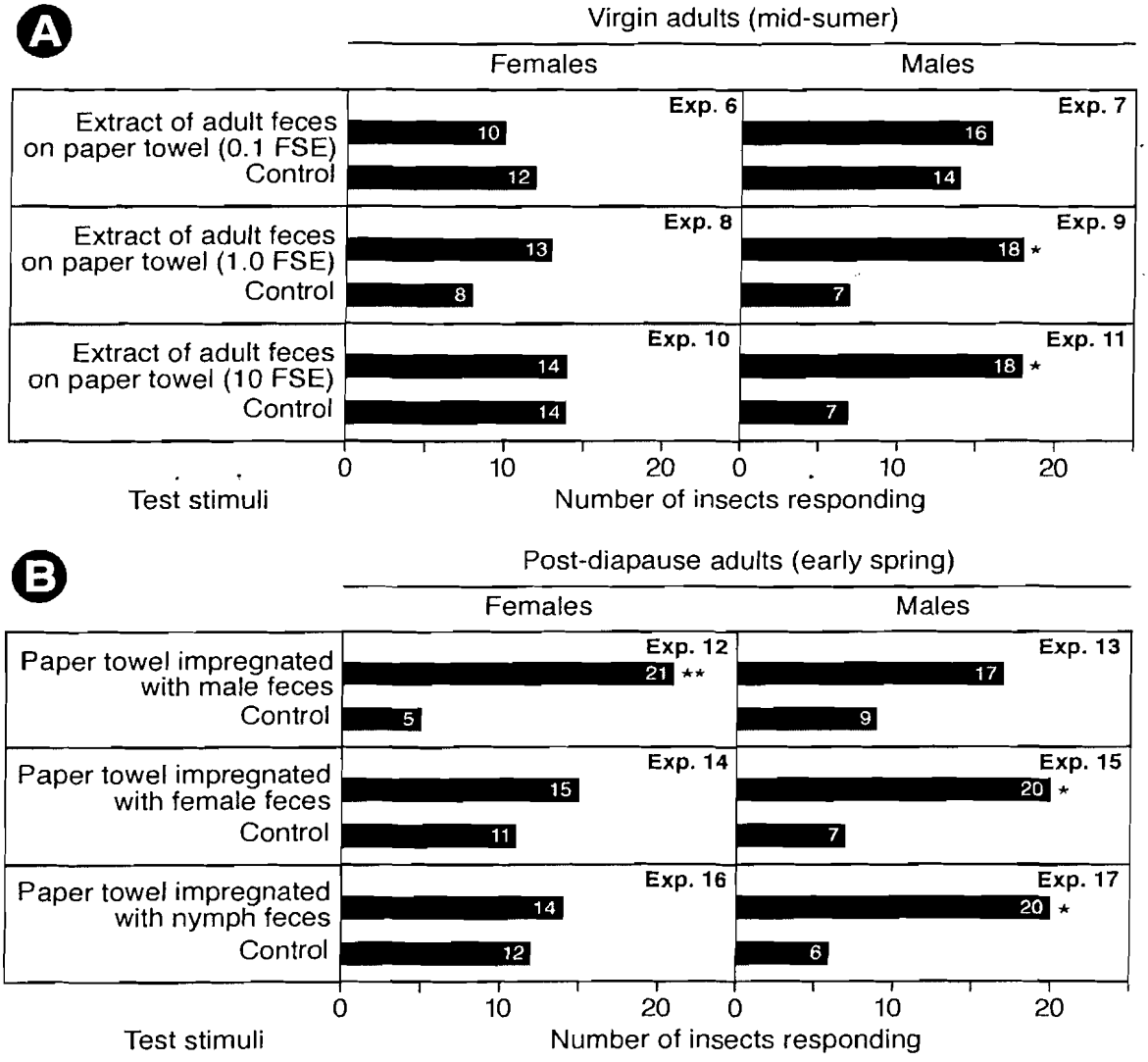
[a]NS = Not significant;
[b]Insect gender: female, male, and 5th-instar nymph;
[c]Insect stage: adults and 5th-instar nymphs;
[d]Reproductive state of adults: Sexually mature but virgin (12-15 days old), and reproductive (4-5 weeks old);
[e]Reproductive state of adults: sexually mature but virgin (12-15 days old), and sexually immature (1-5 days old);
[f]Seasonal state of adult insects: pre-overwintering in reproductive diapause, overwintering, and post-diapause
[g]Bioassay design: Y-tube or 3-chamber (still-air) olfactometers

Fig. 3.1 Response of reproductively-diapausing and pre-overwintering adult, or fifth-instar nymph *Boisea rubrolineata* in Y-tube olfactometers to extracts of paper towels previously exposed to and impregnated with feces from adults (experiments 1, 2), or to paper towels exposed to and impregnated with feces from 5th-instar nymphs (experiments 3-5). Numbers in parentheses indicate numbers of non-responding insects. An asterisk (*) indicates a significant preference for a particular test stimulus ($\chi 2$ test, $^{**}P < 0.01$, $^{***}P < 0.001$).



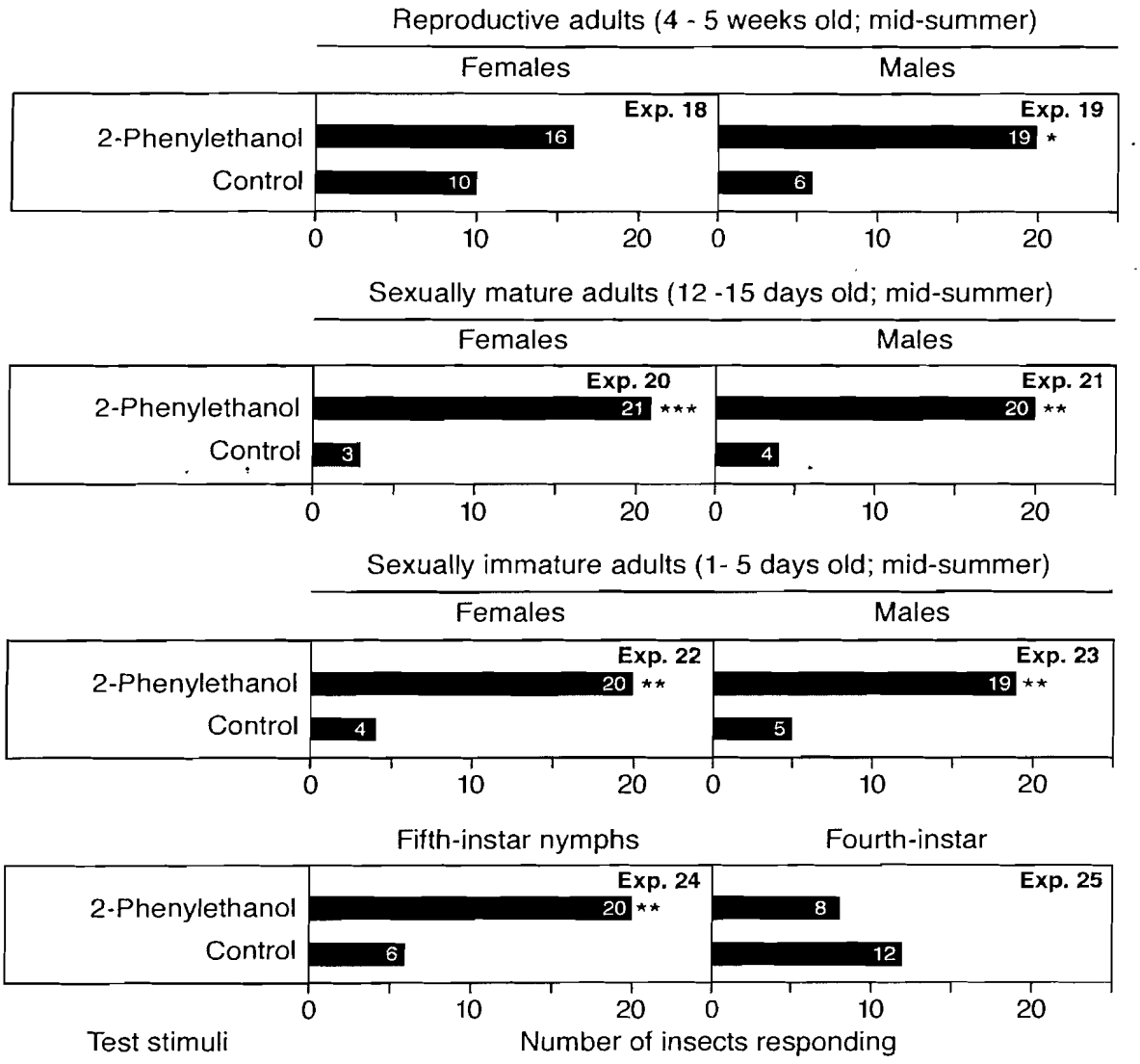
Gries_BEB_pheroAlt_Fig 1

Fig. 3.2 Response of virgin new-generation and post-diapause adult *Boisea rubrolineata* in Y-tube olfactometers to (A) 0.1, 1, or 10 Fecal Spot Equivalents (FSE) of extract of paper towel previously exposed to and impregnated with feces from host tree-fed adults, and (B) paper towels exposed to and impregnated with feces from host tree-fed males, females or nymphs. 1 FSE = amount of analyte in one fresh (< 24-h old) fecal spot. Numbers in parentheses indicate numbers of non-responding insects. An asterisk (*) indicates a significant preference for a particular test stimulus ($\chi 2$ test, $^{*}P < 0.05$, $^{**}P < 0.01$).



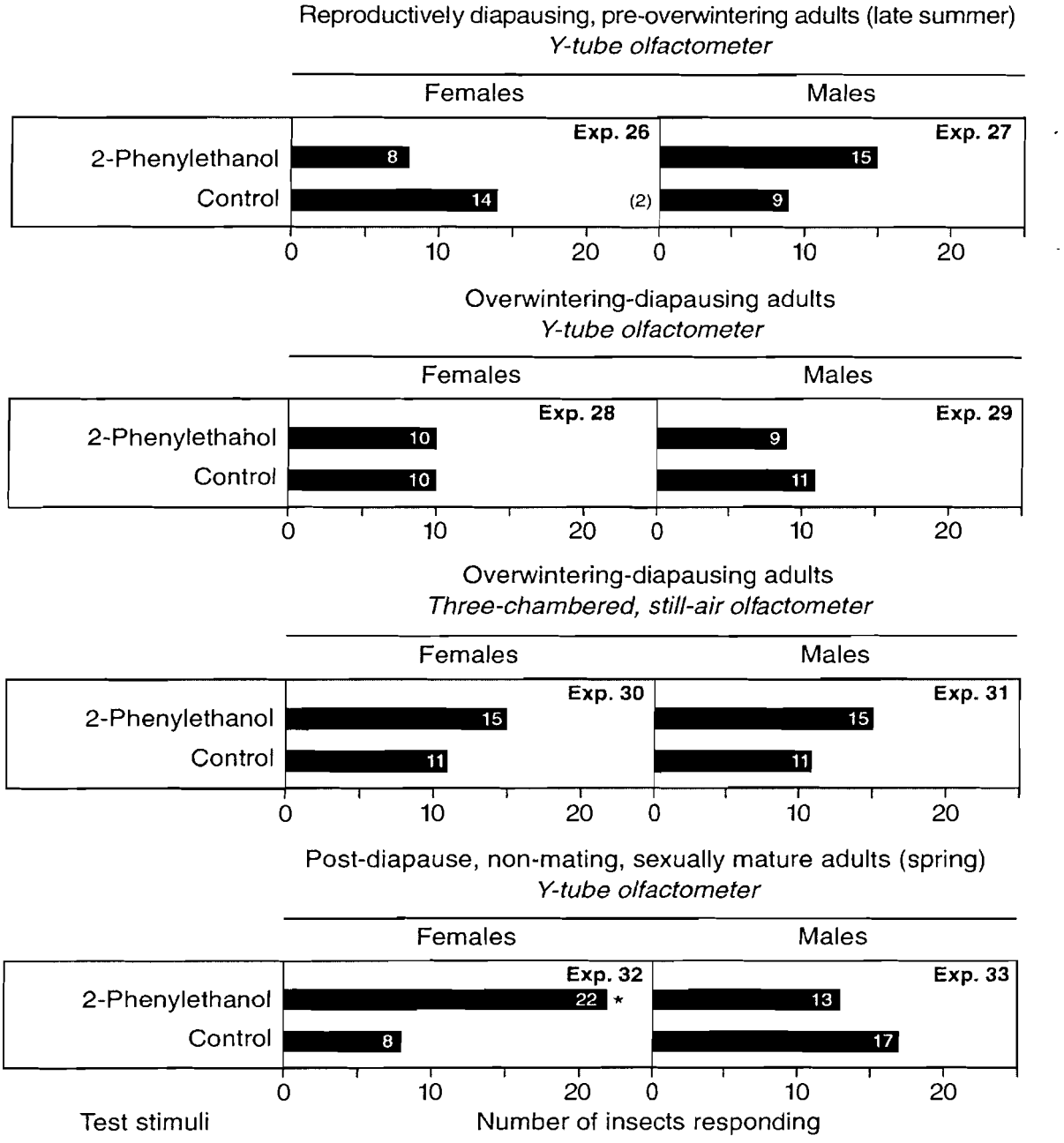
Gries_BEB_pheroAlt_Fig 2

Fig. 3.3. Response of reproductive, sexually mature, and sexually immature adult, or fourth- and fifth-instar nymph *Boisea rubrolineata* in Y-tube olfactometers to synthetic 2-phenylethanol (50 ng). Numbers in parentheses indicate numbers of non-responding insects. An asterisk (*) indicates a significant preference for a particular test stimulus ($\chi 2$ test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).



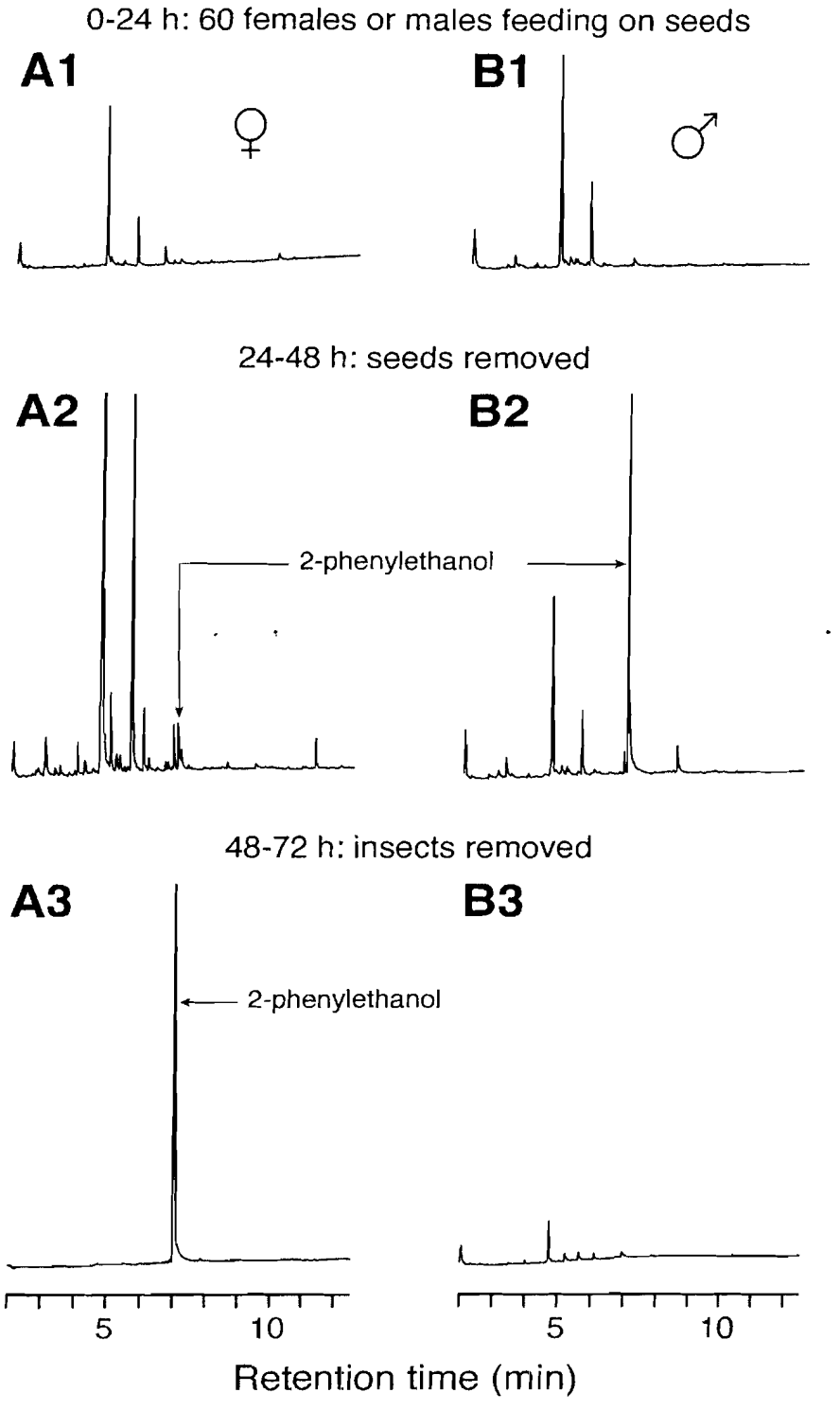
Gries_BEB_pheroAlt_Fig 3

Fig. 3.4 Response of reproductively-diapausing and pre-overwintering, reproductively-diapausing and overwintering (awoken from dormancy), and post-diapause not mated sexually mature adult *Boisea rubrolineata* in Y-tube or three-chambered olfactometers to synthetic 2-phenylethanol (50 ng). Numbers in parentheses indicate numbers of non-responding insects. An asterisk (*) indicates a significant preference for a particular test stimulus ($\chi 2$ test, $^*P < 0.05$).



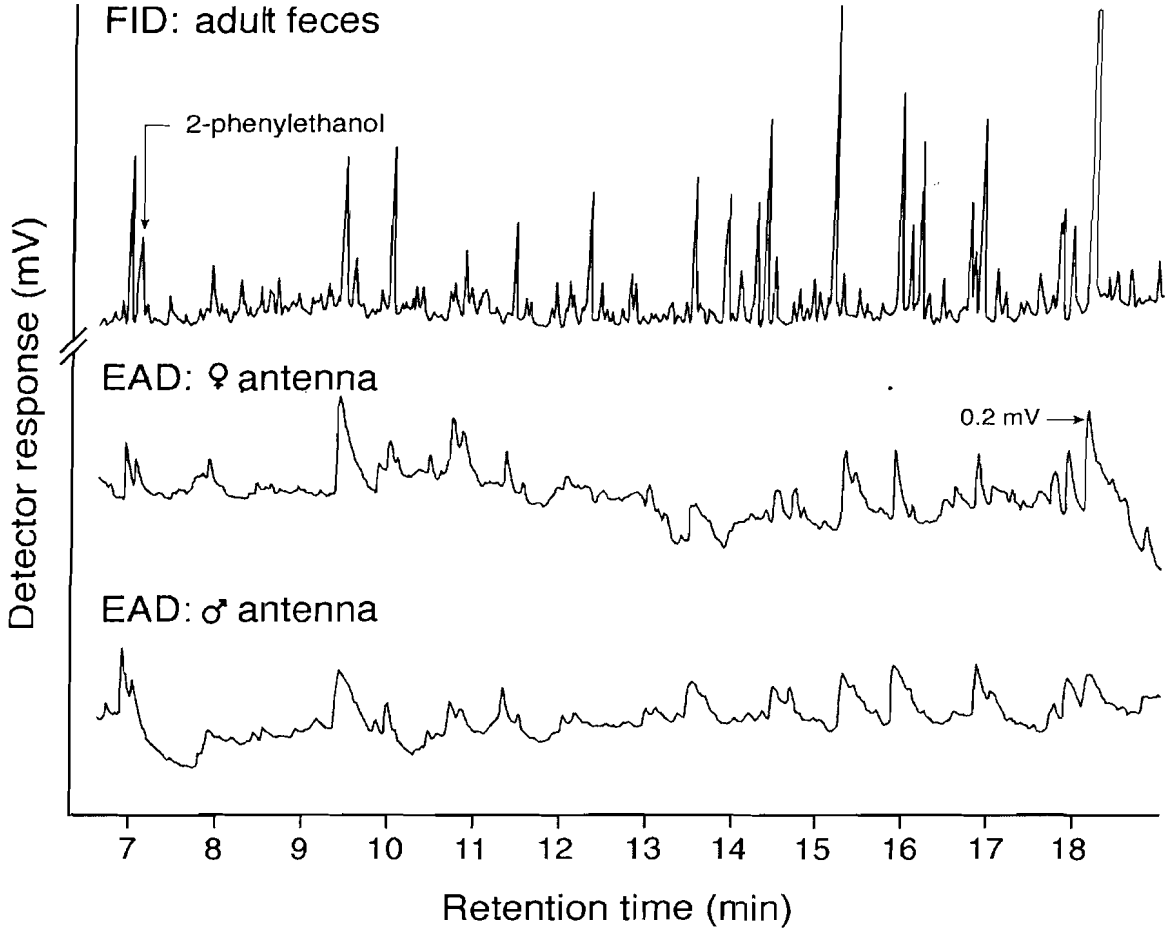
Gries_BEB_pheroAlt_Fig 4

Fig. 3.5 Aliquot chromatograms of headspace volatiles collected in three consecutive 24-hr periods from aeration chambers containing (A1, B1) 60 female or 60 male *Boisea rubrolineata* feeding on host seeds of *Acer negundo*; (A2, B2) the same insects without seeds; and (A3, B3) neither insects nor seeds. Note: females defecated during their removal just prior to the third aeration period. Temperature program: 50°C (for 2 min) 10°C/min to 280°C (held 5 min).



Gries_BEB_Fig5

Fig. 3.6 Flame ionization detector (FID) and electro-antennographic detector (EAD: female or male *Boisea rubrolineata* antenna) responses to aliquots of extracts of paper towels previously exposed to and impregnated with feces from adult *B. rubrolineata*. Temperature program: 50°C (1 min) 10°C/min to 280°C (5 min).



Gries_BEB_Fig6

4 DO BOXELDER BUGS SUNBATH FOR SANITATION?

4.1 Abstract

When boxelder bugs, *Boisea rubrolineata* (Hemiptera: Rhopalidae), form aggregations in warm sunlight, they release from their posterior dorsal abdominal gland an odorous blend of monoterpenes with heretofore unknown biological function. In laboratory analyses and experiments we show that bugs in warm sunlight, but not in shade, exude and spread copious amounts of monoterpenes onto their cuticle. These monoterpenes do not serve as either an attractant or arrestment pheromone, but as a means of sanitation. They inhibit germination of conidia spores of the fungal pathogen *Beauveria bassiana* and halt the growth of germinated spores. This prophylactic defense against pathogens appears adaptive for phytophagous insects, like *B. rubrolineata*, that are prone to infections by pathogens thriving on leaf surfaces and in the insects' overwintering microhabitat.

4.2 Introduction

As extreme thermoregulators, boxelder bugs (Hemiptera: Rhopalidae) form aggregations in warm sunlight (Smith & Shepherd, 1937; Tinker, 1952; Schowalter, 1986; Yoder & Robinson 1990) numbering thousands of specimens (Aldrich et al., 1990b; Schowalter, 1986). On warm winter days, they even break overwintering dormancy to sunbathe gregariously (Smith & Shepherd, 1937; Knowlton, 1944; Schowalter, 1986). Sunbathing boxelder bugs have a higher thoracic temperature

(Tinker, 1952; Yoder & Robinson, 1990; Robinson, 1996; JJS & ST, unpublished data), metabolism and activity than their conspecifics in the shade (Tinker, 1952). They also release an odorous blend of monoterpenes (JJS, unpublished).

Monoterpenes were isolated from the posterior dorsal abdominal gland (PDAG) of *B. rubrolineata* and *B. trivittata* (Aldrich et al., 1990), and the PDAG of other scentless plant bugs including *Jadera* spp. (Aldrich et al., 1990) and *Leptocoris* spp. (Ho et al., 2006). Species-specificity of monoterpene blends in PDAGs suggested that monoterpenes may contribute to pheromonal communication or reproductive isolation of these plant bugs (Aldrich et al., 1990). However, their potential roles as intra- or interspecific warning odors or defensive irritants were also considered (Aldrich et al., 1990; Ho et al., 2006).

Boisea rubrolineata is aposematically colored and apparently sequesters host toxins for secondary defense (see Chapter 2). Such aposematically colored insects gregariously and conspicuously advertise their noxiousness to predators (Rothschild et al., 1984; Guildford, 1987, 1990; Vulinec, 1990; Aldrich et al., 1997; Al Abassi et al., 1998), and they may reinforce the visual warning by semiochemicals (Eisner & Kafatos, 1962; Eisner, 1970; Rothschild et al., 1984; Guilford et al., 1987; Rothschild & Moore, 1987; Moore et al., 1990; Aldrich et al., 1997, 1999; Al Abassi et al., 1998) which may, or may not, concurrently serve as aggregation pheromones (Pasteels et al., 1983; Blum, 1996). For example, the aposematic ladybird beetle *Coccinella septempunctata* produces a pyrazine aggregation pheromone that is also believed to deter predators (Al Abassi et al., 1998). Aposematic milkweed bugs *Tropidothorax cruciger* and *Oncopeltus fasciatus*, in contrast, produce hexyl and octyl acetates as aggregation

pheromones but emit methoxypyrazines as warning odor during aggregations (Aldrich et al., 1997, 1999). Thus, PDAG-derived monoterpenes may serve as a pheromone, predator deterrent or both.

Phytophagous insects, such as *B. rubrolineata*, are exposed to, and risk infection by pathogens thriving on leaf surfaces (Wertheim et al., 2005). Clustering insects affect their microclimate (Howe, 1962) and are prone to infection by pathogenic fungi (Sinha & Wallace, 1966). *Beauveria bassiana* is a pathogenic fungus known to target overwintering insects such as *C. septempunctata* (Hodek et al., 1996; Pettersson et al., 2005). As a biological control agent, it has been deployed to control populations of the boxelder bug *B. trivittata*, and the red-shoulder bug *Jadera haematoloma* (Reinert et al., 1999). Boxelder bugs are most abundant after dry periods but in wet weather suffer high mortality from pathogenic fungi (Hoffman & Pellitteri, 2002). Insects under selection pressure from natural pathogens should favor antimicrobial defense strategies.

In the Hemiptera, the exocrine system may have evolved from semi-aquatic insects that produce antimicrobial compounds (Cobben, 1978) to ground-living terrestrial insects that produce defensive secretions (Schaefer, 1972; Cobben, 1978). In lace bugs, *Corythucha spp.* and *Stephanitis spp.* (Heteroptera: Tingidae), compounds secreted from glandular body hairs are allelopathic to pathogenic bacteria and parasitic roundworm larvae (Neal et al., 1995). When water-bugs groom their bodies they secret substances from meta-thoracic scent glands that contain hydrogen peroxide with antimicrobial activity (Maschwitz, 1971). Secretion-grooming as an antimicrobial defense strategy in aquatic bugs and beetles prevents bacterial buildup from affecting

the hydrophilicity of respiratory hairs which are essential for the functioning of their aquatic respiratory system (Kovac & Maschwitz, 1989, 1990, 1991, 2000).

Monoterpenes have anti-microbial properties (Cole & Blum 1975; Okamoto et al., 1978; Berryman, 1988; Chen et al., 2003; Alma et al., 2004; Grujic-Jovanovic et al., 2004; Sonboli et al., 2005a) as they can penetrate the lipid bilayer of microbes, disrupt their cellular structure and function (Sikkema et al., 1994, 1995), and thus destabilize membrane permeability (Silver & Wendt, 1967; Moleyar & Narasimham, 1987). If sunbathing *B. rubrolineata* were to excrete monoterpenes onto their cuticle, as a form of secretion-grooming (Kovac & Maschwitz, 2000), then monoterpenes could serve as a prophylactic defense against pathogens. Sunlight, or elevated body temperature and metabolic activity associated with it (see above), may trigger the biosynthesis and release of monoterpenes which, in turn, may interfere with fungal spore germination and growth. Such prophylactic microbial defense would differ from the well-known "behavioral fever" of already fungal-infested specimens that actively elevate their body temperature to control the degree of their mycosis (Inglis et al., 1996a, 1997a,b; Karban, 1998; Blanford & Thomas, 2001; Kalsbeek et al., 2001; Elliot et al., 2002; Moore and Freehling, 2002; Ouedrago et al., 2003, 2004; and Roy et al., 2006). Sunlight may not only trigger but also enhance the antimicrobial efficacy of monoterpenes (Cookson et al., 1963; Barany et al., 1978), as does sunlight enhance the toxicity of plant allelochemicals that deter herbivory (Downum & Rodriguez, 1986; Berenbaum, 1987).

Here we report (i) that sun-exposed unlike shade-exposed *B. rubrolineata* release copious amounts of monoterpenes onto their cuticle, (ii) that these PDAG-derived

monoterpenes inhibit *Beauveria bassiana* conidia germination, and (iii) that they serve no function as pheromone signals.

4.3 Materials and Methods

4.3.1 Collection and maintenance of experimental insects

Late-instar nymphs and adults were field-collected weekly on or near pistillate trees of *A. negundo* or *A. saccharinum* in Kelowna, Westbank, and Princeton, British Columbia, Canada from March to October in 2004 through 2006. Insects were maintained at 22-30°C, 30-70% relative humidity, under a natural photoperiod (5000 K daylight spectrum), and were provisioned with water and new-growth shoots of *A. negundo* with intact leaves and seeds that were clipped weekly from field sites and transported to SFU in 19-L buckets of water. Late-instar nymphs were field-collected from June to September, and pre-overwintering adults in reproductive diapause were collected in September and October, and used in bioassays as needed.

4.3.2 Excision and extraction of posterior dorsal abdominal glands (PDAG) from bugs exposed to sun or shade

In each of 5 replicates, 40-60 reproductively inactive adults entering overwintering dormancy were randomly selected from a 76-L glass holding tank kept outdoors and placed into each of two 2-L glass jars exposed to either shade or sun. Prior to experiments, jars were kept for 1-2 h in the shade to ensure that bugs could acclimate and volatiles released due to the bugs' handling disseminated. Temperatures (°C) in- and

outside jars, light intensities (Lux), and the bugs' fecal deposits were recorded. After a 4-h exposure to sun or shade, jars were chilled on ice, and six or ten bugs from each jar were carefully removed, CO_2-anesthetized, and pinned through the dorsal thorax under tap water onto a wax dish (100 mm). Three incisions were made with a dissecting scalpel, one separating the thin membrane between thorax and abdomen, and the other two separating the abdominal dorsum from the ventrum. The dorsum was then pulled back with forceps, exposing the PDAG which was excised with a scissor and forceps. Excess water was absorbed with tissue paper. The PDAG of each bug was placed into a separate vial on dry ice containing 50 µl of hexane. After 10 min of extraction at room temperature, the supernatant was withdrawn, sealed in an ampule, and cold-stored (4°C). Gland equivalents (GE; 1 GE = analyte present in the extract of one gland) were analyzed by chromatography (see below) or bioassayed.

4.3.3 Bodywashes of bugs exposed to sun or shade

After a 4-h exposure to sun or shade (see above), six or ten insects from each treatment were placed separately into vials on dry ice containing 200 µl of hexane. After 1-2 min of extraction the insects were removed, the supernatant was withdrawn, sealed in separate ampules and cold-stored (4°C). Bodywash equivalents (BWE; 1 BWE = analyte present in the bodywash of one bug) were analyzed by chromatography (see below) or bioassayed.

4.3.4 Collection of head-space volatiles

A mixed group of females (80) and males (80) or of fifth-instar nymphs (140) were enclosed in a cylindrical Pyrex® glass aeration chamber (15 × 37.5 cm) with a moist cotton wick. A water aspirator drew charcoal-filtered air at ~ 0.5 L/min for 48 h through each chamber and through a Pyrex® glass column containing 500 mg of Porapak-Q (50-80 mesh, Waters Associates, Inc., Milford, MA) for trapping headspace volatiles. The same protocol was applied to collect headspace volatiles from two groups of 25 females or 25 males each except that (i) chambers with bugs were exposed outdoors to sun or shade, (ii) a 1- to 2-h shade exposure of all bugs preceded volatile collection, and (iii) air was drawn for 1.5 h by an electric pump.

Volatiles were eluted from the Porapak-Q with 2 ml of redistilled pentane. Porapak extracts were concentrated as needed under a stream of nitrogen. Insect-hour equivalents (IHE; 1 IHE = volatiles released from one insect during 1 h) were analyzed or bioassayed. The procedure for obtaining control stimuli were identical to those described above except that aeration chambers did not contain any insects.

4.3.5 Analyses of extracts

Aliquots of pheromone gland, body washes, or headspace volatile extracts were analyzed by coupled gas chromatographic-electroantennographic detection (GC-EAD; Arn et al., 1975; Gries et al., 2002), employing a Hewlett Packard 5890A gas chromatograph equipped with a GC column (30 m × 0.25 ID) coated with DB-5 (J&W Scientific, Folsom, CA). Helium was used as carrier gas (35 cm/sec), with the following temperature program: 50°C (2 min) 10°C/min to 280°C (5 min). Coupled GC-mass spectrometric

(MS) analyses of all monoterpenes employed a Saturn 2000 Ion Trap GC-MS (Varian) fitted with the DB-5 column referred to above. Enantiomeric composition of gland-derived monoterpenes was analyzed by employing a Hewlett Packard 6890 GC equipped with a GC chiral column (30 m × 0.25mm ID) coated with cyclodexB (J&W Scientific, Folsom, CA). Helium was used as a carrier gas (33.9 ml/min), with the following temperature program: 80°C (15 min) 5°C/min to 150°C. Monoterpenes [α-thujene, α-pinene, α-fenchene, camphene, sabinene, β-pinene, myrcene, α-terpinene, limonene, trans-ocimene, γ-terpinene, terpinolene] were identified by comparing their retention indices (Van den Dool and Kratz, 1963) and mass spectra with those reported in the literature (Adams, 1989) and with those of authentic standards. Authentic α-thujene (unknown enantiomeric composition, 99% chemically pure) was collected from a natural plant source followed by HPLC; (+)- and (-)-α-pinene (98% each), (+)- and (-)-camphene (80% each), myrcene (91%), (+)- and (-)-β-pinene (98% each), α-terpinene (89%), (+)- and (-)-limonene (99 and 96%, respectively), and γ-terpinene (97%) were purchased from Sigma-Aldrich; (+)-sabinene (96%) was purchased from Indoline; trans-ocimene was purchased from IFF followed by silica-gel/$AgNO_3$ fractionation (88%), and terpinolene (90%) from Fluka. Fenchene, thujene, and (-)-sabinene were not purchasable.

4.3.6 Behavioral experiments

The potential role of monoterpenes as attractant or arrestment pheromones was tested in Y-tube and still-air olfactometer experiments.

Anemotactic responses of walking males and females to aliquots of test stimuli were bioassayed at 24-27°C in a horizontal Y-shaped Pyrex® glass olfactometer (stem 20 cm ×

23 mm ID; side arms at 120°; 18 cm long) illuminated from above by a fluorescent mercury lamps (GE™, Ecolux; 5000 K, 32 W). For each replicate a treatment and solvent (pentane) control stimulus were randomly assigned to and pipetted onto a Whatman® filter paper disc (1.5 cm) at the orifice of either side arm. In all replicates, a water aspirator drew charcoal-filtered and humidified air through the Y-tube at ~ 0.5 L/min carrying volatiles from the filter papers towards the insect. For each replicate, a new Y-tube and a different insect were employed. An insect was scored as a responder if within 5 min it approached a test stimulus within 2.5 cm. Non-responding insects were excluded from statistical analysis. Olfactometers were washed in warm water with Sparklene™ detergent, rinsed with cold tap and distilled water, and were oven-dried at 125°C for at least 1 hr.

Arrestment responses of adults or nymphs to 10-µl aliquots of test stimuli were bioassayed at 25-28°C in a horizontal three-chambered Pyrex® glass still-air olfactometer (Tremblay and Gries, 2003) illuminated from above by fluorescent "wide-spectrum grow light". Olfactometers consisted of a central Pyrex® glass Petri dish connected to two lateral Petri dishes (each dish 9 × 3 cm, and covered with a lid) via Pyrex® glass tubing (2 × 2.5 cm). For each replicate, a volatile treatment and solvent (pentane) control stimulus were randomly assigned to, and pipetted onto a Whatman® filter paper disc (42.5 mm) placed into each of the two lateral chambers. Each chamber was fitted with a Pyrex® glass Petri-dish lid. The bioassay insect was placed into the central chamber 1 h prior to scotophase and its position was recorded 3 h later. Non-responding insects were excluded from statistical analysis. Olfactometers were washed in warm water with

Sparklene™ detergent, rinsed with cold tap and distilled water, and were oven-dried at ca. 125°C for at least 1 h.

I have predicted that females, males and nymphs respond similarly to monoterpene headspace volatiles from, or extracts of the PDAG, but have tested them separately not to miss any gender- or stage-specific behavior. Similarly, we have tested insects at different times of year in the unlikely event that the insects' response to monoterpenes was season-dependent.

4.3.7 Specific experiments

Experiments 1-8 (Table 4.1) tested the response of walking females and males during their peak breeding season to various doses of headspace volatiles of aggregating adults. Experiments 9-14 (Table 4.1) tested the response of females and males ready to form large overwintering aggregations to various doses of PDAG extract in Y-tube or still-air olfactometers (Exps. 11, 12). Two types of olfactometers were used to test for both anemotactic attraction and arrestment behavior. Experiments 15-17 (Table 4.1) tested the response of fifth-instar nymphs to various doses of headspace volatiles of aggregating fifth-instars in still-air olfactometers

4.3.8 Ability of monoterpenes to inhibit germination of *B. bassiana* conidia spores

In each of two replicates, 100-µl aliquots of suspended *B. bassiana* conidia in sterilized water (replicate 1: 1.65×10^6 /100 μl^{-1}; replicate 2: 1.80×10^6 /100 μl^{-1}) were added to a 900-µl potato dextrose broth in separate quartz test tubes (13 × 100 mm) or in 4-mL vials, vortexing the mixtures. Treatment unlike control tubes or vials were then admixed with a

synthetic blend of neat monoterpenes [α-thujene, α-pinene, (+)-sabinene, (-)-β-pinene, myrcene, α-terpinene, (-)-limonene, *trans*-ocimene, γ-terpinene, terpinolene] in a quantity (1.5 µl = 1.5 mg) as found in ~ 100 BWE, which equates to 0.1 BWE per 1 µl of broth. We employed 100 BWE because the conidia concentration in the broth was high and the 1-mL broth volume greatly exceeded the ~ 0.1-µl volume one turgid PDAG gland might secret. Excluded from the blend were α-fenchene, which was unavailable and camphene, which occurred in amounts too low for aliquoting in neat form. Moreover, (+)- instead of (-)-sabinene (present in body washes) was formulated in the blend because the latter was unavailable. After admixture of the monoterpene blend, treatment and control tubes/vials were sealed, vortexed and then subjected for 1 h to either (i) direct sunlight, (ii) direct sunlight with UV radiation photochemically excluded, (iii) open shade outdoors, or (iv, v) 25°C or 50°C indoors in darkness (Table 4.2). The photochemical filter was prepared by mixing 75 g of $NaNO_2$ in 100 mL of distilled water (Parker, 1968), and by submerging 4-ml treatment or control vials (see above) into an 80-ml beaker filled with 60 ml of the photochemical filter solution.

4.3.9 Ability of monoterpenes to suppress growth of *B. bassiana*

In each of three replicates, 100-µl aliquots of suspended Bb conidia in sterilized water (2.7×10^{-6} /100 μl^{-1}) were added to a 900-µl potato dextrose broth in separate 4-ml vials which were sealed, vortexed and then stored at 25°C in darkness. Unlike control vials, treatment vials received a synthetic blend of monoterpenes (1.5 µl = 1.5 mg) upon which they were sealed, vortexed and stored. Monoterpenes were administered: (1) at 0 h; (2)

after 18 h of spore germination; and (3) after 18 h of spore germination followed by evaporation of monoterpenes 24 h later, and observations of growth at hour 42 and 90.

4.3.10 Statistical Analyses

Quantitative analyses of bodywashes and extracts of PDAGs were subjected to Student's t-test ($\alpha = 0.05$) (Zar, 1999). Data of Y-tube or still-air olfactometer experiments were analyzed with the χ^2 goodness-of-fit test, using Yates correction for continuity ($\alpha = 0.05$) (Zar, 1999).

4.4 Results

4.4.1 Analyses of Extracts

Adults and fifth-instar nymphs release from their PDAG a plethora of monoterpenes (Table 4.3; see Table 4.4 for enantiomeric composition), of which α-thujene, sabinene, β-pinene, myrcene, limonene, *trans*-ocimene, γ-terpinene and terpinolene elicit responses from adult antennae in GC-EAD recordings (data not shown).

Sun-exposed bugs have significantly greater amounts of monoterpenes on their cuticle than in their PDAG (Figure 4.1, A), whereas shade-exposed bugs have similar amounts of monoterpenes on their cuticle as in their PDAG (Figure 4.1, B). There are similar amounts of monoterpenes in the PDAG of sun- and shade-exposed bugs (Figure 4.2, A), but sun-exposed bugs have significantly higher amounts on their cuticle than shade-exposed bugs (Figure 4.2, B). Sun-exposed bugs produce overall greater amounts

of monoterpenes than shade-exposed bugs as is evident by subtracting the amount of monoterpenes in the PDAG from that on cuticle (Figure 4.2, C). Moreover, there is a significantly greater amount of monoterpenes in the headspace of sun-exposed bugs than shade-exposed bugs (Figure 4.3).

4.4.2 Y-tube and still-air olfactometer experiments

In Y-tube olfactometers, neither females nor males were attracted to various doses of (i) headspace volatile extract of aggregating adults (Table 4.1, experiments 1-8; see Table 4.3 for IHE) or (ii) PDAG extracts of adults (Table 4.1, experiments 9, 10, 13, 14, 15, 16). In still-air olfactometers, adults were not arrested by PDAG extracts of adults (Table 4.1, experiments 11, 12), and fifth-instars were not arrested by headspace volatiles of aggregating nymphs (Table 4.1, experiments 17-19; see Table 4.3 for IHE).

4.4.3 Effect of monoterpenes on *B. bassiana*

The presence of monoterpenes completely inhibited germination of *B. bassiana* conidia (Table 4.5). Following evaporation of monoterpenes, conidia germination ensued. Exposure of germinated conidia to monoterpenes halted further growth of germ tubes (Figure 4.4). In the absence of monoterpenes, exposure of conidia in darkness to hot (50°C) temperature or to direct sunlight reduced viability to 16-22.1% and 33.1-43.3%, respectively (Table 4.5). There was no considerable reduction in the viability of conidia when they were kept in darkness at 25°C, in open shade, or in direct sunlight without UV radiation (Table 4.5).

4.5 Discussion

Our data support the conclusion that sunbathing by *B. rubrolineata* triggers a sharp increase in biosyntheses and release of monoterpenes from the posterior dorsal abdominal gland (PDAG) (Figure 4.1). Larger amounts of monoterpenes on the cuticle (Figures 4.2B, C) and in the headspace (Figure 4.3) of sun-exposed bugs compared to their counterparts in the shade, coupled with similar amounts of monoterpenes in the PDAG of sun- or shade-exposed insects (Figure 4.2A), all support the conclusion that sun exposure sparks monoterpene biosyntheses and secretion.

Compared to shade-exposed insects, sun-exposed insects experience higher temperature and light intensity (Table 4.2) and exhibit enhanced activity (JJS and GG observations), defecation, and thus, likely metabolism (Table 4.2) (Tinker, 1952), possibly yielding the energy required for monoterpene biosyntheses. Similar to the heterogenic rhopalids *Niesthrea louisianica* and *Jadera haematoloma* (Aldrich et al., 1979), *B. rubrolineata* likely biosynthesizes monoterpenes *de novo* because (i) bugs were food-deprived during sun-exposure and could not have acquired potential biosynthetic precursors from host-plants and (ii), headspace volatile constituents from host plants lack these monoterpenes except for *trans*-ocimene (Chapter 2).

Sunbathing and monoterpene emission could play a role and be adaptive in the context of (1) pheromonal signaling, (2) microbial defense, and (3) aposematic warning.

There is strong experimental evidence that the PDAG-derived monoterpenes do not have pheromonal characteristics. Various doses of monoterpene headspace volatiles from aggregating adults, or of extracts of the PDAG, failed to attract or arrest females

and males in Y-tube or still-air olfactometers (Table 4.1, experiments 1-8 and 9-16). Similarly, fifth-instar nymphs were not arrested by monoterpenes in still-air olfactometers (Table 4.1, experiments 17-19) and instead may respond to chemical constituents from their anterior dorsal abdominal gland (ADAG), which is not retained in adults. Indeed, ADAG constituents of first-instar nymph *N. viridula* elicit aggregation or repellent behavior in first instars (Fucarino et al., 2004), and attract second instars (Pavis et al., 1994). Homologous ADAG constituents of nymph *B. trivittata* (Aldrich et al., 1990), and perhaps *B. rubrolineata*, may thus also have pheromonal activity.

If PDAG-derived monoterpenes were to function as aggregation pheromone, then bugs in the shade, which hardly produce any monoterpenes (Figures 4.1, B; 4.2, B, C; 4.3), would be incapable communicators. Moreover, if monoterpenes mediated aggregation, then one might expect strong attraction instead of indifferent behavior in olfactometer experiments, which yielded more non-responding than responding bugs (Table 4.1). In contrast, strong stimuli such as the host-tree semiochemicals phenylacetonitrile and 2-phenethyl acetate (Chapter 2) or the aggregation pheromone 2-phenylethanol (Chapter 3), elicited strong attraction of bugs with minimal numbers of non-responders.

It was still conceivable that PDAG monoterpenes function as alarm rather than aggregation pheromone. Then, however, one would otherwise expect that the copious amounts of monoterpenes released during sunbathing elicit some kind of alarm or dispersal behavior that would interfere with the bugs' forming and maintaining of aggregations on and off host trees. Such interference was not observed.

The fact that PDAG monoterpenes do not attract conspecifics is not surprising when one considers the many cues and signals that assemble *B. rubrolineata* on or near resources, including host-tree semiochemicals phenylacetonitrile and 2-phenethyl acetate (Chapter 2), the aggregation pheromone 2-phenylethanol (Chapter 3), and sex attractant pheromones of unknown molecular structure (Chapter 3). Yet another attractive pheromone from the PDAG would appear redundant in a semiochemical system that already is very complex.

Insects, such as *B. rubrolineata*, that overwinter in aggregating clusters are prone to infection by pathogenic fungi (Howe, 1962; Sinha & Wallace 1966; Hodek et al., 1996) and thus should have countermeasures to alleviate their impact. We present evidence that release of monoterpenes by *B. rubrolineata* during sunbathing constitutes such a countermeasure. In the presence of monoterpenes, germination of *B. bassiana* conidia spores was completely inhibited (Table 4.5) and growth of germ tubes that were already present before monoterpene administration, was halted (Figure 4.4, E). The adverse effect of monoterpenes on conidia germination exceeded that of elevated temperature (50°C) or sunlight exposure (Table 4.5), suggesting that monoterpenes greatly contribute to the bugs' microbial defense. Moreover, just moderate ambient temperature (Table 4.2), below the optimal temperature of 25°C for *B. bassiana* growth (Fargues et al., 1997), triggers biosyntheses and release of monoterpenes by *B. rubrolineata* (Figures 4.1; 4.2). The phototoxicity of monoterpenes is enhanced by UV-radiation (Cookson et al., 1963), and high temperature may increase their formation of phototoxic products (Barany et al., 1978). If monoterpenes do contribute to the bugs' microbial defense, it would explain our observations that sunbathing bugs readily

"groom" with their legs as if they were spreading monoterpene exudates from the PDAG over the body surface. It may also explain why *B. rubrolineata* repeatedly interrupts winter dormancy and basks in the sun (Smith & Shepherd, 1937; Knowlton, 1944; Schowalter, 1986) only to return to harborage thereafter. With a cuticular shield of sequestered flavonoids (plant pigments) and perhaps carotinoids (Palmer & Knight, 1924) protecting against oxidative damage of the suns' UV radiation and of phototoxic chemicals (Atkins, 1987; Harborne, 1988; Aucoin et al., 1990; Carroll et al., 1997; and Carroll and Berenbaum, 2006), *B. rubrolineata* appears pre-adapted to sunbathing, which triggers release of PDAG exudates as part of their prophylactic defense against pathogens.

Our data, revealing that both UV radiation and heat appears to have affected germination of *B. bassiana* conidia (Table 4.5), are consistent with previous results (Inglis et al., 1995, 1996, 1997a, b). Pathogen-infected insects that up-regulate their body temperature to 38-40°C (Gardner & Thomas, 2002; Thomas & Blanford, 2003) adversely affect, but do not completely eliminate pathogens (Inglis et al., 1996, 1997b; Arthurs & Thomas, 2001; Elliot et al., 2002; Ouedraogo et al., 2004). The insects' "behavioral fever" enhances the efficacy of their trans-membrane defense-complex in the hemolymph (Green & Vermeulen, 1994; Ouedraogo et al., 2002) which entails removal of fungal blastospores through multiplication of hemocytes and increased phagocytosis (Ouedraogo et al., 2003). The best defense against pathogens for *B. rubrolineata*, however, may lie in the combined and possibly synergistic actions of UV radiation, heat and monoterpenes put into effect by sunbathing (this study) and secretion-grooming (see Kovac and Maschwitz, 1989, 1990a,b, 1991, 1993, 2000) prior

to infection. This concept, however, awaits thorough experimental testing. Once infected, however, specimens may resort to thermal energy to stage a "behavioral fever" that governs the degree of their mycosis (Inglis et al., 1996a, 1997a, b; Karban, 1998; Blanford & Thomas, 2001; Kalsbeek et al., 2001; Elliot et al., 2002; Moore and Freehling, 2002; Ouedrago et al., 2003, 2004; and Roy et al., 2006).

We argue, but have yet to test experimentally, that PDAG-derived monoterpenes add to the bugs' aposematic cues. Chemical constituents, including monoterpenes, in abdominal glands of heteropteran bugs have interspecific defensive functions as irritants and repellents (Eisner, 1970; Aldrich, 1988, 1995; Whitman et al., 1990). Conceivably, this may also be applicable to the PDAG monoterpenes of *B. rubrolineata*. If so, monoterpenes might be considered a semiochemical reinforcement of the aposematic warning coloration that advertises the bugs' apparent noxiousness to potential predators.

4.6 References

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TABLE 4.1 Response of female, male or nymph *Boisea rubrolineata* in Y-tube or three-chambered, still-air olfactometers to various doses of (i) headspace volatile extracts of aggregating adults or 5th-star nymphs, or to extracts of the posterior dorsal abdominal gland (PDAG).

Exp.	Test stimuli: Monoterpenes in headspace of adults	Insects tested	Olfactometer	n[c]	T[d]	C[e]	NR[f]	Month
1	0.1 IHE[a]	females	Y-tube	16	3	1	12	mid-July
2	0.1 IHE	males	Y-tube	16	0	1	15	mid-July
3	1 IHE	females	Y-tube	16	2	1	13	mid-July
4	1 IHE	males	Y-tube	16	3	1	12	mid-July
5	10 IHE	females	Y-tube	15	2	2	11	mid-July
6	10 IHE	males	Y-tube	15	2	3	10	mid-July
7	100 IHE	females	Y-tube	16	1	3	12	mid-July
8	100 IHE	males	Y-tube	19	2	4	13	mid-July
	Extracts of adult PDAG							
9	0.1 GE[b]	females	Y-tube	24	2	6	16	mid-Oct
10	0.1 GE	males	Y-tube	24	5	3	16	mid-Oct
11	1 GE	females	3-chamber	10	0	0	10	mid-Oct
12	1 GE	males	3-chamber	10	0	0	10	mid-Oct
13	1 GE	females	Y-tube	12	3	4	5	mid-Oct
14	1 GE	males	Y-tube	12	4	3	5	mid-Oct
15	10 GE	females	Y-tube	27	10	12	5	mid-Oct
16	10 GE	males	Y-tube	28	11	10	7	mid-Oct
	Monoterpenes in headspace of 5th-instar nymphs							
17	1 IHE	5th-instars	3-chamber	66	18	11	37	mid-Sept to Oct
18	10 IHE	5th-instars	3-chamber	66	15	13	38	mid-Sept to Oct
19	100 IHE	5th-instars	3-chamber	66	13	21	32	mid-Sep to Oct

[a]IHE = Insect Hour Equivalent; 1 IHE = amount of volatiles released by 1 insect during 1 h.
[b]GE = Gland Equivalent; 1 GE = analyte present in the extract of one posterior dorsal abdominal gland (PDAG);
[c]n = Number of insects bioassayed;
[d]T = Number of insects responding to the treatment stimulus;
[e]C = Number of insects responding to the control (solvent) stimulus;
[f]NR = Number of non-responding insects.

TABLE 4.2 Numbers of female and male *Boisea rubrolineata* in each of replicates 1-5 exposed to sun or shade, processed for acquisition of body washes or excision of the posterior dorsal abdominal gland (PDAG), and physical parameters (light intensity, temperature) measured at the onset and end of each replicate.

	Number of Insects						Light Intensity (Lux)				Temperature (°C)							
											sun				shade			
	PDAG		Bodywash		Fecal Deposits		sun		shade		in-jar		out-jar		in-jar		out-jar	
Rep.	sun	shade	sun	shade	sun	shade	onset	end	onset	end	onset	end	onset	end	onset	end	onset	end
1	6	5	0	0	25	2	580000	310000	27000	8700	20	16	12	11	09	10	09	10
2	10	10	10	10	28	0	500000	330000	26000	9000	19	16	09	11	09	09	09	09
3	6	6	10	10	21	0	550000	410000	19000	11000	20	17	13	12	10	11	10	11
4	6	6	6	6	22	4	470000	56000	38000	3900	23	12	12	07	07	7.5	08	7.5
5	6	6	6	6	24	0	440000	410000	16000	13000	19	17	08	07	05	05	05	05
Total	34	33	32	32	120	6												
Gender	♀18 ♂16	♀17 ♂16	♀12 ♂20	♀15 ♂17														

TABLE 4.3 Composition of monoterpenes in the headspace of a mixed group of 80 female and 80 male *Boisea rubrolineata* or in a group of 140 fifth-instar nymphs.

		ADULTS	5th-INSTARS
Monoterpene	RI[a]	ng/1 IHE[b]	ng/1 IHE[b]
α-thujene	933	0.02	0.01
α-pinene	940	0	0
α-fenchene	950	0.005	0.005
camphene	953	0.002	0.005
sabinene	974	0.08	0.08
β-pinene	980	1.1	0.85
myrcene	987	0.17	0.07
α-terpinene	1018	0.005	0.01
limonene	1032	0.2	0.79
trans-ocimene	1047	0.05	0.05
γ-terpinene	1061	0.02	0.03
terpinolene	1087	0.01	0.03
TOTALS		1.66	1.93

[a]RI = Retention Index;
[b]IHE = Insect Hour Equivalent; 1 IHE is the amount of volatiles released by 1 insect during 1 h

TABLE 4.4 Enantiomeric composition of PDAG-derived monoterpenes of *Boisea rubrolineata* (mixture of 117 PDAGs, 40 body washes, and volatiles from 190 adults aggregating)

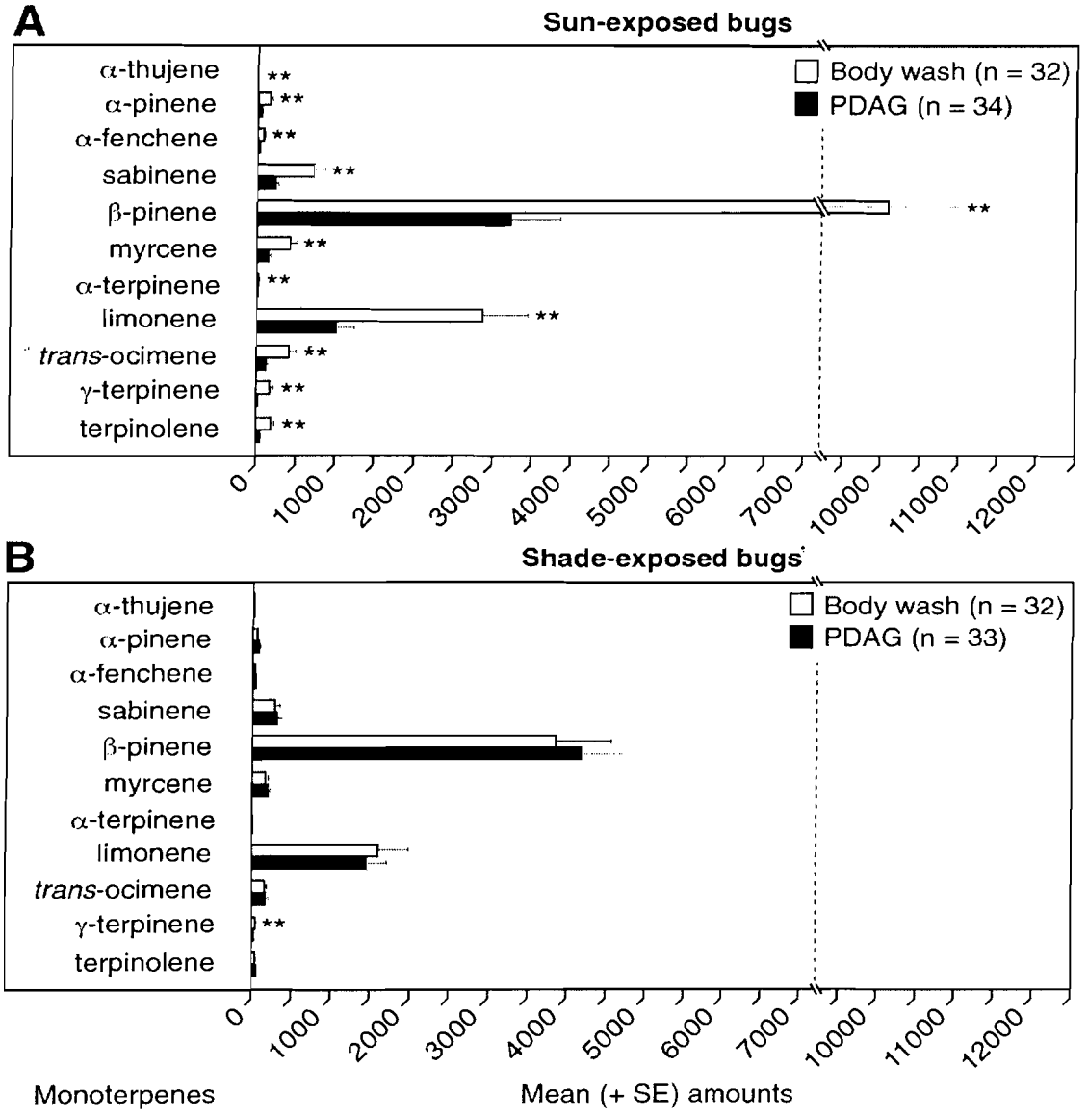
Monoterpene	RI[a]	Chiral Ratios
α-thujene	933	UC[b]
α-pinene	940	1(+) : 2.5(-)
α-fenchene	950	UC
camphene	953	0(+) : 1(-)
sabinene	974	1(+) : 5(-)
β-pinene	980	1(+) : 38(-)
myrcene	987	NC[c]
α-terpinene	1018	NC
limonene	1032	1(+) : 16(-)
trans-ocimene	1047	NC
γ-terpinene	1061	NC
terpinolene	1087	NC

[a]RI = Retention Index;
[b]UC = Unknown Chirality;
[c]NC = Not Chiral

TABLE 4.5 Viability of conidia spores of the fungal pathogen *Beauvaria bassiana* exposed indoors in darkness to warm (25°C) or hot (50°) temperature or outdoors to shade, direct sunlight or sunlight with UV-radiation photochemically excluded (Sun + NaNO2), with all treatments tested either in the absence or presence of monoterpenes. The 1.5-mg blend of synthetic monoterpenes [α-thujene (0.1%), α-pinene (1%), (+)-sabinene (4.7%), (-)-β-pinene (66.5%), myrcene (2.8%), α-terpinene (0.2%), (-)-limonene (19%), trans-ocimene (2.8%), γ-terpinene (1.2%), terpinolene (1.2%)] was equivalent to the amount of monoterpenes present in the body wash of one insect.

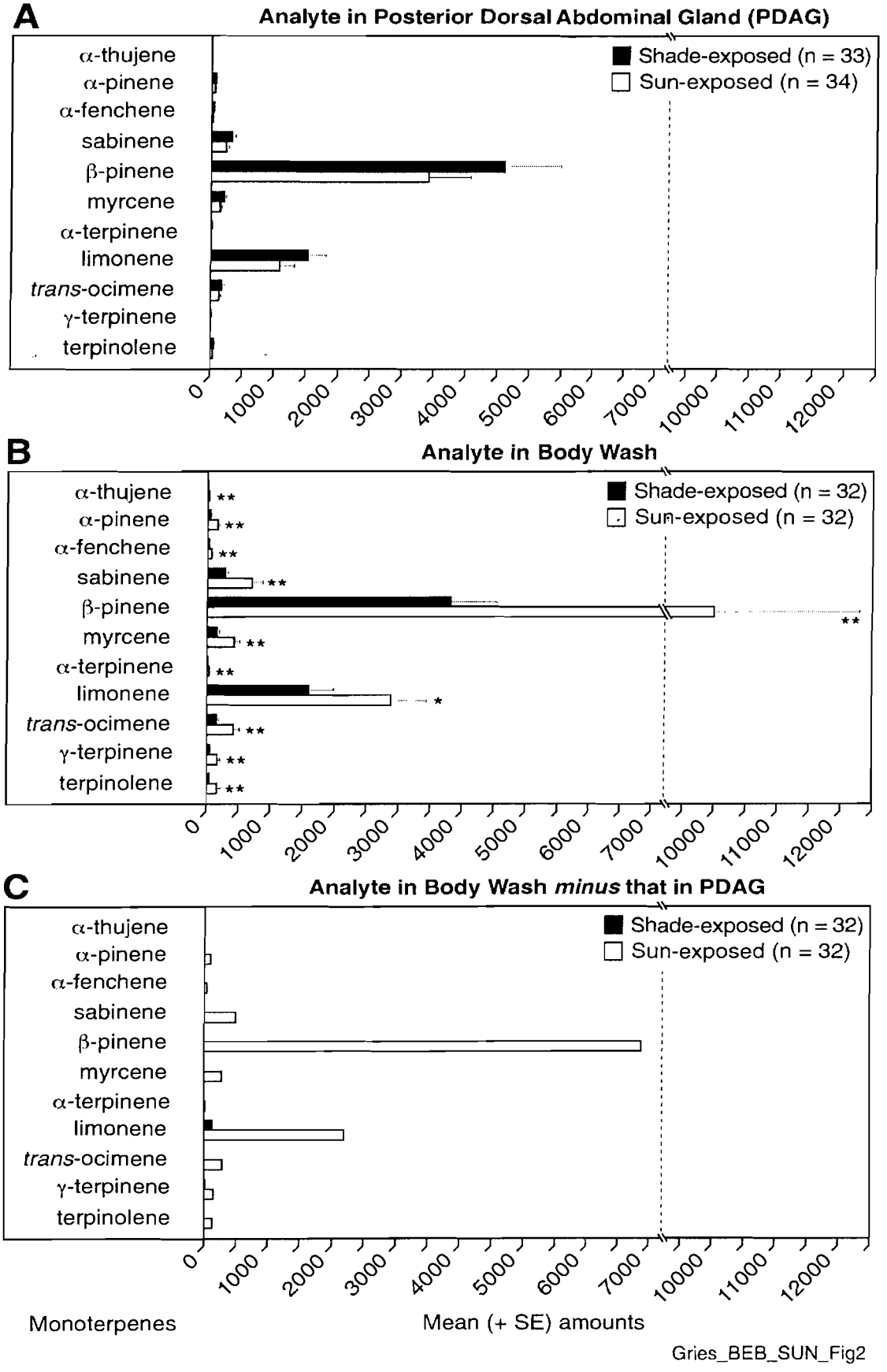
	Monoterpenes (rep. 1)			Monoterpenes (rep. 2)	
Treatment	absent	present	Treatment	absent	present
25°C	95.0% (400/421)	0%	25°C	94.7% (500/528)	0%
50°C	22.1% (102/462)	0%	50°C	16.0% (117/733)	0%
Shade (22-22.5°C)	95.8% (457/477)	0%	Shade (28-29°C)	94.5% (464/491)	0%
Sun (28-29°C)	33.1% (118/356)	0%	Sun (35-37°C)	43.3% (239/552)	0%
Sun + NaO_2 (29°C)	92.7% (369/398)	0%	Sun + NaO_2 (37°C)	94.0% (514/587)	0%

Fig. 4.1 Mean (+ SE) amount of monoterpene analyte present in body washes or excised Posterior Dorsal Abdominal Glands (PDAG) of individual boxelder bugs, *Boisea rubrolineata*, exposed to sun (A) or shade (B) (see Table 2). An asterisk (*) indicates a significant preference for a particular test stimulus (Student's *t*-test, *$P < 0.05$, **$P < 0.01$)



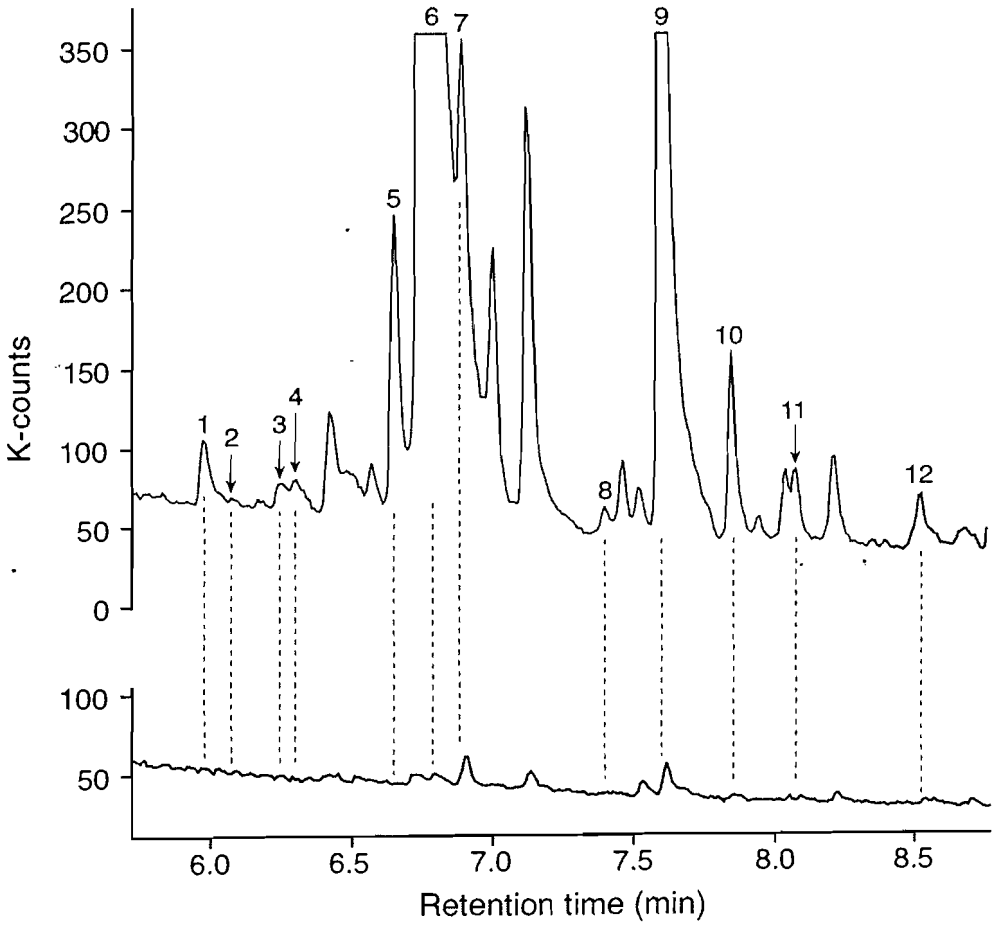
Gries_BEB_SUN_Fig1

Fig. 4.2 Mean (+ SE) amount of monoterpene analyte in the posterior dorsal abdominal gland (**A**), the body wash (**B**), and the body wash minus that in the posterior dorsal abdominal gland (**C**) of individual shade- and sun-exposed boxelder bugs, *Boisea rubrolineata*. An asterisk (*) indicates a significant preference for a particular test stimulus (Student's *t*-test, *$P < 0.05$, **$P < 0.01$)



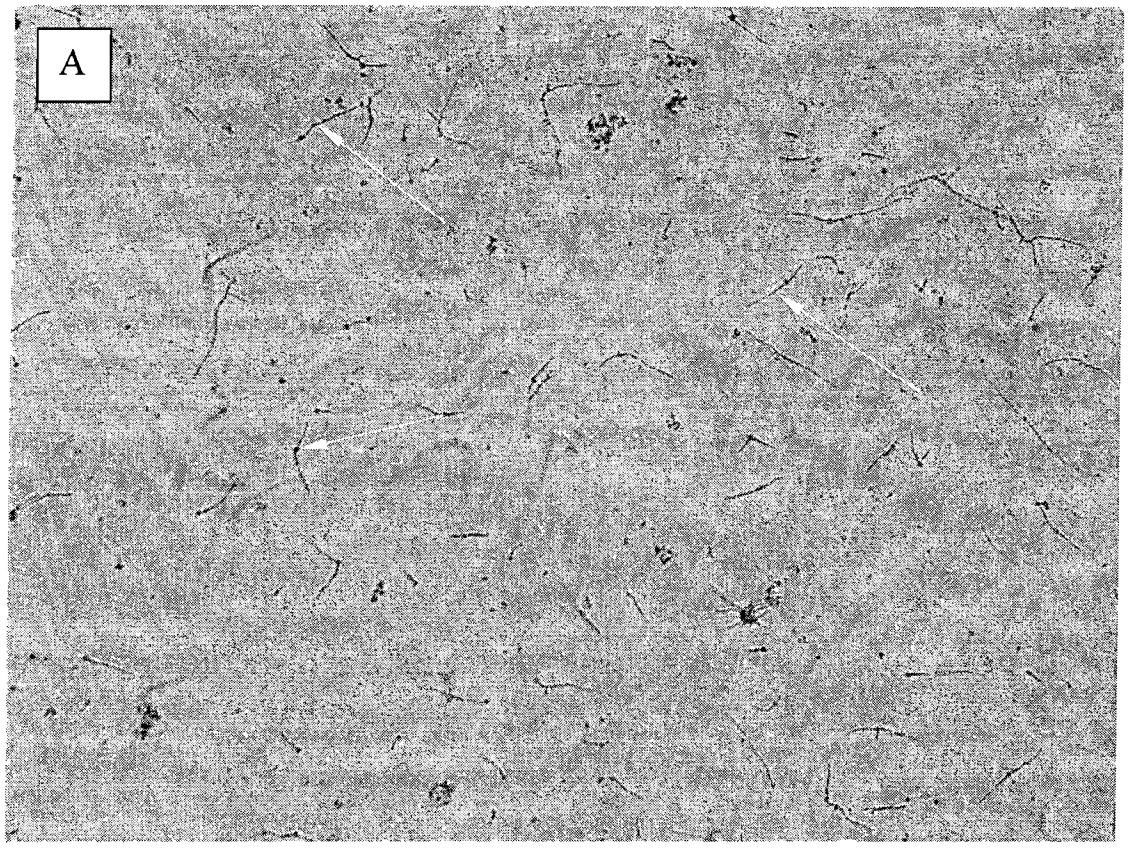
Gries_BEB_SUN_Fig2

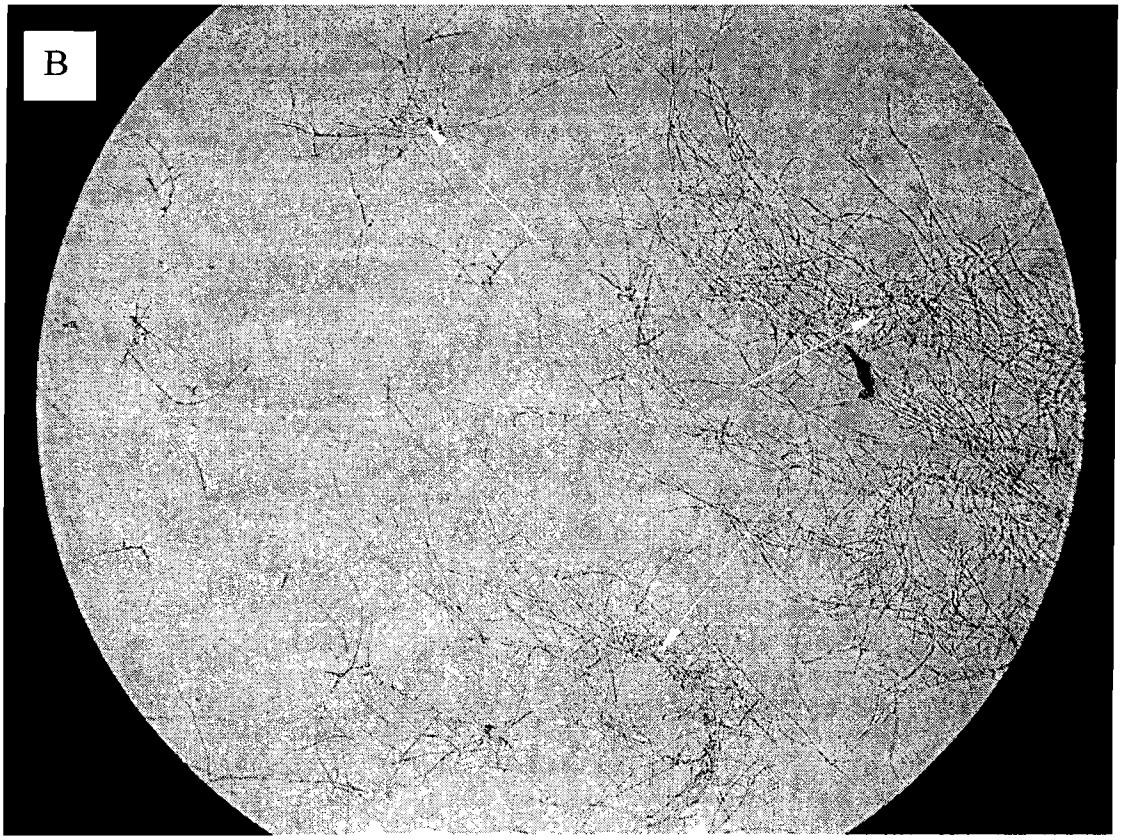
Fig. 4.3 Coupled gas chromatographic-mass spectrometric comparison of monoterpenes present in headspace volatiles of boxelder bugs, *Boisea rubrolineata*, exposed to sun (top) or shade (bottom). Nomenclature: 1 = α-thujene, 2 = α-pinene, 3 = α-fenchene, 4 = camphene, 5 = sabinene, 6 = β-pinene, 7 = myrcene, 8 = α-terpinene, 9 = limonene, 10 = trans-ocimene, 11 = γ-terpinene, 12 = terpinolene.



Gries_BEB_SUN_Fig3

Fig. 4.4 Representative photographs depicting germination and growth of the fungal pathogen *Beauveria bassiana* in a potato dextrose broth (A, B) in darkness at 25˚C in the absence of monoterpenes for 20 h and 72 h, (C) when monoterpenes were added at 0 h and retained for 72 h, (D) when monoterpenes were added after 18 h of germination for a 24-h period, and (E) when monoterpenes were added after 18 and evaporated off 24 h later, with the photograph taken at 90 h. Arrows point to (A, B) germinated conidia at 20 h and 72 h, respectively; (C) monoterpene-encapsulated conidia that failed to germinate at 72 h; (D, E) germinated conidia with no apparent further growth at 42 h and 90 h.


A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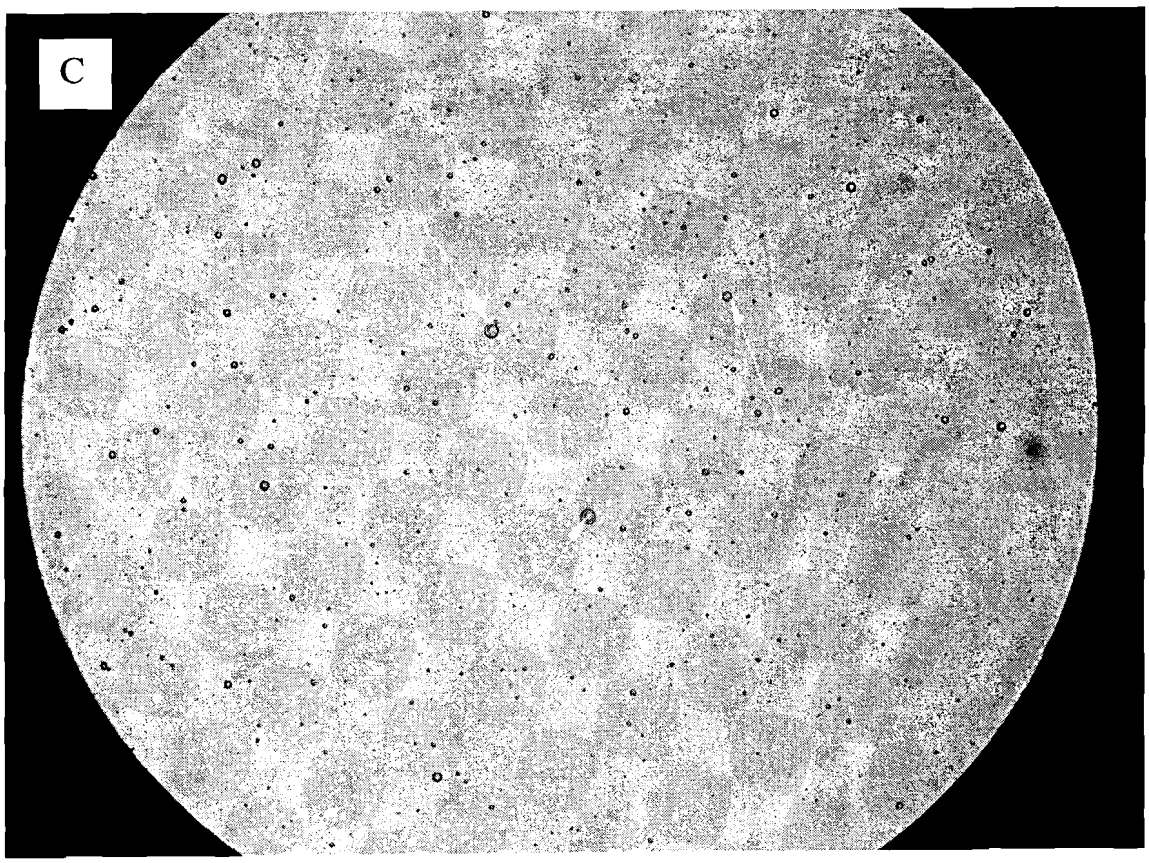
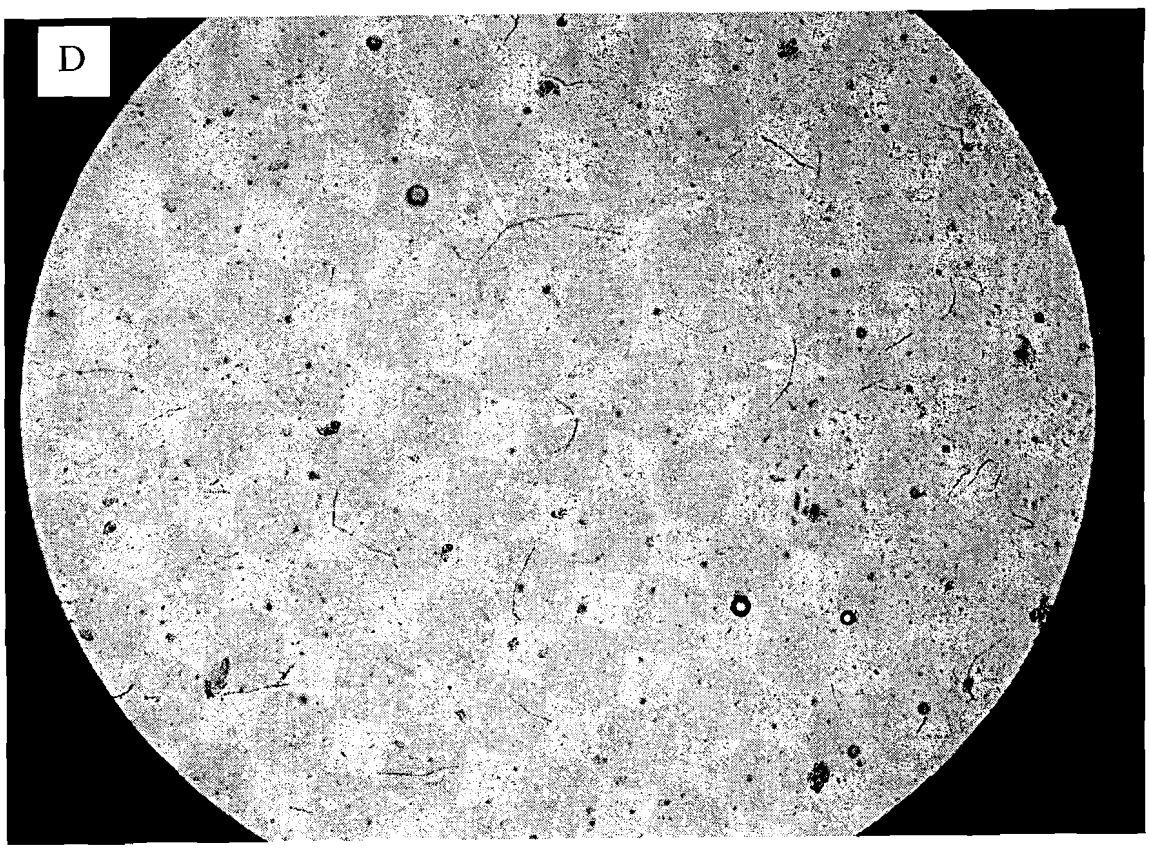
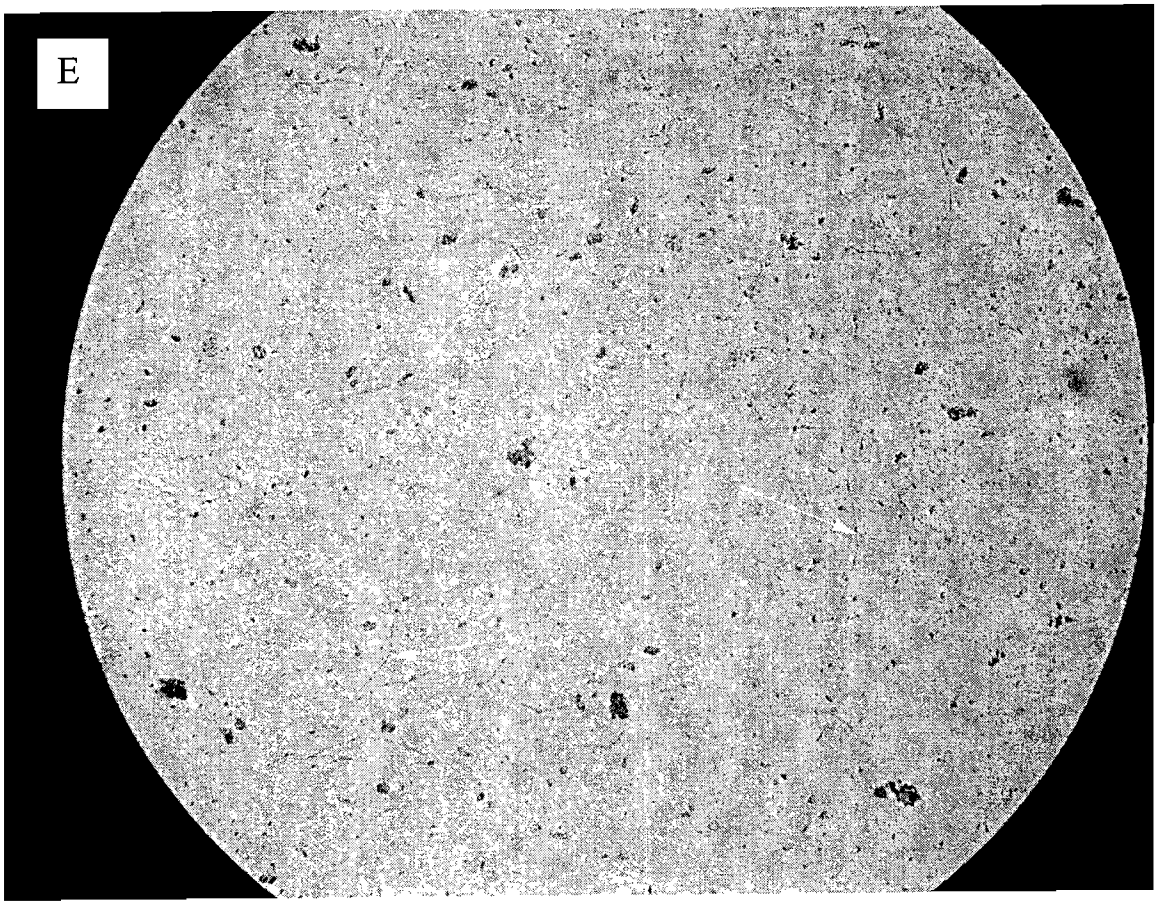

C


D


E

5 CONCLUSIONAL SUMMARY

1) Boxelder bugs, *Boisea rubrolineata*, appear to track the changing chemical phenology of their boxelder tree host tree, *Acer negundo*, from early spring to fall by responding to volatile semiochemicals of both staminate and pistillate trees.
2) *Boisea rubrolineata* track and exploit the availability of protein-rich food sources by responding to phenylacetonitrile emitted from pollen-bearing staminate trees and pistillate trees with maturing seeds. This suggests that the insects' reproductive ecology is synchronized to the phenology of *A. negundo*.
3) The host semiochemical phenylacetonitrile is attractive to males, females and nymphs, whereas the host semiochemical 2-phenethyl acetate is attractive to adults only.
4) When *A. negundo* senesces in the fall and seeds ripen and enter the state of dormancy, phenylacetonitrile and 2-phenethyl acetate decrease below detectable limits. As a result, the attractiveness of *A. negundo* to *B. rubrolineata* declines. In turn, *B. rubrolineata* enters reproductive diapause, cease feeding, and depart from their host trees.
5) Plants producing phenylacetonitrile are known to store secondary metabolites that specialist herbivores sequester for secondary (aposematic) defense. This suggests that *A. negundo*-produced phenylacetonitrile is likely linked to the trees' production of secondary metabolites (i.e., flavonoids, cyanogenic

glycosides, glucosinolates) that aposematic *B. rubrolineata* may obtain for aposematic coloration and defense against predators. If true, phenylacetonitrile may constitute a kairomone of *A. negundo* that conveys information to *B. rubrolineata* where and when to obtain food and secondary defense.

6) *Boisea rubrolineata* form spectacular aggregations on pistillate *A. negundo* with mature seeds, in and around shelters in fall and winter, and in response to warm sunlight. Aggregations are mediated, in part, by pheromone(s) that are derived from feces and/or cuticular hydrocarbons, and appear to include a female-specific sex pheromone that attracts males and a male-specific sex pheromone that attracts females.
7) 2-Phenylethanol serves as an aggregation pheromone in mid-summer for females, males and 5th-instar nymphs, and as a female attractant pheromone in early spring.
8) As an aggregation pheromone, 2-phenylethanol originates from the feces of seed-feeding females, males, and/or the ventral abdominal gland of males.
9) As a sex pheromone, 2-phenylethanol originates from the ventral abdominal gland of males that emerge from overwintering diapause.
10) 2-Phenylethanol is likely biosynthesized by *B. rubrolineata* from host-derived phenylalanine.
11) As extreme thermoregulators, *B. rubrolineata* forms highly conspicuous aggregations in warm sunlight. Moreover, *B. rubrolineata* breaks winter dormancy and sunbath on warmer winter days. While sunbathing, the bugs

exude from their posterior dorsal abdominal gland an odorous blend of monoterpenes that they spread over their cuticle.

12) These monoterpenes do not serve as an attractant or arrestment pheromone. Rather, they inhibit germination of conidia spores of the insect-pathogenic fungus *Beauveria bassiana* and halt further growth of germinated spores. This prophylactic defense against such pathogens appears adaptive for insects like *B. rubrolineata* that are prone to pathogen infection during overwintering dormancy.