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Semiochemistry of Cerambycid Beetles: Interactions Among Pheromones, Host Plant Volatiles, and Density Dependent Effects

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

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in

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by

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ABSTRACT OF THE DISSERTATION

Semiochemistry of Cerambycid Beetles:
Interactions Among Pheromones, Host Plant Volatiles,
and Density Dependent Effects

by

Robert Maxwell Collignon

Doctor of Philosophy, Graduate Program in Entomology
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Dr. Jocelyn Millar, Chairperson

Longhorn beetles (Coleoptera: Cerambycidae) are wood-boring insects that infest trees and woody plants of varying conditions, from healthy to dead. Pheromones have now been identified for a number of cerambycid species, but our understanding of pheromone use in the family is limited. The first section of my research investigated the role of host plant volatiles on attraction to pheromones, and sought to identify new host volatiles that may be attractive or synergistic, particularly for oak-infesting species. My results corroborated previous work demonstrating that conifer volatiles are synergists for some species of conifer-infesting cerambycids; no such effect was found for oak volatiles and oak-infesting species, however. The second portion of this dissertation examined how

cerambycids utilize their pheromones, focusing on several species that use male-produced pheromones, which attract both sexes and are often not species-specific compounds. Several possible pheromone partitioning mechanisms were tested, and I found that diel activity periods and pheromone blends have roles in reproductively and/or competitively isolating cerambycid species that use similar or the same pheromone components. Although not directly tested, there is evidence that host plant volatiles may play a role in partitioning and isolation as well. Evidence was also found that males of one species, *Phymatodes grandis*, may exploit the volatiles emitted by males of another species, *Phymatodes obliquus*, as a kairomone. Response to increasing release rates of two common pheromone components, 2-methylbutan-1-ol and 3-hydroxyhexan-2-one, was also tested and I found that responses increased with release rates for four of the five test species, even at the highest rates tested (~1,450 μg/h and ~720 μg/h, respectively). However, responses of the fifth species, *P. obliquus,* did plateau with increasing release rates. I also tested the effect of density of conspecific males on per capita pheromone production. Headspace volatiles of male *P. grandis* were sampled from individuals, pairs, or groups of three or four beetles held in 500 ml Teflon chambers. Emission rates of their pheromone (*R*)-2-methylbutan-1-ol were found to decrease with increasing density, suggesting that cerambycids may use optimal density strategies, as has been shown for other coleopteran species that utilize male-produced sex-aggregation pheromones.

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Chapter 1: Introduction to the chemical ecology of Cerambycidae.

Cerambycid beetles, commonly known as longhorn beetles, are wood-boring insects whose larvae – and sometimes adults – feed in and on woody plants. Longhorn beetles have the potential to be ecologically and economically damaging when they are introduced into new habitats as invasive species (Parry and Teale 2011; Haack et al. 2010). Detection of invasions by wood-boring insects is particularly problematic because larvae can persist cryptically within lumber, wooden shipping materials – such as pallets, shipping crates, and dunnage – and finished wooden products – such as furniture – being shipped internationally (Haack 2006). Conversely, native cerambycids are ecologically important to forest ecosystems because they play a major role in primary decomposition of woody material, allowing carbon and other nutrients to cycle back into the ecosystem (Haack and Byler 1993; Ulyshen 2014; Chen and Foschler 2016). In addition, they cull weak, sickly, and stressed trees within forests, maintaining baseline mortality and turnover of trees, an important factor for healthy forest ecosystems (Manion and Griffin 2001; Teale and Castello 2011). Among wood-boring insects, the Cerambycidae are one of the most diverse families (~30,000 species), rivalled only by the speciose Curculionidae (~51,000 species; Ślipiński et al. 2011).

Due to the cryptic nature of cerambycids, the options for sampling them are limited. Use of semiochemical attractants could be a valuable management option for several complementary reasons. For example, from a conservation perspective,

effective lures for biodiversity monitoring would be useful, especially considering the diversity of the family and the large proportion of species diversity within forest systems that consists of wood-boring insects (Grove 2002). Conversely, semiochemically-baited traps may be the most effective means of detecting low-density populations of pest and invasive species, providing a means of detection of longhorn beetles, for example at ports of entry or in sensitive ecosystems, as well as providing effective means of delineating the range of infestations of invasive or pest cerambycid species (Brockerhoff et al. 2006; Shea 1995).

Host-plant volatiles (HPVs) and blends mimicking HPVs also attract cerambycids, either alone or in combination with pheromones. In addition, fermenting baits have been reported as cerambycid attractants (Linsley 1959), and early work using neat ethanol established it as a general attractant for these beetles (Montgomery and Wargo 1983). The attraction of wood-boring beetles to ethanol is likely due to it being a volatile cue associated with trees stressed by damage, disease, flooding or drought, or other factors (Kimmerer and Kozlowski 1982; Gara et al. 1993; Kelsey 1994; Kelsey and Joseph 2002; Kelsey et al. 2013). Ethanol is also attractive to secondary wood-boring species (those that infest a tree after it has died; Klimetzek et al. 1986), the hypothesis being that it is a cue of microbial activity in decaying woody material (Byers 1989).

Turpentine had long been used as an attractant for longhorn beetles (Gardiner 1957). This led to the investigation of various monoterpenes as

attractants for cerambycids, with α-pinene found to be the more attractive than more complex terpene blends for some species (Chénier and Philogène 1989). Thus, blends utilizing α-pinene and ethanol have been widely used as general attractants for cerambycids, with conifer-infesting species typically most strongly attracted to these compounds (Allison et al. 2001; Sweeney et al. 2004; Sweeney et al. 2006; Miller 2006). Whereas monoterpenes are constituents of the volatiles of nearly all tree species, conifers generally emit more monoterpenes than woody angiosperms.

As well as being primary attractants for some cerambycids, HPVs are also known to modulate attraction of some cerambycid species to their pheromones. Ethanol, α-pinene, and other HPVs have been shown to have additive or synergistic effects when deployed in combination with pheromone lures for conifer-infesting species in the subfamilies Spondylidinae, Lamiinae, and Cerambycinae (Hanks et al. 2012; Hanks and Millar 2013). For example, a number of *Monochamus* species are more strongly attracted to combinations of their pheromone monochamol with α-pinene and ethanol than to the pheromone alone (Teale et al. 2011; Fierke et al. 2012; Allison et al. 2012). Similarly, the spondylidines *Tetropium fuscum* and *Tetropium cinnamopterum* respond only weakly to their pheromones alone, with attraction being significantly increased by ethanol plus a synthetic blend of five monoterpenes from spruce trees (*Picea*), their primary hosts (Silk et al. 2007).

The interplay of HPVs and pheromones has received less research attention for angiosperm-infesting species, but there is evidence of additive or synergistic

interactions for some species (Hanks et al. 2012; Hanks and Millar 2013). The lamiine species *Hedypathes betulinas* is one such example. Its host plant green maté (*Ilex paraguariensis*; Aquifoliales: Aquifoliaceae) was not attractive by itself, but volatiles from the host did increase attraction to the male-produced pheromone. The authors also noted that males would only produce pheromone in the presence of host-plant material (Fonseca et al. 2010).

Angiosperm HPVs have also been found to be attractive in the absence of pheromones, in some cases, seemingly eschewing cerambycid species' need to produce their own long-range pheromones, including the cerambycine *Phoracantha semipunctata* (Hanks et al. 1996; Hanks 1999) and the lamiine *Anoplophora chinensis* (= *A. malasiaca*; Yasui 2009). Even angiosperm-infesting species that likely use long-range pheromones also may be attracted to angiosperm HPVs. For example, newly-cut white oak (*Quercus alba*) logs and ethanol have been shown to be attractive for the cerambycines *Xylotrechus colonus* – which attacks stressed trees, and was most attracted to ethanol and oak logs – and *Elaphidion mucronatum* – which attacks dying trees, and was most attracted to ethanol with cardboard simulated logs (Dunn and Potter 1991). These results support the importance of changes in HPV profiles for cerambycid orientation to hosts, but also in indicating the appropriate physiological condition of the host tree to the beetle. Another angiosperm-infesting species, the lamiine cocoa beetle, *Steirastoma breve*, displayed antennal responses to cocoa plant (*Theobroma cacao*;

Malvales: Malvaceae) volatiles in electroantennography tests, and both females and males preferentially oriented to the volatiles in Y-tube assays (Liendo et al. 2005). Finally, females and males of three species of cerambycines, *Xylotrechus colonus*, *Megacyllene caryae*, and *Neoclytus mucronatus mucronatus*, were attracted to volatiles released by bolts of a known host, shagbark hickory (*Carya glabra*; Fagales: Juglandaceae) in Y-tube assays (Ginzel and Hanks 2005). All three of these species have since had pheromones identified (Hanks et al. 2007; Lacey et al. 2007, 2008), with ethanol and α-pinene increasing attraction to a generic pheromone blend for *X. colonus* and *N. m. mucronatus* (Hanks et al. 2012). These studies indicate an important role of host volatiles for angiosperm-attacking cerambycid species in host- and possibly mate-seeking.

Taken together, it is clear that HPVs are important factors in the chemical ecology of cerambycids, and so the first section of my dissertation research examined how HPVs modulate attraction to pheromones for a community of cerambycids in the San Bernardino National Forest (SBNF) in southern California. My objectives were to develop comprehensive synthetic blends representative of conifers and oaks generally, and to test these blends alone and together with a generic blend of cerambycid pheromones. The goals of these experiments were: A) to determine how conifer-infesting species responded to complex and simple HPV mimics, and B) to attempt to find a generic HPV blend or a compound that is attractive to oak-infesting species (Chapter 2).

In conducting this research, however, it became clear that ecological interpretation of the results was difficult because our understanding of how most cerambycids utilize their pheromones is rudimentary at best. Whereas the past decade has seen a rapid increase in the identification of cerambycid pheromones and pheromone candidates, the context of how these pheromones are being used has received much less research attention.

Of the five subfamilies for which pheromones have been identified, species in the subfamilies Prioninae and Lepturinae seem to use female-produced sex pheromones (Barbour et al. 2011; Ray et al. 2011, 2012a, 2012b; Wickham et al. 2016a, 2016b). In contrast, species in the subfamilies Cerambycinae, Lamiinae, and Spondylidinae, utilize male-produced, aggregation sex-pheromones, attractive to both females and males, and which are often shared among species (reviewed in Hanks and Millar 2016). The primary purpose of these male-produced pheromones (MPPs) is generally assumed to be as sex pheromones, emitted for the purpose of attracting female mates, and male responders are likely eavesdropping as opposed to expressing aggregative behavior (Landolt 1997; Cardé 2014; Hanks and Millar 2016). The pheromones and pheromone candidates identified from these species represent approximately twenty compounds (not including enantiomers), but have been shown to attract ~200 species from these three subfamilies (Hanks and Millar 2016, Supp. Table 1, and unpub. data). Some of the attraction may be kairomonal attraction as opposed to pheromonal (i.e. interspecies vs. intraspecies). Two

apparent examples of this are the attraction of the lamiines *Monochamus carolinensis* and *Monochamus titillator* to the cerambycine 2,3-alkanediol pheromones co-deployed with α-pinene (Allison et al. 2012), and the attraction of the cerambycine *Phymatodes grandis* to another cerambycine pheromone, 3-hydroxyhexan-2-one, a compound it apparently does not produce (Hanks et al. 2007). This attraction may be an indirect mechanism of increasing offspring fitness due to facultative predation by cerambycid larvae on smaller intraguild larvae (Anbutsu and Togashi 1997; Dodds et al 2001; Ware and Stephen 2006; Schoeller et al. 2012), and/or it may represent a means to locate suitable host trees. It is unclear how much of the co-attraction to pheromone components in field bioassays is due to the compounds acting as kairomones as opposed to pheromones (Hanks et al. 2007, 2012; Mitchell et al. 2011; Wong et al. 2012; Hanks and Millar 2013; Wickham et al. 2014), but there are examples of compounds later being confirmed as pheromones produced by an attracted species (Mitchell et al. 2011; Hughes et al. 2016; Meier et al. 2016). Thus, in Chapter 3, I examined if and how a community of cerambycids might utilize overlapping pheromones, for example by various means of partitioning their usage. Previous research indicates that both diel and seasonal temporal partitioning are used by cerambycid species (Mitchell et al. 2015; Skabeikis et al. 2016), and that blend composition may play a role as well (Hanks et al. 2012; Hanks and Millar 2013; Mitchell et al. 2015). As well as testing these factors for the SBNF community of cerambycines, I was particularly interested in

the responses of *P. grandis*, because of its previously reported kairomonal attraction to a pheromone it does not produce (Hanks et al. 2007).

In the process of reviewing other work that has been done on the chemical ecology of insect taxa that utilize MPPs, I noticed several important parallels among the ecology of these taxa, many of which are coleopteran species. Most notable is the additive or synergistic effect HPVs have on response to or production of MPPs, and the correlation between MPP use and the exploitation of scarce and/or ephemeral resources (Woods 1982; Landolt 1997). Further, as with cerambycids, many MPP-utilizing species use pheromone components that are shared among many species (Wood 1982; Williams et al. 1995; Landolt 1997; Arnaud et al. 2002).

Although the effects of density- and dose-dependence on MPP pheromone emission and responses to pheromones has received limited attention for cerambycids (Lacey et al. 2004, 2007; Sweeney et al. 2010; Lemay et al. 2010), such effects have been reported for other MPP-species (dose = release or emission rate). For example, males of several *Carpophilus* species produced less pheromone when in large groups than when held individually, possibly due to a negative feedback loop to reduce group sizes and conspecific competition (Bartelt and James 1994; Petroski et al. 1994; Bartelt et al. 1995). As well, whereas small groups of *Prostephanus truncatus* males were more attractive to conspecifics than single males, large groups of ~60 beetles were not attractive (Scholz et al. 1998), and plum curculio males were most attractive in groups of two rather than as individuals or

in larger groups (Hock et al. 2014). Similarly, males of the bark beetles *Ips paraconfusus* and *Ips typographus* were less attracted to high rates of release of MPPs, likely because high emission rates indicate a fully exploited host (Byers 1983; Schlyter et al. 1987). Other scolytine species utilize various pheromonally-mediated mechanisms for density control (Raffa 2001). These findings suggest optimal density response strategies that vary with species and context, and which could be relevant to a better understanding of the ecology of cerambycid beetle species that may use analogous MPP-based strategies. For this reason, I wanted to explicitly test how a broad range of pheromone release rates might influence responses by females and males for a number of cerambycine species. Conversely, I also wanted to test the effect of conspecific male density on pheromone per capita emission rates, using *P. grandis* as a model species for the latter experiment. This research was conducted to determine if cerambycid species could also be utilizing MPPs as part of a larger optimal density strategy, as other MPP-taxa appear to be doing (Chapter 4).

In my final data chapter, I explore the pheromones of a number of spondylidine species found in the SBNF, as relatively few pheromones have actually been confirmed from this subfamily (Chapter 5; Silk et al. 2007; Sweeney et al. 2010). I also discuss my progress on a number of pheromone identifications at varying levels of completion in the Appendix.

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Chapter 2: The influence of host plant volatiles on the attraction of longhorn beetles to pheromones.

Introduction

Cerambycid beetles are ecologically important in forest ecosystems as primary decomposers of woody material, allowing carbon and other nutrients to cycle back into the ecosystem (Grove 2002; Ulyshen 2014). In addition, they cull weak, diseased, and stressed trees within forests, maintaining baseline mortality and turnover of trees, an important factor for healthy forest systems (Haack and Byler 1993; Teale and Castello 2011). In counterpoint to their beneficial role in coevolved systems, longhorn beetles have the potential to be ecologically and economically damaging when they are introduced into new habitats as invasive species (Haack et al. 2010; Parry and Teale 2011). Preventing introductions of wood-boring insects is particularly difficult because larvae can persist cryptically within lumber, other wooden materials such as shipping crates and dunnage, and finished wooden products such as furniture, being shipped internationally (Haack 2006).

Prior to 2004, almost nothing was known about the pheromones of cerambycids (Hanks 1999; Allison et al. 2004), but within the last decade a large number (>100) of pheromones and pheromone candidates have been identified (Millar and Hanks 2016). Within the five subfamilies for which at least some

pheromones have been identified, patterns in pheromone use are emerging. For example, all identified pheromones from the subfamilies Prioninae (Barbour et al. 2011; Ray et al. 2012a; Wickham et al. 2014, 2016) and Lepturinae (Ray et al. 2011, 2012b, 2014) are female-produced sex pheromones, whereas all known examples of volatile pheromones from the subfamilies Cerambycinae, Lamiinae, and Spondylidinae are male-produced aggregation pheromones that attract both sexes. Common pheromone motifs for cerambycine species include 2,3-hexanediols, 3-hydroxyhexan-2-ones (Schröder et al. 1994; Lacey et al. 2004, 2008, 2009; Hanks et al. 2007; Ray et al. 2009, 2015) and their C_8 analogs (Leal et al. 1995; Narai et al. 2015), whereas fuscumol [(*E*)-6,10-dimethyl-5,9-undecadien-2-ol] and its ester, fuscumol acetate [(*E*)-6,10-dimethyl-5,9-undecadien-2-yl acetate], are common pheromone components for the subfamilies Spondylidinae (Silk et al. 2007; Sweeney et al. 2010) and Lamiinae (Fonseca et al. 2010; Mitchell et al. 2011; Hughes et al. 2013). In addition, for the lamiine genus *Monochamus* and some related genera, monochamol [2-(undecyloxy)-ethanol] is a proven or likely pheromone component for ~15 species (Pajares et al. 2010; Teale et al. 2011; Allison et al. 2012; Fierke et al. 2012; Macias-Samano et al. 2012; Hanks and Millar 2013; Pajares et al. 2013; Ryall et al. 2014; Wickham et al. 2014). There are also several examples of attraction of cerambycid species to pheromones of other cerambycid species that they themselves do not produce, possibly exploiting these

signals as kairomones to locate suitable habitats (Hanks et al. 2007, 2012; Mitchell et al. 2011; Wong et al. 2012; Hanks and Millar 2013; Wickham et al. 2014).

Before pheromones were identified for cerambycid beetles, mimics of naturally produced suites of host plant volatiles (HPVs), such as α-pinene, turpentine, and related materials, were used as generic attractants for cerambycids (reviewed in Allison et al. 2004, Millar and Hanks 2016). Fermenting sugar-based baits also have been used as lures (Linsley 1959), with early work establishing ethanol as a general attractant for cerambycids and other saproxylic wood-boring insects that infest stressed, dying, and dead trees (Kimmerer and Kozlowski 1982; Montgomery and Wargo 1983; Klimetzek et al. 1986; Gara et al. 1993; Kelsey 1994). Comparing the ubiquitous monoterpene α-pinene with more complex blends of monoterpenes, α-pinene has been found to be more attractive for some species of cerambycid (Chénier and Philogène, 1989; Morewood et al. 2002; Sweeney et al. 2004). Thus, blends utilizing α-pinene and ethanol have been widely used as general attractants for cerambycids (e.g. Miller 2006), with conifer-infesting species typically being most strongly attracted to such blends.

More recent studies have shown that HPVs may strongly synergize the attraction of cerambycid species to their pheromones. Among species in the subfamily Lamiinae, a number of *Monochamus* species are more strongly attracted to combinations of monochamol with α-pinene and ethanol than to monochamol alone (Teale et al. 2011; Fierke et al. 2012; Allison et al. 2012; Ryall et al. 2014).

Similarly, *Tetropium fuscum* (F.) and *Tetropium castaneum* (L.) (subfamily Spondylidinae) respond only weakly to their pheromones alone, with attraction being significantly increased by a blend of five monoterpenes from their hosts (spruce; *Picea* species) with ethanol (Silk et al. 2007).

In contrast, much less is known about the possible role of angiosperm HPVs as attractants or co-attractants (with pheromones) for cerambycids. In one of the few reported examples where angiosperm HPVs were tested, volatiles from green maté (*Ilex paraguariensis* A. St. Hill; Aquifoliales: Aquifoliaceae), the host plant of the lamiine species *Hedypathes betulinus* (Klug), were not attractive to the beetles in Y-tube assays, but they did increase attraction of female beetles to the male-produced pheromone (Fonseca et al. 2010). HPVs also have been intensively studied as possible attractants or co-attractants for the invasive Asian longhorned beetle, *Anoplophora glabripennis* Motschulsky, but the results have been mixed, with lures being relatively weakly attractive overall (Wickham 2009; Meng et al. 2014; Nehme et al. 2014). For the congeneric species *Anoplophora malasiaca* (Thomson), in which the reproductive behaviors have been studied intensively (reviewed in Yasui 2009), the evidence suggests that this species relies heavily on HPVs for mate-/host-location, eschewing the need for long-range pheromones. Although *A malasiaca* is highly polyphagous, populations reared from mandarin orange (*Citrus unshiu* Marc.; Sapindales: Rutaceae) are most strongly attracted to citrus HPVs, whereas willow-infesting (*Salix schwerinii* E. Wolf; Malphigiales:

Salicaceae) populations orient preferentially towards willow HPVs (Yasui et al. 2011). Within the subfamily Cerambycinae, both sexes of *Xylotrechus colonus* (F.), *Megacyllene caryae* (Gahan), and *Neoclytus mucronatus mucronatus* (F.) were attracted to odors from logs of their host, shagbark hickory (*Carya glabra* Miller; Fagales: Juglandaceae) in Y-tube assays (Ginzel and Hanks 2005). In sum, these studies suggest that host volatiles may also be involved in attraction of angiosperm-infesting cerambycids.

Overall, these studies indicate that our knowledge of the interactions among HPVs and pheromones in the reproductive behaviors of cerambycids is limited. Thus, the goal of the work reported here was to obtain an overview of the influence of HPVs on attraction of cerambycids to male-produced pheromones, for species infesting both conifers and angiosperm trees (specifically oaks). Our main objectives were:

1) To determine whether volatiles from fresh host plant material affected the attraction of cerambycids to their pheromones, for species attacking either conifers or oaks;

2) To analyze the volatiles from fresh host materials, with the goal of reconstructing blends of host volatiles from synthetic chemicals;

3) To test those reconstructed blends of HPVs at various release rates with and without pheromones.

We also conducted follow-up experiments to test the importance of blend complexity for conifer-infesting species, and the role of ethanol alone as a possible host tree cue for oak-infesting species. Here, we report the results from these experiments.

Methods and Materials

Field Sites and General Experimental Design Field bioassays were conducted at two sites in the San Bernardino National Forest (SBNF) in San Bernardino Co., California, USA. The first site was near Jenks Lake (34°09'45.8"N 116°54'08.6"W) and was dominated by Ponderosa pine (*Pinus ponderosa* Douglas) and white fir (*Abies concolor* [Gordon]) (Pinales: Pinaceae), with some western black oak (*Quercus kelloggii* Newbury), canyon live oak (*Quercus chrysolepis* Liebm.) (Fagales: Fagaceae), bigcone Douglas-fir (*Pseudotsuga macrocarpa* [Vasey]) (Pinales: Pinaceae), and incense cedar (*Calocedrus decurrens* Torr.) (Pinales: Cupressaceae). This site was used for all three experiments. The second site was near the community of Seven Oaks (34°11'08.0"N 116°51'57.4"W) and was dominated by canyon live oak and interior live oak (*Quercus wislizeni* A. DC.), with some single-leaf pinyon pine (*Pinus monophylla* Torr. & Frém.) and Jeffrey pine (*Pinus jeffreyi* Balfour); this site was used for the first two experiments. Black cross-vane traps (Alpha Scents, Portland OR, USA) coated with Fluon® (Graham et al. 2010) were hung on tree branches at a height of ~1.5-2 m (first experiment)

or on 1.5 m tall, L-shaped stands made from PVC pipe (second and third experiments). For all experiments, traps were placed 10-15 m apart in transects, with treatments initially assigned randomly to traps. Traps were checked twice weekly, with lures changed once weekly, at which time the trap order was rerandomized.

To attract a broad range of species, a blend of known cerambycid pheromones was used, as done in previous studies (Wong et al. 2012; Hanks and Millar 2013). The blend was formulated in isopropanol, using 25 mg/ml for pure compounds (i.e., monochamol) or 50 mg/ml for racemic compounds (all others). The blend contained the following compounds: racemic 3-hydroxyhexan-2-one (Bedoukian Research, Danville CT, USA); (2*R**,3*R**)-2,3-hexanediol (synthesized as described in Lacey et al. 2004); racemic fuscumol (Bedoukian Research); racemic fuscumol acetate (Bedoukian Research); monochamol (Bedoukian Research); and racemic 2-methylbutanol (Aldrich Chemical Co., Milwaukee WI, USA). The blend used in the first two experiments contained all six compounds, whereas (2*R**,3*R**)-2,3-hexanediol was omitted from the blend in the final experiment, because none of the cerambycine species that we had trapped in the first two experiments use 2,3-hexanediols as their pheromones. Lures consisted of 1 ml of the blend deployed in 2 mil wall thickness, low-density polyethylene resealable baggies (~5 × 7.6 cm; Fisher Scientific, Pittsburgh PA, USA).

Beetles were live trapped so that they could be used for pheromone collection and electrophysiological assays in the laboratory. Excess beetles were released at least 100 m from the field sites. Voucher specimens of all species captured in statistically significant numbers have been deposited in the Entomology Museum at UC Riverside.

Bioassay of Pheromones with Crude Host Plant Material The first experiment was designed to test the effects of crude tree volatiles in combination with the pheromone blend. The experiment was run from 4 May to 11 September 2012 at both study sites, with six spatial replicates (i.e., six trap transects). Host tree species included four conifer species (Jeffrey and Ponderosa pines, white fir, bigcone Douglas-fir) and three oak species (western black, interior live, and canyon live oak), all of which occur in the SBNF and are known hosts to a diversity of cerambycid species (Linsley and Chemsak 1997). Branches were harvested from 2-4 healthy trees of each of the species from April through June, and were chipped with an industrial chipper to increase surface area and enhance the release of volatiles. Chipped material was stored in sealed jars in a -20ºC freezer until deployed in the field. To make HPV lures, one liter of chipped material was bundled in aluminum window screen and tied to the top of the trap. Chipped material was replaced weekly, with a different species of conifer and oak being tested each week. The treatments were: 1) pheromone blend, 2) pheromone blend + chipped conifer

material, 3) pheromone blend + chipped oak material, 4) chipped conifer material, 5) chipped oak material, 6) blank control.

Identification of Host Plant Volatiles and Reconstruction of HPV Blends Compounds were identified from the seven species of trees used in the first experiment, i.e. Jeffrey pine, Ponderosa pine, white fir, bigcone Douglas-fir, western black oak, interior live oak, and canyon live oak. Approximately 250 ml of chipped tree branches of a given species were placed in a 250 ml mason jar, with the lid fitted with air inlets and outlets. Charcoal-filtered air was pulled through the container at 250 ml per min, collecting the headspace volatiles on ~100 mg of thermally-desorbed activated charcoal (50-200 mesh; Fisher Scientific) held between glass wool plugs in a short piece of glass tubing. Volatiles were collected for 24-48 h at a time, with consecutive samples being taken to follow temporal changes in the emitted blend of volatiles. Volatiles were collected from 2-4 trees of each species. Adsorbed volatiles were eluted from the collector with 1 ml of dichloromethane, and samples were analyzed with an HP 6890 gas chromatograph (Hewlett-Packard, now Agilent, Santa Clara CA, USA) fitted with a DB-17 column (30 m × 0.25 mm ID × 0.25 micron film; J&W Scientific, Folsom CA, USA), coupled to an HP 5973 mass selective detector. The temperature program used was 40ºC/1 min, then increased 5ºC/min to 280ºC, hold 5 min. To improve the chromatography of early eluting compounds, the injector temperature was set to

125ºC and injections were made in split mode. Spectra were taken in full scan mode with electron impact ionization (70 eV).

Compounds were tentatively identified by matches with database spectra (NIST 98, Agilent), and then confirmed by matching retention times and mass spectra with those of authentic standards. The absolute configurations of chiral compounds were determined by GC analysis using Cyclodex-B and Beta-Dex columns (both 30 m × 0.25 mm ID × 0.25 micron film; J&W Scientific). All samples were run on both columns, matching retention times with those of standards of known absolute configuration. Analyses were conducted using a temperature program of 50ºC/1 min, then 5ºC/min to 220ºC, hold 20 min, with an injector temperature of 100ºC. Samples were run in split mode.

Authentic standards of the host plant compounds were obtained from the following sources: Sigma-Aldrich (Milwaukee, WI, USA): (*R*)- and (*S*)-α-pinene, racemic 3-carene, (*R*)-limonene, racemic camphene, (*Z*)-3-hexenol; Alfa-Aesar (Ward Hill, MA, USA): (*S*)-β-pinene, (*S*)-limonene, 1,8-cineole, (*S*)-borneol, (*R*)- and (*S*)-camphor, 4-allylanisole, benzaldehyde, hexanol, hexanal; Acros Organics (Geel, Belgium): myrcene, (*E*)-2-hexenal, (*E*)-2-hexenol; TCI Americas (Portland, OR, USA): (-)-*trans*-β-caryophyllene, methyl salicylate.

For the two synthetic blends of HPVs, the primary criteria used to decide which compounds to use in the blends were: 1) commonality of the compound among all conifer or all oak species sampled, 2) commercial availability of the

compound (e.g., β-phellandrene was excluded because it was not readily available), 3) high abundance compounds, 4) compounds known to act as host plant cues in other insect-plant systems [e.g., (-)-*trans*-β-caryophyllene; Yasui et al. 2008], and 5) compounds that elicited strong antennal responses during coupled gas chromatography-electroantennogram detection (GC-EAD) screening tests (e.g., 4-allylanisole, see below). The ratios of compounds for the two blends were estimated from the ratios found in GC-MS chromatograms of the sampled volatiles, as determined by integrated peak areas (Tables 2.1 and 2.2). To correct for the relative volatilities of each compound in the blend, the compounds were initially blended in equal amounts and then were placed in the medium release rate device (7.5 ml glass vial, see below for details) and the headspace volatiles were sampled for 24 h from a 250 ml mason jar in the same manner as the chipped material. These data were then used to adjust the amounts of each compound added to the blends so that the resulting profile approximately matched that obtained from the sampled chipped material. Because the percent abundance among the monoterpene hydrocarbons was similar between conifers and oaks, the same ratio of these terpenes was used in the two blends to standardize this variable between the two blend compositions. The reconstructed blends are listed in Table 2.3.

To determine which compounds beetles can perceive, GC-EAD analyses were conducted with the HPV samples and with synthetic compounds. GC-EAD analyses were carried out with antennae of the oak-infesting species *Phymatodes*

obliquus Casey, *Phymatodes grandis* Casey, *Brothylus conspersus* Leconte, *Brothylus gemmulatus* Leconte, and *Neoclytus modestus modestus* Fall, and the conifer-infesting species *Neospondylis upiformis* (Mannerheim), *Asemum striatum* [L.], *Asemum caseyi* [L.], *Asemum nitidum* [L.], and *Monochamus clamator* (Leconte). The objective was not to determine every compound that may or may not be perceived by the beetles, but to screen for low abundance compounds that elicited strong antennal responses that might otherwise have been overlooked. GC-EAD analyses were conducted on an HP 5890 Series II GC fitted with a DB-17 column (30 m × 0.25 mm ID × 0.25 micron film) programmed from 50°C/1 min, then increased 5°C/min to 250°C, hold 10 min. The injector temperature was 250°C, and injections were made in splitless mode. A glass X-cross split the effluent between the flame-ionization detector (FID) and EAD, with helium being added through the fourth arm of the cross as makeup gas. The column effluent was directed into a humidified air stream that then was directed over the antennal preparation. Antennae were prepared by excising the distal three to five segments of a live beetle's antenna and then removing the tip of the antenna with a razor blade to provide electrical contact. The antennal section was suspended between two saline-filled (7.5 g NaCl, 0.21 g $CaCl_2$, 0.35 g KCl, and 0.20 g $NaHCO_3$ in 1 L Milli-Q purified water) glass capillary electrodes with 0.2 mm diameter gold wire connections between the electrodes and a custom-built electroantennogram

amplifier. The signals from the GC and the EAD were recorded simultaneously using Peak-Simple software (SRI International, Menlo Park, CA, USA).

Table 2.1. Mean (± 1 SE) relative abundances of volatile chemicals in headspace aerations of conifer species and enantiomeric composition for chiral compounds. Relative abundance of chemicals in the reconstructed synthetic blend is also included.

	Ponderosa pine (n=2)		Jeffrey pine (n=2)		Bigcone Douglas-fir (n=1)		White fir (n=1)		All conifers (n=6)	Synthetic conifer blend
Compound	Mean ± SE	Chirality	Mean± SE	Chirality	Rel. abund -ance	Chirality	Rel. abund -ance	Chirality	Mean ± SE	Rel. abundance
α-pinene	44.9 ± 5.7	*S*[1]	35.9 ± 6.2	*S*	8.59	*R*,*S*	12.0	2:1 *S*:*R*	30.4 ± 6.9	21.4
camphene	5.0 ± 0.6	(-)	3.6 ± 2.1	(-)	0.35	(-),(+)	0.57	(-)	3.0 ± 1.0	3.6
β-pinene	13.3 ± 1.0	1:2 *R*:*S*	17.3 ± 1.9	*S*	37.4	*S*	45.3	*S*	24.0 ± 5.7	19.9
myrcene	4.4 ± 0.4	-	5.6 ± 0.1	-	2.84	-	2.1	-	4.1 ± 0.6	13.1
3-carene	6.9 ± 1.1	-	7.5 ± 5.5	-	4.36	-	3.1	-	6.1 ± 1.6	19.0
D-limonene	3.6 ± 0.03	*R*	12.6 ± 4.6	*R*	7.69	*R*	5.1	*R*	7.5 ± 2.1	14.4
β-phellandrene	6.4 ± 0.4	-	3.7 ± 0.1	-	19.0	-	20.9	-	10.0 ± 3.2	-
eucalyptol	2.9 ± 0.1	-	0	-	0	-	0	-	0.97 ± 0.61	4.5
borneol	0.13 ± 0.04	*S*	0.16 ± 0.09	*S*	0.29	*S*	0.26	*S*	0.19 ± 0.04	0.45
camphor	0.08 ± 0	2:1 (-):(+)	0.08 ± 0.03	(-)	0.21	2:1 (-):(+)	0.10	(-)	0.10 ± 0.02	1.1
1,8-cineole	3.8 ± 1.0	-	2.4 ± 1.8	-	4.29	-	-	-	3.3 ± 0.7	1.1
(-)-*trans*-β-caryophyllene	1.6 ± 0.5	-	0.20 ± 0.20	-	1.85	-	3.4	-	1.8 ± 0.5	-
unidentified	7.2 ± 3.8	-	11.1 ± 3.2	-	13.1	-	7.3	-	9.5 ± 1.7	-

[1]Unless otherwise specified, indicates dominant isomer.

Table 2.2. Mean (± 1 SE) relative abundances of volatile chemicals in headspace aerations of oak species and enantiomeric composition for chiral compounds. Relative abundance of chemicals in the reconstructed synthetic blend is also included.

	Canyon live oak (n=2)		Interior live oak (n=3)		Western black oak (n=2)		All oak samples (n=7)	Synthetic oak blend
Compound	Mean ± SE	Chirality	Mean ± SE	Chirality	Mean ± SE	Chirality	Mean ± SE	Rel. abundance
hexanal	0.24 ± 0.07	-	0.29 ± 0.11	-	0.08 ± 0.08	-	0.22 ± 0.06	0.87
1-hexanol	3.1 ± 0.8	-	2.3 ± 1.0	-	1.9 ± 1.2	-	2.4 ± 0.5	2.2
(*E*)-3-hexen-1-ol	18.6 ± 3.7	-	4.5 ± 1.5	-	7.3 ± 2.8	-	9.3 ± 2.7	5.9
(*Z*)-2-hexen-1-ol	0	-	1.2 ± 0.9	-	1.8 ± 1.0	-	1.4 ± 0.5	-
α-pinene	29.3 ± 3.3	*S*,*R*	9.9 ± 1.4	2:1 *S*:*R*	9.4 ± 2.7	*S*,*R*	15.3 ± 3.8	16.6
(*Z*)-2-hexenal	2.2 ± 1.4	-	3.4 ± 2.6	-	4.1 ± 2.0	-	3.3 ± 1.2	11.4
camphene	1.1 ± 0.2	(-)	0.30 ± 0.13	(-)	0.30 ± 0.14	(-)	0.53 ± 0.17	2.6
β-pinene	16.4 ± 2.9	1:2 *R*:*S*	7.6 ± 5.5	1:2 *R*:*S*	1.7 ± 1.0	*R*	8.4 ± 3.2	13.7
myrcene	4.0 ± 0.5	-	3.5 ± 2.9	-	1.1 ± 0.6	-	3.0 ± 1.2	8.4
3-carene	2.2 ± 0.3	-	1.3 ± 0.9	-	0.55 ± 0.38	-	1.3 ± 0.4	12.5
D-limonene	11.5 ± 2.3	*R*[1]	9.4 ± 6.9	*R*	2.6 ± 1.0	2:1 *R*:*S*	8.0 ± 3.0	9.1
β-phellandrene	2.7 ± 0.5	-	3.7 ± 2.7	-	2.9 ± 1.5	-	3.2 ± 1.1	-
1,8-cineole	5.2 ± 3.1	-	69.1 ± 9.1	-	56.0 ± 1.7	-	43.5 ± 11.8	14.4
benzaldehyde	0.13 ± 0.02	-	0.15 ± 0.09	-	0.09 ± 0.01	-	0.12 ± 0.03	0.77
4-allylanisole	0.55 ± 0.02	-	0.54 ± 0.29	-	0.34 ± 0.13	-	0.49 ± 0.12	1.1
methyl salicylate	0.19 ± 0.02	-	0.38 ± 0.21	-	3.3 ± 0.5	-	1.2 ± 0.6	0.55
(-)-*trans*-β-caryophyllene	0.10 ± 0.01	-	0.07 ± 0.07	-	0.08 ± 0.01	-	0.08 ± 0.03	-
unidentified	2.5 ± 1.2	-	5.5 ± 5.5	-	6.6 ± 2.1	-	4.9 ± 2.2	-

[1]Unless otherwise specified, indicates dominant isomer.

Table 2.3. Synthetic blend compositions for the conifer and oak blends, with each compound's contribution expressed as milliliters per 100 ml. Ratio of enantiomers listed.

	Conifer blend		Oak blend	
Compound	enantiomeric ratio	ml / 100 ml	enantiomeric ratio	ml / 100 ml
hexanal	-	-	-	0.4
hexanol	-	-	-	7.3
(*Z*)-3-hexen-1-ol	-	-	-	14.7
(*E*)-2-hexen-1-ol	-	-	-	1.7
α-pinene	2:1 (*S*:*R*)	17.2	2:1 (*S*:*R*)	7.3
(*E*)-2-hexenal	-	-	-	14.7
camphene	racemic	1	racemic	0.4
β-pinene	pure (*S*)	17.2	pure (*S*)	7.3
myrcene	-	17.2	-	7.3
3-carene	racemic	17.2	racemic	7.3
limonene	2:1 (*R*:*S*)	17.2	2:1 (*R*:*S*)	7.3
1,8-cineole	-	4	-	14.7
benzaldehyde	-	-	-	0.4
borneol	pure (*S*)	2	-	-
camphor	2:1 (*S*:*R*)	2	-	-
4-allylanisole	-	1	-	3.7
methyl salicylate	-	-	-	3.7
(-)-*trans*-β-caryophyllene	-	4	-	1.8

Bioassay of Pheromones with Reconstructed Blends of Host Plant Volatiles

The goal of this experiment was to test synthetic blends of volatiles mimicking the odors of oaks and conifers respectively (see above), with and without pheromones. Three release rates were tested: ~0.02, ~0.4, and ~3 g per day of the total blend for the low, medium, and high release rates, respectively. The release devices consisted of a screw-top 2 ml glass vial (low rate; 3 cm tall × 11 mm outer diameter × 5 mm opening), a 7.5 ml glass vial (medium rate; 3.7 cm tall × 22 mm outer diameter ×

13 mm opening), and a 25 ml glass jar (high rate; 4.3 cm tall × 4.3 cm outer diameter × 3.1 cm opening). All three release devices were left uncapped for deployment, and vials were never more than half-filled to control for higher release rates of compounds closer to the rim of the container (Weatherston et al. 1985). One and 5 ml, respectively, of the synthetic blends were loaded into the low and medium release devices weekly, and 10 ml was loaded into the high release rate dispensers twice weekly. Before replenishing the tree volatile blends, the remaining blend in the vials was poured into a waste container for disposal.

Release rates from the devices were estimated in the laboratory by measuring weight loss from devices held in a fume hood. These approximations were confirmed by observing the amount of each blend that evaporated from the release devices in the field. The ratio of the compounds in the blend remained consistent until the meniscus of the blend reached the base of the vial, at which point lower volatility compounds were in higher abundance (determined by GC-MS analysis of blends sampled in the laboratory). During the experiment, the high-release rate device was occasionally reduced to the meniscus when site temperatures were high.

The field experiment was run from 15 April to 3 September 2013 at both field sites, with four spatial replicates. The treatments were: 1) pheromone blend + ethanol, 2) pheromone blend + ethanol + low release rate synthetic conifer blend, 3) pheromone blend + ethanol + medium release rate conifer blend, 4) pheromone

blend + ethanol + high release rate conifer blend, 5) pheromone blend + ethanol + low release rate synthetic oak blend, 6) pheromone blend + ethanol + medium release rate oak blend, 7) pheromone blend + ethanol + high release rate oak blend, 8) ethanol + medium release rate conifer blend, 9) ethanol + medium release rate oak blend, 10) ethanol control. Ethanol was included in this experiment because it is known to occur in stressed trees and because of its known efficacy in attracting cerambycids (see Introduction). The ethanol was released from 10 × 15 cm low density polyethylene resealable bags (2 mil wall thickness; Fisher Scientific) loaded with 100 ml ethanol. These devices had a consistent release rate of ~0.2 g per day as long as the bags contained enough ethanol to coat the interior surface area of the bag. The release rate was determined in the laboratory gravimetrically.

Bioassay of Pheromones with Subsets of Host Plant Volatiles To minimize the overall number of traps that had to be deployed and to use the available field sites most efficiently, this experiment combined two parts. The objective of the first part was to test whether a relatively complex blend of HPVs could be sufficiently mimicked by a single major component, α-pinene, for the species infesting conifers. Thus, we tested the full synthetic conifer blend used in the second experiment versus α-pinene, with and without ethanol. The second objective was to test whether ethanol increased attraction of oak-infesting species to their pheromones. The experiment was run from 29 April to 11 August 2014 at the Jenks Lake site, using

three spatial replicates. The high release rate of the conifer blend was used because this had proven to be most attractive to conifer-infesting species in the second experiment. α-Pinene as a single component was released from the high-release device, and gravimetric measurements in the laboratory confirmed that the release rate was comparable to that of the full synthetic conifer blend (i.e., ~3 g/day). α-Pinene was deployed in a 2:1 ratio of the (*S*)- and (*R*)-enantiomers, as was used in the full conifer blend. Ethanol was released from 100 ml plastic bags as described above. Thus, the treatments were: 1) pheromone blend + α-pinene, 2) pheromone blend + ethanol + α-pinene, 3) pheromone blend + full conifer blend, 4) pheromone blend + ethanol + full conifer blend, 5) pheromone blend, 6) pheromone blend + ethanol. Cerambycid species were classified as either conifer- or oak-infesting (Linsley and Chemsak 1997) and then analyzed for differences between treatments 1-4 or 5-6, respectively. The oak-infesting species do not necessarily specialize solely on oaks, but all typically utilize oaks as primary hosts in the SBNF.

Statistical Analyses Species with less than five individuals trapped were excluded from analysis. In the first experiment using chipped host plant material, our initial attempts to analyze the trap catch data by tree species were not successful because of the high variability among trap catches and relatively low numbers of beetle captured for some species. Thus, the data from the four conifer and three oak species, respectively, were pooled for analysis. Replicates for each experiment were

based on both spatial and temporal replicates, with temporal replicates equalling the number of times the traps were checked. Temporal replicates where no beetles of a given species were trapped – usually due to inclement weather or being outside the species' flight period – were not included in analyses for that species. Data were not normally distributed as determined by the Shapiro-Wilks Test, and so were analyzed with the non-parametric Kruskal-Wallis Test followed by Dunn's Test due to rank-ties (Elliott and Hynan 2011; SAS 9.3 and 9.4, SAS Institute), using $\alpha = 0.05$. For Dunn's Test, the *q*-value must be greater than the *q* (0.05)-value to be statistically significantly different. The *q* (0.05)-value is 2.94 for six treatments (first and third experiments), 3.26 for 10 treatments (second experiment), and 1.96 for two treatments (third experiment).

Results

Bioassays of Pheromone Blends with Crude Host Plant Volatiles A total of 1,046 cerambycid beetles of species that typically infest oak were caught, with four cerambycine species being caught in sufficient numbers to warrant statistical analysis, including *Brothylus conspersus* (23 beetles), *Brothylus gemmulatus* (20 beetles), *Phymatodes grandis* (98 beetles), and *Phymatodes obliquus* (905 beetles). The entire list of species caught is provided in the supplementary information (Table 2.6). There were no significant differences in the responses of any of the four species to pheromones alone versus the pheromones plus chipped oak (*P.*

obliquus, $q = 1.05$; *P. grandis*, $q = 0.07$; *B. conspersus*, $q \leq 2.19$; *B. gemmulatus*, $q \leq 2.35$; Fig. 2.1a, b). Attraction of both *Phymatodes* species to the pheromone blend was inhibited by volatiles from the chipped conifer branches (*P. obliquus,* $q = 3.55$; *P. grandis*, $q = 4.11$), whereas there were no significant differences in the responses of either *Brothylus* species to the pheromone versus pheromone + conifer material treatments. None of the four species were significantly attracted to the chipped oak or conifer materials alone.

Three conifer-infesting species were caught in significant numbers during this experiment: *Tetropium abietis* Fall (Spondylidinae) (20 beetles), *Monochamus clamator* (Lamiinae) (27 beetles), and *Tragosoma depsarium* sp. nov. Laplante (Prioninae) (38 beetles) (Table 2.6). *Tetropium abietis* were significantly more attracted to pheromone blend plus conifer chips than to any non-pheromone blend treatments ($q = 4.17$), but not to the pheromone alone or to the pheromone + chipped oak material ($q \leq 2.83$; Fig. 2.1c). *Monochamus clamator* were equally attracted to the pheromone + conifer and pheromone + oak treatments ($q = 0.83$), significantly more so than to all other treatments ($q \geq 3.01$; Fig. 2.1c). *Tragosoma depsarium* were equally attracted to pheromones alone or pheromones with either conifer or oak chips, but not to the remaining treatments ($q \leq 1.73$; Fig. 2.1c).

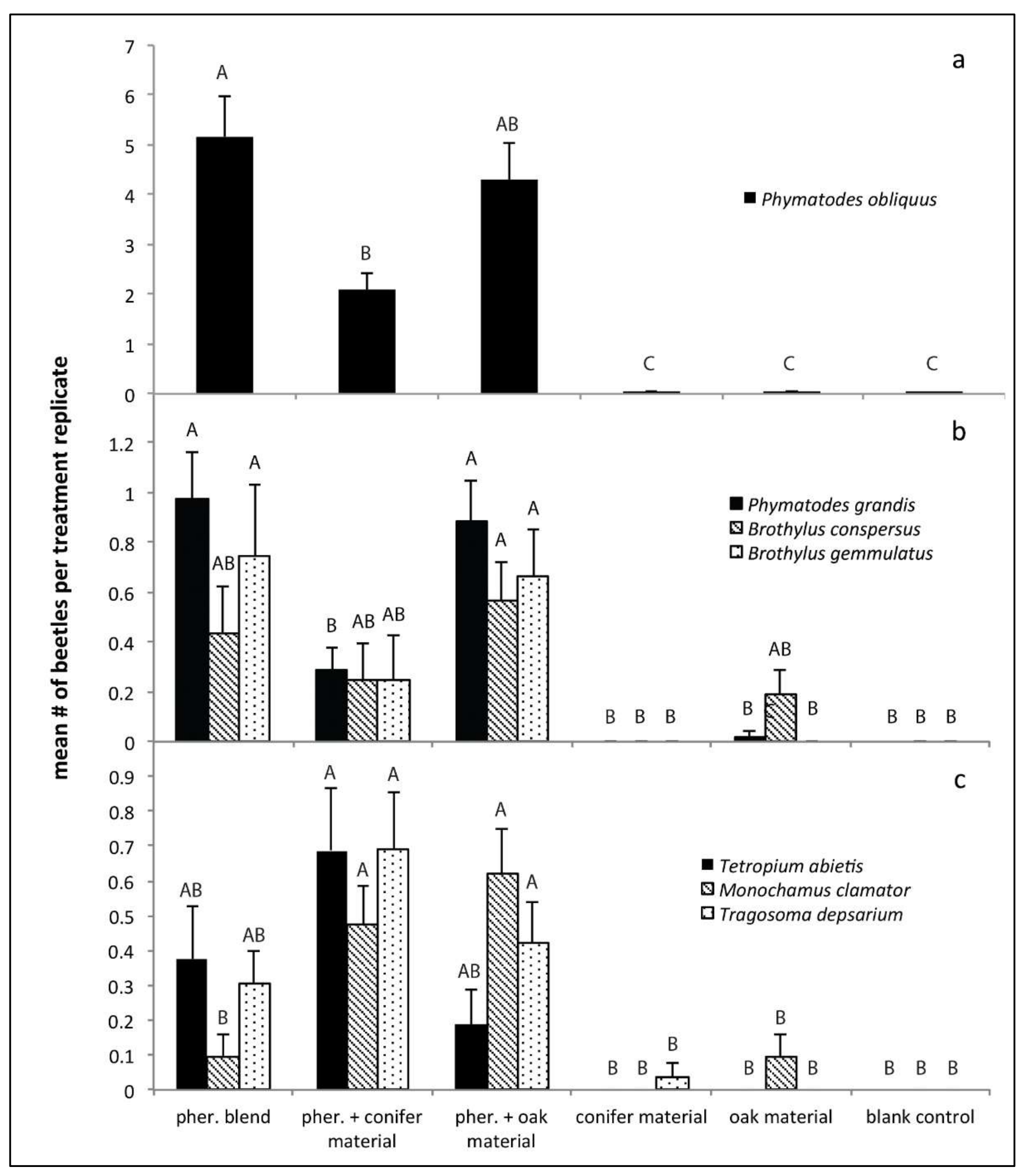

Fig. 2.1. Mean (± 1 SE) numbers of beetles caught in traps baited with the pheromone blend and chipped host plant materials. a) Oak-infesting species, *P. obliquus*, b) oak-infesting species *P. grandis*, *B. conspersus*, and *B. gemmulatus* (Cerambycinae), c) conifer-infesting species *T. abietis* (Spondylidinae), *M. clamator* (Lamiinae), and *T. depsarium* sp. nov. Laplante (Prioninae). Within each species, means with the same letter are not significantly different (Dunn's test $P > 0.05$).

Analysis of Host Plant Volatiles, and Reconstruction of Synthetic Blends Mimicking Host Plant Volatiles The extracts from both conifers and oaks shared a number of monoterpenes, including α-pinene, β-pinene, myrcene, 3-carene, limonene, and β-phellandrene (Fig. 2.2; Tables 2.1 and 2.2). However, only the oak samples contained green-leaf volatiles such as hexanal, hexanol, (*Z*)-3-hexenol, (*E*)-2-hexenol, and (*E*)-2-hexenal. The conifer and oak samples also showed qualitative differences among low-abundance sesquiterpenes and oxygenated monoterpenoids. Many of the chiral compounds were present as non-racemic and variable ratios of enantiomers, in which case we set the ratios in the synthetic blends to 2:1 to standardize the differences between samples. 3-Carene was not resolved on either the Cyclodex-B or the Beta-Dex column, and so racemic 3-carene was used in the synthetic blends. The final ratios of compounds used in the synthetic conifer and oak blends are shown in Table 2.3.

Among species trapped in the field bioassays, antennae of five oak-infesting species (*P. obliquus*, *P. grandis*, *B. conspersus*, *B. gemmulatus*, and *N. m. modestus*) and five conifer-infesting species (*N. upiformis*, *Asemum striatum*, *Asemum caseyi*, and *Asemum nitidum*, and *M. clamator*) were tested for their responses in GC-EAD analyses to samples of the HPVs collected by aeration of chipped host plant material. The results from these analyses clearly indicated that cerambycid species can detect non-host compounds (Tables 2.4 and 2.5).

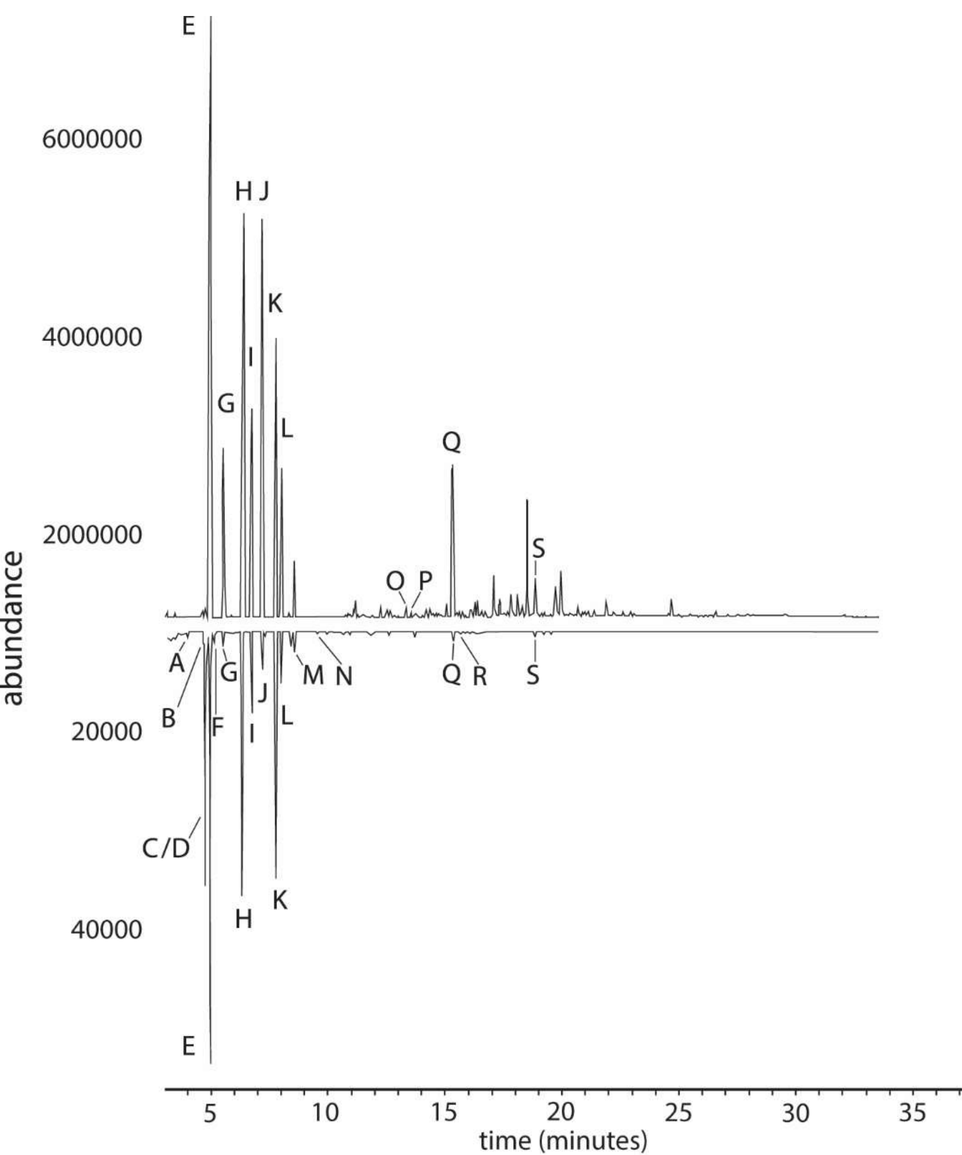

Fig. 2.2. Representative gas chromatograms for conifers and oaks. Top chromatogram: sample of volatiles from Jeffrey pine; bottom: sample of volatiles from canyon live oak. Labelled peaks are as follows: A) hexanal, B) 1-hexanol, C) (*Z*)-3-hexen-1-ol, D) (*E*)-2-hexen-1-ol (co-eluted shoulder of (*Z*)-3-hexen-1-ol), E) α-pinene, F) (*E*)-2-hexenal, G) camphene, H) β-pinene, I) myrcene, J) 3-carene, K) limonene, L) β-phellandrene, M) 1,8-cineole, N) benzaldehyde, O) borneol, P) camphor, Q) 4-allylanisole, R) methyl salicylate, S) (-)-*trans*-β-caryophyllene.

Table 2.4. Volatiles identified from oak and conifer species that elicited responses from antennae of oak-infesting Cerambycinae species, in coupled gas chromatography-electroantennogram detection (GC-EAD) analyses.

	hexanal	hexanol	(*Z*)-3-hexenol	(*E*)-2-hexenol	α-pinene	(*E*)-2-hexenal	camphene	β-pinene	β-myrcene	3-carene	limonene	β-phellandrene	1,8-cineole	benzaldehyde	borneol	camphor	4-allylanisole	methyl salicylate	(*E*)-β-caryophyllene	unidentified
Brothylus conspersus ♀		x	x	x																1
Brothylus conspersus ♂		x	x	x	x			x	x				x					x		3
Haplidus testaceus ♀			x		x			x	x	x	x	x					x			16
Haplidus testaceus ♂																				
Molorchus eberneus ♂		x	x	x				x	x	x	x		x		x	x	x	x		1
Neoclytus modestus ♀	x	x	x	x	x	x	x	x	x	x	x	x	x				x	x		8
Neoclytus modestus ♂																				
Phymatodes grandis ♀																				
Phymatodes grandis ♂		x	x					x	x			x		x			x			1
Phymatodes infuscatus ♀		x	x	x	x								x	x			x	x		5
Phymatodes infuscatus ♂																				
Phymatodes obliquus ♀		x	x	x	x	x		x	x	x	x		x		x	x	x	x	x	1
Phymatodes obliquus ♂		x	x	x	x	x							x				x	x		

Table 2.5. Volatiles identified from oak and conifer species that elicited responses from antennae of conifer-infesting cerambycid species, in coupled gas chromatography-electroantennogram detection analyses.

	hexanal	hexanol	(*Z*)-3-hexenol	(*E*)-2-hexenol	α-pinene	(*E*)-2-hexenal	camphene	β-pinene	β-myrcene	3-carene	limonene	β-phellandrene	1,8-cineole	benzaldehyde	borneol	camphor	4-allylanisole	methyl salicylate	(*E*)-β-caryophyllene	unidentified
Asemum caseyi		x	x	x	x	x		x	x	x	x			x		x	x	x	x	1
Asemum nitidum		x	x	x		x											x	x		
Asemum striatum										x	x					x	x	x		1
Callidium sp. ♀		x	x	x	x		x	x	x	x		x	x			x	x	x		1
Callidium sp. ♂		x	x	x	x		x	x	x	x		x		x		x		x		2
Monochamus clamator ♀				x	x			x	x	x		x	x					x		1
Monochamus clamator ♂																				
Ortholeptura valida								x			x		x			x	x			1
Poliaenus oregonus ♀																				
Poliaenus oregonus ♂		x	x	x	x					x								x		1
Pseudostylopsis sp.			x	x	x			x	x	x	x	x			x	x	x		x	17

Bioassays of Pheromones with Reconstructed Blends of Host Plant Volatiles

Four oak-infesting species, *P. obliquus* (896 beetles), *P. grandis* (243 beetles), *B. gemmulatus* (322 beetles), and *N. m. modestus* (20 beetles), all in the subfamily Cerambycinae, were caught in significant numbers (Table 2.7). Trap catches of *P. obliquus* and *P. grandis* decreased with increasing release rates of both synthetic conifer and oak volatiles, with trap catches at the highest release rates of either being no different from catches in traps baited with the various control treatments without pheromones (Fig. 2.4a, b). *Brothylus gemmulatus* trap catches appeared to show similar trends, but high variability within the various treatments prevented any meaningful interpretation of the results (Fig. 2.4a). *Neoclytus m. modestus* was equally attracted to ethanol alone and the pheromone + ethanol treatment ($q = 1.68$), indicating that attraction was primarily due to ethanol. Addition of any of the host volatile blends at any rate to the pheromone + ethanol blend decreased captures ($q \geq 3.83$; Fig. 2.4b).

Conifer-infesting species were represented in this experiment in significant numbers by the spondylidines *N. upiformis* (51 beetles), *A. striatum* (20 beetles), *A. caseyi* (11 beetles), and *A. nitidum* (18 beetles), the lamiine *M. clamator* (18 beetles), and the cerambycine *Xylotrechus albonotatus* Casey (10 beetles) (Table 2.7). *Neospondylis upiformis* were most attracted by the pheromone + high release rate conifer volatiles treatment, with significant separation from all other treatments except pheromone plus medium release rate conifer ($q \geq 3.39$, $q = 2.38$; Fig. 2.4c).

Responses of *M. clamator* followed a similar pattern, with significant separation of pheromone blend + high release rate conifer blend from all other treatments ($q \geq$ 3.36; Fig. 2.4d); no other treatments were significantly different from the controls. For *X. albonotatus*, treatment means did not separate statistically, although a Kruskal-Wallis test indicated differences among all the treatments (P = 0.024; Fig. 2.4d). In this experiment, the spondylidines *A. striatum*, *A. caseyi*, and *A. nitidum* were caught in low numbers overall (see above), and their responses to the various treatments also did not separate statistically. However, all but *A. striatum* exhibited differential responses to the treatments when analyzed by Kruskal-Wallis tests (*A. nitidum*, P = 0.015; *A. striatum*, P = 0.059; *A. caseyi*, P = 0.043; Fig. 2.3).

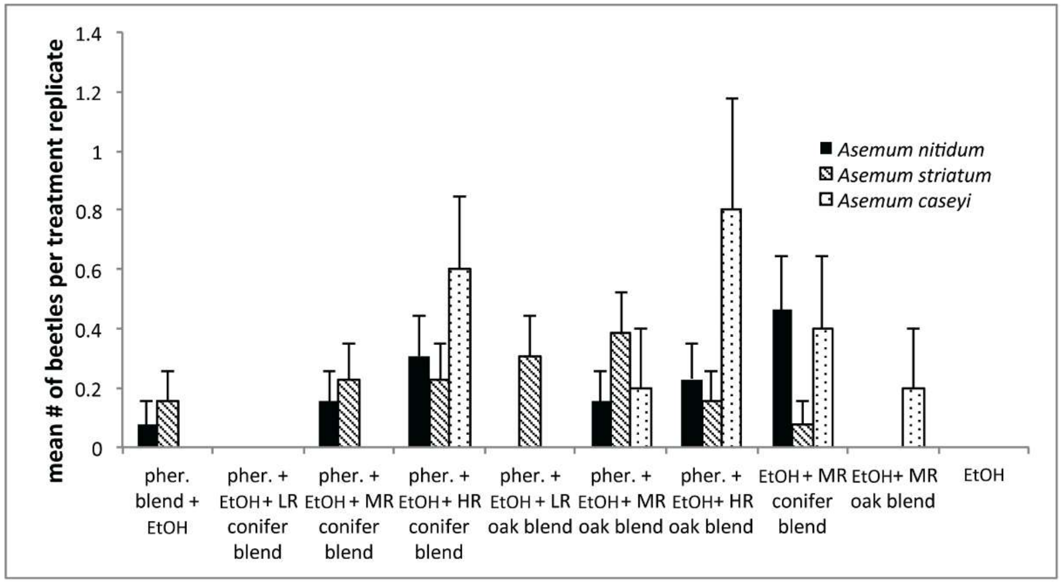

Fig. 2.3. Mean (± 1 SE) numbers of *A. nitidum, A. striatum,* and *A. caseyi* (Spondylidinae) caught in traps baited with the pheromone blend, low (LR), medium (MR), and high (HR) release rates of reconstructed blends of host plant compounds, and ethanol. There were no significant means separations among the treatments (Dunn's Test).

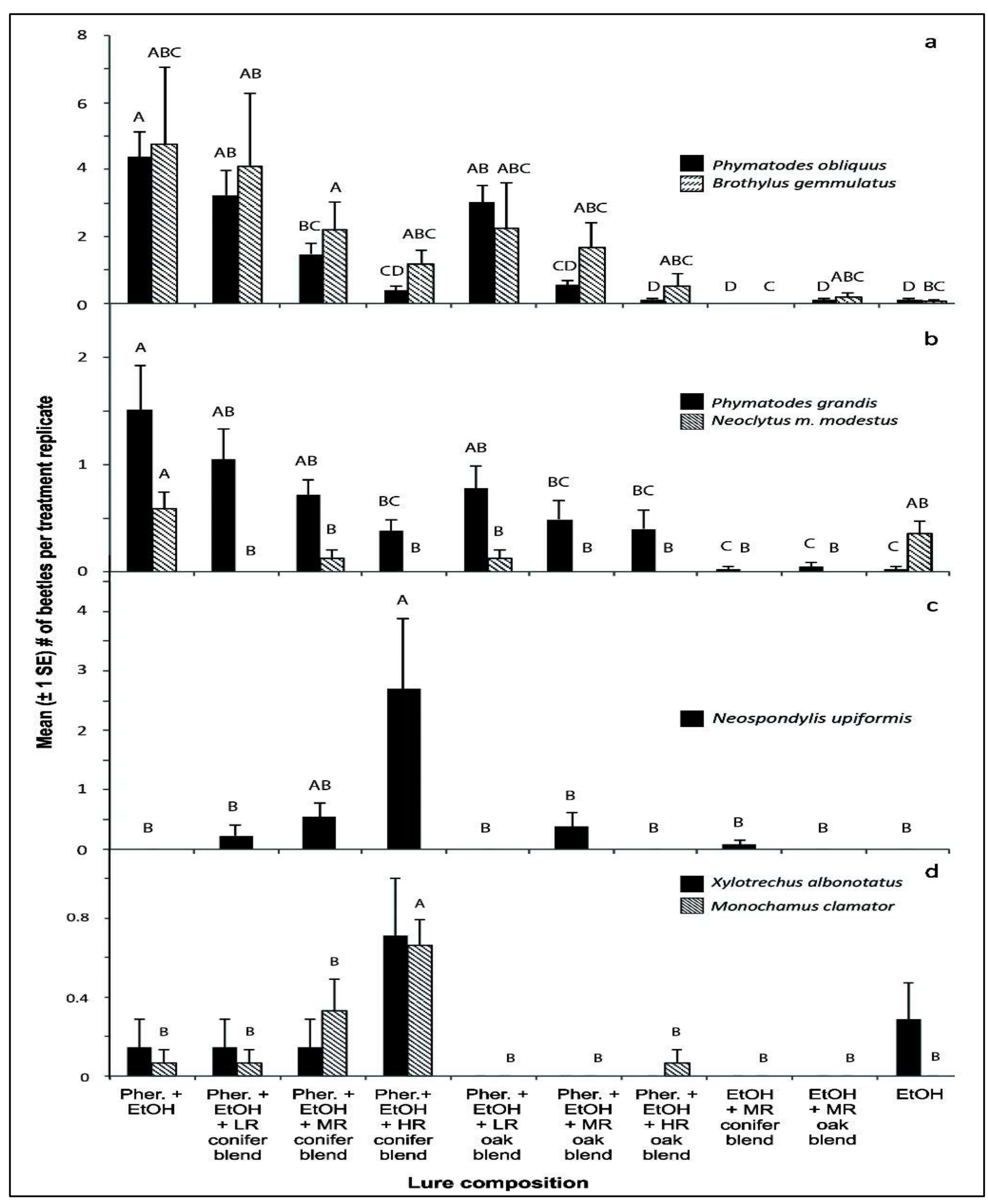

Fig. 2.4. Mean (± 1 SE) numbers of beetles caught in traps baited with the pheromone blend, low (LR), medium (MR), and high (HR) release rates of reconstructed blends of host plant compounds, and ethanol. a) Oak-infesting species *P. obliquus* and *B. gemmulatus*, b) oak-infesting species *P. grandis* and *N. m. modestus* (Cerambycinae), c) conifer-infesting *N. upiformis* (Spondylidinae), d) conifer-infesting *X. albonotatus* (Cerambycinae) and *M. clamator* (Lamiinae). Within each species, means with the same letter are not significantly different (Dunn's test $P > 0.05$).

Bioassay of Pheromones with or without Ethanol, for Oak-Infesting Species

The oak-infesting cerambycines *P. grandis* (208 beetles), *P. obliquus* (458 beetles), *B. conspersus* (8 beetles), and *B. gemmulatus* (17 beetles) were trapped during this experiment (Table 2.8). The treatment effect was significant only for *P. grandis*, with attraction to the pheromone blend increased by ethanol (q = 2.91; Fig. 2.5). None of the remaining species discriminated between pheromone with or without ethanol (Fig. 2.5).

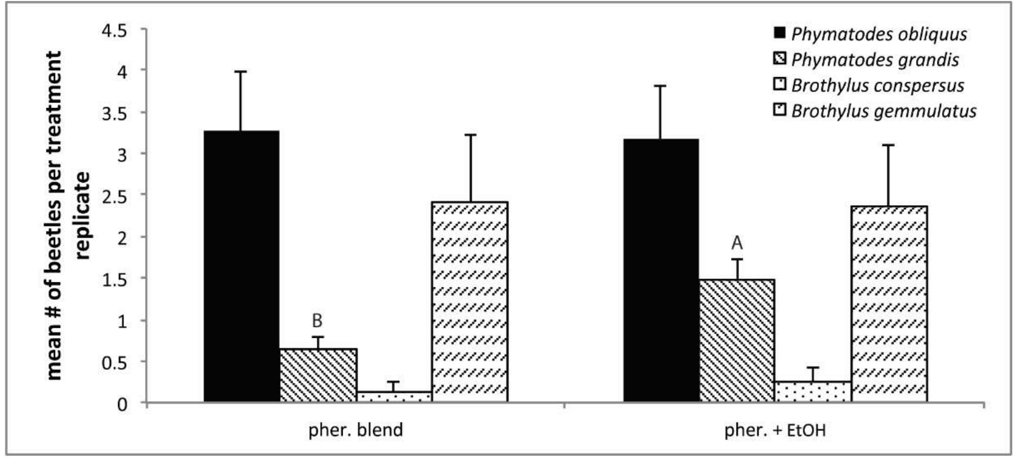

Fig. 2.5. Mean (± 1 SE) numbers of oak-infesting *P. obliquus*, *P. grandis*, *B. conspersus*, and *B. gemmulatus* (Cerambycinae) beetles caught in traps baited with the pheromone blend with and without ethanol. Within each species, means with the same letter are not significantly different (Dunn's test $P > 0.05$).

Bioassay of Pheromones with Conifer Volatiles or α-Pinene The spondylidines *A. nitidum* (142 beetles), *A. striatum* (25 beetles), *N. upiformis* (96 beetles), and *T. abietis* (13 beetles), the lamiine *M. clamator* (34 beetles), and the cerambycine *X. albonotatus* (8 beetles) were trapped during this experiment (Table 2.8). For *N. upiformis*, the pheromone + conifer blend + ethanol treatment was most attractive and statistically separated from all treatments except the pheromone plus conifer blend without ethanol ($q \geq 3.36$, $q = 1.68$; Fig. 2.6a); no other treatments separated from each other. *Asemum nitidum* and *A. striatum* were most strongly attracted to the pheromone + α-pinene treatments, particularly the pheromone + α-pinene without ethanol treatment, which was significantly more attractive than the two conifer blend treatments (*A. nitidum*, $q \geq 3.91$; *A. striatum*, $q = 2.87$; Fig. 2.6a, b). *Monochamus clamator* was most strongly attracted to pheromone blend + conifer blend, and addition of ethanol to this mixture had no significant effect; only pheromones + α-pinene + ethanol was significantly less attractive than pheromone + conifer blend ($q = 2.83$; Fig. 2.6b).

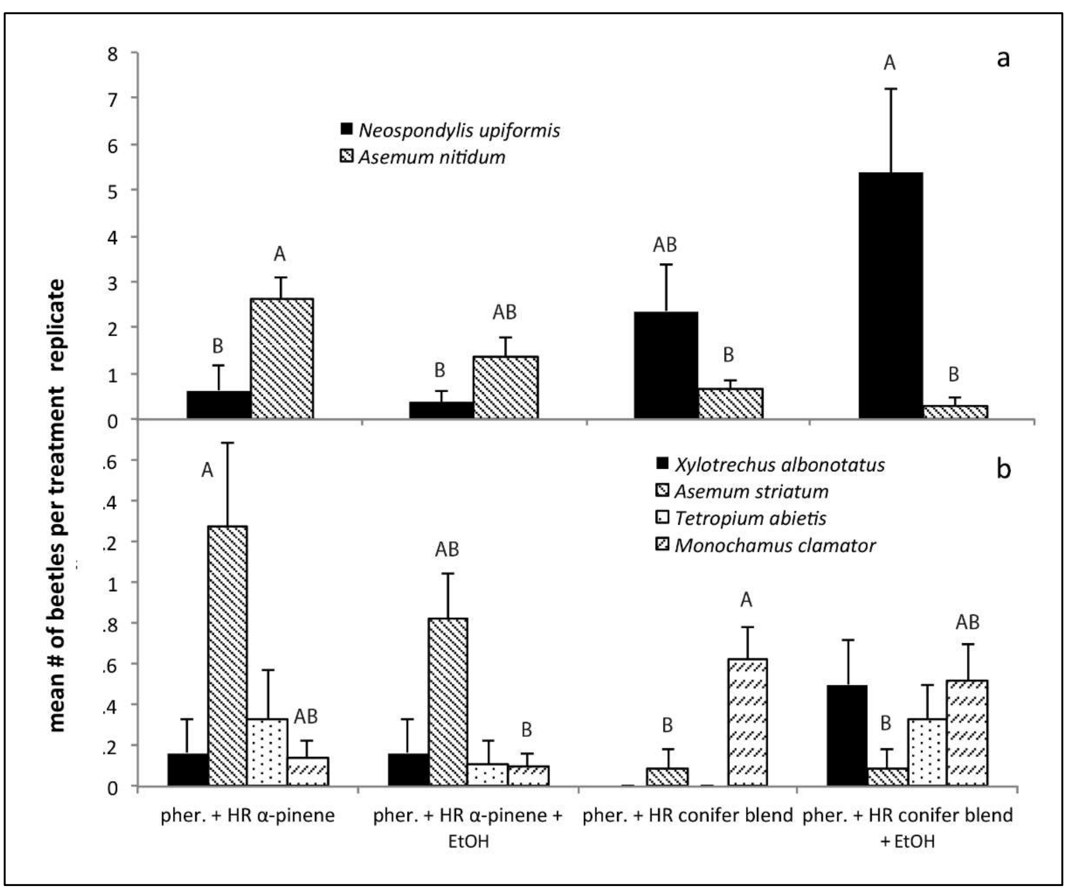

Fig. 2.6. Mean (± 1 SE) numbers of conifer-infesting species in traps baited with the pheromone blend, high release rate (HR) conifer volatiles or α-pinene, and ethanol. a) Conifer-infesting *N. upiformis* and *A. nitidum* (Spondylidinae), b) conifer-infesting *X. albonotatus* (Cerambycinae), *A. striatum* and *T. abietis* (Spondylidinae), and *M. clamator* (Lamiinae). Within each species, means with the same letter are not significantly different (Dunn's test $P > 0.05$).

Discussion

In the first experiment, testing the influence of crude chipped host tree material on the attraction of cerambycids to their pheromones, the data suggested that HPVs enhanced attraction of conifer-infesting cerambycids to pheromone lures. In contrast, for oak-infesting species, attraction to pheromone lures was not

influenced by oak volatiles. However, attraction of some of the oak-infesting species to the pheromone lures was inhibited by odors from non-host conifer material.

The reconstructed HPV blends developed for the second and third experiments were based on analyses of the volatiles collected from the same stock of chipped tree materials used in the first round of bioassays. Whereas we anticipated that the profiles of the various conifer and oak species respectively would be similar within these two groups, we found that qualitatively, the profiles of monoterpenes between these two groups were similar as well. However, in addition to monoterpenes, the chipped oak material produced substantial amounts of green leaf volatiles that were not present in the conifer volatiles, and the sesquiterpene profiles of the conifers and oaks also differed (Fig. 2.2). Furthermore, the overall abundance of volatile compounds differed between the two groups, as demonstrated by the two orders of magnitude difference in compound abundance seen in Fig. 2.2, and typical of the other conifer and oak volatile samples.

In the second round of bioassays testing reconstructed blends of HPVs with pheromones, the three HPV release rates were not intended to exactly replicate the emission rate from conifers or oaks, but to test a range (approximately three orders of magnitude) of release rates for practical deployment in traps. In these experiments, the reconstructed blend of conifer volatiles appeared to be a satisfactory mimic of the odors released by the crude plant material, with the

conifer-infesting species generally exhibiting increased responses with increasing release rates of the conifer volatiles blend. In addition, two of the species, *N. upiformis* and *M. clamator*, showed minimal responses to treatments containing pheromone alone, clearly indicating that the HPVs form an important part of the overall attractant for these species.

In contrast, the responses of the oak-infesting species generally decreased with increasing release rates of the reconstructed blends of host volatiles, for either the conifer or oak volatile blends. Given that the oak-infesting species had shown no response to volatiles from chipped oak branches, and were in some cases repelled by the chipped conifer material, these results were not unexpected. However, the lack of response to oak volatiles in the first experiment and the repellence to high release rates of oak volatiles in the second experiment may be due in part to the emitted oak volatiles not being representative of appropriate hosts for these species. That is, the oak branches used as sources of volatiles were fresh, and were chipped shortly after collection. Consequently, they may not have been representative of the host condition typically utilized by our study species, which infest dying and dead oaks (Linsley and Chemsak 1997). For example, Dunn and Potter (1991) found that *Elaphidion mucronatum* (Say) (Cerambycinae), which attacks dying and dead oaks, were not attracted to freshly cut oak logs plus ethanol, but only to a cardboard log mimic from which ethanol was released.

In the first part of the third field experiment, we tested the hypothesis that the more complete reconstruction of the conifer HPV blend would be a better pheromone synergist than the single host component α-pinene, which has been used extensively as a generic lure for cerambycids (see Introduction). However, we found that different species responded differently to α-pinene versus the more complex blend. Thus, adult *N. upiformis* were significantly more attracted to the blend of pheromone + conifer volatiles + ethanol than to the corresponding blend with α-pinene as a single host component. Similarly, *M. clamator* was significantly more attracted to the blend of pheromone + conifer volatiles than to pheromone + α-pinene. Thus, for these two species, minor components of the conifer volatiles result in increased attraction. In contrast, the two *Asemum* species were more attracted to lures with α-pinene alone than to those with the full conifer blend, indicating that one or more of the minor components may be antagonistic. This could also explain why these species did not show a preference for the conifer blend treatments in the second experiment. These differences among ecologically similar conifer-infesting species may be related to their specific host preferences. For example, *M. clamator* and the SBNF population of *N. upiformis* prefer pines, whereas the *Asemum* species all prefer fir (Linsley and Chemsak 1997). In prior work, Chénier and Philogène (1989) and Sweeney et al. (2004) tested reconstructed conifer blends in the absence of pheromones, and similarly found that *A. striatum* was more attracted to traps baited with only α-pinene than to a blend of conifer

terpenes. Conversely, *Spondylis buprestoides* L. was more attracted to the conifer blend than to α-pinene (Sweeney et al. 2004). Further bioassays of subsets of conifer blends will be required to determine the specific components which are responsible for the added or decreased attraction, respectively, for the different cerambycid species.

The second part of the third experiment tested the hypothesis that oak-infesting cerambycid species utilized ethanol as a host cue, as per Dunn and Potter (1991), and would be more attracted to pheromones when they were co-released with ethanol. We again found that the responses were nuanced, because ethanol significantly increased attraction to pheromones for *P. grandis*, but not for any of the other oak-infesting species. There were also indications that ethanol might mitigate the antagonistic effects of conifer volatiles or α-pinene on attraction of both *P. grandis* and *B. gemmulatus* to pheromones, with all pheromone treatments containing both ethanol + conifer volatiles or α-pinene attracting more than twice as many beetles as the corresponding treatments without ethanol (Table 2.8, Fig. 2.7).

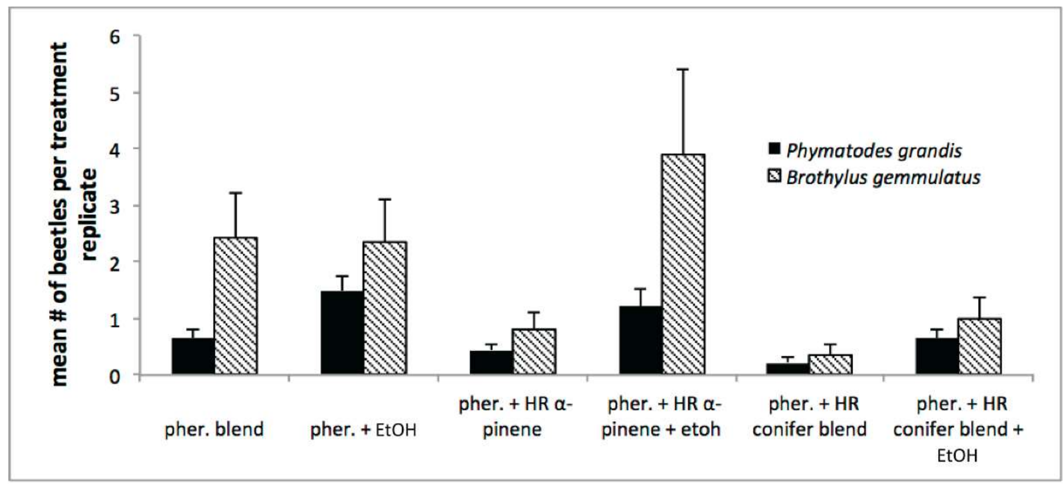

Fig. 2.7. Mean (± 1 SE) number of beetles per treatment replicate for *P. grandis* and *B. gemmulatus* oak-infesting species. There was no significant means separation via Dunn's Test, but both species showed a similar increased response to treatments with ethanol versus the same treatment without (i.e. α -pinene with ethanol vs. α-pinene without ethanol, conifer blend with ethanol vs. conifer blend without ethanol).

To our knowledge, this is the first experiment which has tested the combined effects of reconstructed host plant volatiles with a generic blend of pheromones designed to attract a number of species within a community of cerambycids. Silk et al. (2007) had previously conducted a more focused study by testing reconstructed HPV blends with the pheromones of two conifer-infesting *Tetropium* species. Similar to our results with conifer-infesting species, both *Tetropium* species were more strongly attracted to pheromone + conifer volatiles than to either lure component alone. Based on this and other studies in which HPVs have been shown to enhance attraction of conifer feeders to pheromone lures (see Introduction), increased attraction of conifer-infesting cerambycids to pheromones

when they are released in combination with HPVs is likely to be a general phenomenon.

In contrast, we found no evidence that host plant volatiles other than ethanol for *P. grandis* increased the attraction to pheromones for the oak-infesting cerambycids described here. It was also interesting to note that the oak-infesting species in our study were generally repelled by conifer volatile blends, suggesting that these species are using olfactory cues to avoid non-hosts. GC-EAD analyses with several of the oak-infesting species confirmed that their antennae do indeed perceive non-host volatiles. Non-host volatiles have been reported to repel or inhibit orientation to otherwise attractive compounds for other wood-boring beetles, such as green-leaf volatiles inhibiting attraction of the conifer-infesting cerambycid *Arhopalus tristis* (F.) (Suckling et al. 2001) or conifer-infesting bark beetles (Zhang and Schlyter 2004). Thus, it is reasonable to assume that the reciprocal case is occurring with the oak-infesting species in our study.

For all of our test species, there was no indication that the HPVs were more attractive than controls in the absence of pheromones, despite the fact that HPVs like α-pinene and ethanol are commonly used as generic attractants for cerambycids (reviewed in Allison et al. 2004). Clearly, such blends are by no means attractive to all cerambycid species. However, in the first field experiment in which the chipped material by itself was not attractive, the lack of attraction may have been due in part to the relatively small amounts of chipped material deployed (~1 L of

chips) and the fact that the traps were deployed in field experiments where there would be competition from natural sources of volatiles. In addition, GC-MS analyses of field-aged chips showed that the release rate of volatiles declined rapidly within a few days (data not shown).

In summary, our results have shown that host plant volatiles are important attractants for many cerambycid species that infest conifers, whereas they appear to be much less important for species infesting dying and dead oak trees. Release rates of HPVs will need to be considered when developing generic lures for cerambycids because conifer-infesting species are likely to be most strongly attracted to high release rates of HPVs, whereas species infesting angiosperms are likely to be increasingly repelled with increasing HPV release rates. Comparison of a reconstructed blend of conifer volatiles versus α-pinene produced mixed results, with some species prefering the full blend, whereas others preferred α-pinene alone. The effect of ethanol on species infesting oak was also mixed, with attraction to the pheromone of one species clearly being synergized by ethanol, whereas other species were unaffected. Thus, the choice of which HPVs to use for trapping a given species will require careful consideration. Nevertheless, for many species, due to the overlap in both pheromones and host plant volatiles, it should still be possible to formulate relatively generic lures that will attract a number of species simultaneously, as suggested by several previous studies of blends of cerambycid pheromones with α-pinene and ethanol (e.g., Hanks and Millar 2013).

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Chapter 3: Partitioning mechanisms that limit cross-attraction of sympatric longhorned beetle species (Coleoptera: Cerambycidae) to shared pheromone components.

Introduction

Insect species that use long-range pheromones for mate-finding, particularly moth species, have evolved highly specific signals to avoid the costs of wasted mating effort directed towards heterospecific females (Cardé and Baker 1984). This suggests that the use of similar pheromone blends – or other species-specific signals for that matter – should be partitioned in some way. However, 'generic' pheromones or pheromone blends that attract and are produced by sympatric and syntopic (= same host) species are relatively common (Wood 1982; Williams et al. 1995; Arnaud et al. 2002; Hanks and Millar 2016). This begs the question of if and how these species are partitioning pheromone use, especially among groups of species that share pheromone components, hosts, and ranges (e.g., Symonds and Elgar 2004). Further, when partitioning occurs, is it as a means of reproductive character displacement, as a prezygotic mating barrier for very closely related species, or as ecological character displacement, as competition between heterospecifics (Cardé 1986)?

Beetles in the family Cerambycidae, commonly known as longhorn borers are ubiquitous and usually valuable members of forest ecosystems, where they act

as primary decomposers of woody material (Haack and Byler 1993; Ulyshen 2016; Chen and Forschler 2016), and cull stressed and sickly trees (Manion and Griffin 2001; Teale and Castello 2011). However, cerambycids also have the potential to be ecologically and economically damaging when they are introduced into new habitats as invasive species (Haack 2006; Parry and Teale 2011; Haack et al. 2010). Over the past 15 years, numerous examples of cerambycids sharing pheromone components have been discovered, and many subsets of these species also share hosts and ranges (Hanks and Millar 2016). Pheromone components may be shared within and across genera – e.g., numerous species in the subfamily Cerambycinae utilize C_6 and C_8 3-hydroxyalkan-2-ones and 2,3-alkanediols (Sakai et al. 1984; Iwabuchi et al. 1986; Fettkother et al. 1995; Leal et al. 1995; Lacey et al. 2004, 2008, 2009; Hanks et al. 2007; Ray et al. 2009, 2015; Wong et al. 2012; Imrei et al. 2013; Wickham et al. 2014; Narai et al. 2015; Hayes et al. 2016; Zou et al. 2016). Pheromone components also may be shared across subfamilies, such as fuscumol ((*E*)-6,10-dimethyl-5,9-undecadien-2-ol) being shared by species in the subfamilies Spondylidinae and Lamiinae (Silk et al. 2007; Sweeney et al. 2010; Chapter 5; Fonseca et al. 2010; Mitchell et al. 2011; Meier et al. 2016) – and even across subfamilies that utilize different pheromone emission strategies, that is, male- versus female-produced pheromones. For example, 2,3-alkanediols that are produced by male beetles in the Cerambycinae as sex-aggregation pheromones, are

also produced as sex pheromones by females in the subfamily Prioninae (above refs.; Ray et al. 2012; Wickham et al. 2016).

In order for these shared pheromone components to be able to still provide species-specific signals, they must be used in combination with other mechanisms that limit counterproductive interspecific attraction. In some cases these mechanisms are obvious, for example, when sympatric species have different seasonal activity periods, so that they never overlap in time (Mitchell et al. 2015). Alternatively, sympatric species which are seasonally synchronic may have different daily activity periods (Mitchell et al. 2015; Skabeikis et al. 2016). There is also increasing evidence for signal specificity being encoded by using pheromone blends rather than single components (Hanks et al. 2012; Hanks and Millar 2013; Mitchell et al. 2015), and subtler nuances such as the use of different enantiomers, or even specific ratios of enantiomers (Hanks et al. 2012; Meier et al. 2016). However, there are also examples of cross-attraction between species with no obvious partitioning mechanisms (e.g., *Phymatodes aereus* and *Anelaphus pumilis*; Mitchell et al. 2015), or attraction of species to cerambycid pheromones they do not even produce (e.g., *Phymatodes grandis* to 3-hydroxyhexan-2-one; Hanks et al. 2007), possibly representing eavesdropping on the pheromones of other guild members as a mechanism to locate suitable hosts. Thus, the primary objective of this study was to determine if a group of cerambycids from the subfamily Cerambycinae, that all occur sympatrically and mostly syntopically, are

partitioning pheromone use. The species all overlap phenologically (Collignon et al 2016; this chapter; unpub. data), and all infest oaks in the habitats used in this study, with the exception of *Xylotrechus albonotatus*, which infests white fir (Linsley and Chemsak 1997). I tested for evidence of two possible partitioning mechanisms: pheromone blend composition and differing diel activity periods. The main six study species were *Neoclytus modestus*, whose confirmed pheromone is (*R*)-3-hydroxyhexan-2-one (Hanks et al. 2007), *Phymatodes grandis* (formerly *P. lecontei*), whose confirmed pheromone is (*R*)-2-methylbutan-1-ol (Hanks et al. 2007), *Phymatodes obliquus*, *Brothylus conspersus*, *Brothylus gemmulatus*, and *Xylotrechus albonotatus*. The male-produced pheromones of the latter four species were identified during the course of this study. In addition, the possible role of acetoin (= 3-hydroxybutan-2-one) as a pheromone component was investigated. This compound is an analog of the common 3-hydroxyhexan-2-one motif that has been identified in extracts from several cerambycid species (*P. obliquus*, reported here; *Callimoxys fuscipennis* (LeConte); Millar, unpub. data), but which has received limited testing in field trials (Ray et al. 2015).

Methods

Field sites The two main field experiments were conducted at two sites approximately 500 m apart in the San Bernardino National Forest (SBNF) in San Bernardino Co., California, USA. The sites were near Jenks Lake (34°09'45.8"N

116°54'08.6"W) and are dominated by ponderosa pine (*Pinus ponderosa*; Pinales: Pinaceae) and white fir (*Abies concolor*; Pinaceae), with some western black oak (*Quercus kelloggii*), canyon live oak (*Quercus chrysolepis*; Fagales: Fagaceae), bigcone Douglas-fir (*Pseudotsuga macrocarpa*; Pinaceae), and incense cedar (*Calocedrus decurrens*; Pinales: Cupressaceae).

Field sites at Emerson Oaks Reserve, James San Jacinto Mountains Reserve (James Reserve), Santa Rosa Plateau Ecological Reserve, and Santa Margarita Ecological Reserve were also utilized for the diel trapping assay. Emerson Oaks Reserve is dominated by coast live oak (*Quercus agrifolia*), and includes coastal sage scrub habitat. James Reserve is dominated by ponderosa pine and canyon live oak, and also has some western black oak present. The Santa Rosa Plateau site was dominated by Engelmann oak (*Quercus engelmannii*) and coast live oak. The Santa Margarita site was at the intersection of oak chaparral dominated by coast live oak, and a riparian community dominated by western sycamore (*Platanus racemosa*; Proteales: Platanaceae) and western black cottonwood (*Populus trichocarpa*; Malpighiales: Salicacaea).

Pheromone blend field testing For field bioassays, black cross-vane intercept traps (Alpha Scents, Portland OR, USA) coated with Fluon® (Graham et al. 2010) were hung on 1.5 m tall, inverted L-shaped stands made from PVC irrigation pipe mounted on sections of steel reinforcing bars driven into the ground. Traps were

placed 10-15 m apart in transects to minimize potential interactions among treatments, with treatments initially assigned randomly to traps; transects were deployed at least 50 m apart. Traps were checked twice weekly and their order was rerandomized at every check. Treatments consisted of all possible single, binary, or tertiary blends of racemic acetoin (AC), racemic 2-methylbutan-1-ol (MB) (both Aldrich Chemical Co., Milwaukee WI, USA), and racemic 3-hydroxyhexan-2-one (HH) (Bedoukian Research, Danville CT, USA), for a total of seven treatments. No blank controls were utilized, because previous work by our group has not found them to capture by-catch of the species in this study. Lure blends consisted of 50 mg/ml of all compounds within a given treatment blend, dissolved in 2-propanol. One ml of the blend was deployed in 2 mil wall thickness, low-density polyethylene resealable baggies (~5 × 7.5 cm; Fisher Scientific, Pittsburgh PA, USA). The experiment was run from 6 June through 26 August 2014 at the Jenks Lake (JL) site. Beetles were live trapped so that they could be used for pheromone collection. Excess beetles were released at least 200 m from the field sites. Voucher specimens of all species have been deposited in the Entomology Museum at UC Riverside.

Diel trapping assay Fluon-coated black cross-vane intercept traps as described above were fitted onto a BioQuip timer trap (model #2850; BioQuip Products, Rancho Dominguez, CA, USA), which allowed catches to be segregated into eight 3 h time periods (12:01am to 3am, etc.) covering an entire day. The timer

traps were deployed at five different sites. A trap was deployed from 28 May through 27 June 2010 and checked three times, 1, 16, and 27 June at Emerson Oaks Reserve (EO), and again from 21 June through 13 July 2011, also checked three times, 30 June, 6, and 13 July. A timer trap was deployed from 27 June through 28 July 2010 at James Reserve (JR), and checked four times, 6, 14, 22, and 28 July. At Santa Rosa Plateau Reserve (SR), a trap was deployed from 16 June through 20 July 2010, checked four times, 23 June, 5, 13, and 20 July. Finally, a trap was deployed from 20 June to 29 June 2011 at the Santa Margarita Reserve (SM), and checked once on 29 June. The diel trapping at this site was conducted by Ann M. Ray.

For the Jenks Lake trial, two timer traps were set up, one at each of the two adjacent sites listed above. The traps were deployed approximately 50 m from any other experiments being run concurrently in 2015 and 2016. The traps were setup for 24 h at a time, starting at noon. The two traps were deployed 27 April, 28 May, 8 June, and 16 July 2015, and 2, 9, 21, and 23 June 2016. Voucher specimens of all species caught have been deposited in the Entomology Museum at UC Riverside.

Identification of beetle-emitted volatiles Live beetles were non-invasively sexed via light abdominal squeezing to extrude the ovipositor in females, and further confirmed with secondary sexually dimorphic characteristics, such as antennal

compared to body length, and enlarged femora for male *Phymatodes* species. Sexed individuals were sampled for pheromone emission individually or in single-sex groups. Collections of headspace volatiles from beetles during 2012-2014 were often made with host material present (western black and canyon live oak twigs held 1-2 wk under ambient laboratory conditions), but this did not seem to increase likelihood of pheromone production (unpub. data) and so from 2015 onwards, most collection chambers were simply fitted with wire mesh as perches. In 2015, beetles were also provided ad libitum access to 10% sugar water. However, collections from *X. albonotatus* were always made in the presence of twigs of its host white fir because host plant volatiles appear to be important in the chemical ecology of conifer-infesting species (Collignon et al. 2016). Headspace volatiles controls of the three hosts and the sugar water were made as well, to confirm compounds as strictly beetle-produced. Whenever new host twigs were harvested, they were sampled alongside beetle aerations, and sugar water controls also were run periodically. Headspace collections from 2012-2014 were made by aerating beetles in 250 ml wide-mouth glass canning jars with the lid replaced with a 1/16" thick Teflon sheet and fitted with Swagelok bulkhead unions (Swagelok, Solon OH, USA) to connect inlet and outlet tubes. For 2015-2016, volatile collections were conducted in wide-mouth 500 ml Teflon jars (Thermo Scientific, Fisher Scientific; #24030250), with the screw-cap lids fitted with bulkhead unions as described for the lids of the glass chambers. Aerations were conducted under ReptiSun 10.0 UVB

lights (Zoo Med Labs. Inc., San Luis Obispo CA, USA) with a 16:8 h L:D cycle. Air was pulled through the system by vacuum at 250 ml/min for the glass chambers and 500 ml/min for the Teflon ones. Incoming air was cleaned by passage through granulated activated charcoal (14-16 mesh, Fisher Scientific). Volatiles were collected on ~50 mg of thermally-desorbed activated charcoal (50-200 mesh; Fisher Scientific) held between glass wool plugs in a short piece of glass tubing. Volatiles were eluted from the charcoal with 0.5-1 ml dichloromethane.

For analysis of extracts from 2012-2014, one microliter of the sample was injected onto an HP 6890 gas chromatograph (GC; Hewlett-Packard, now Agilent, Santa Clara CA, USA) coupled to an HP 5973 mass selective detector (GC-MS). The GC was fitted with a DB-17 column (30 m × 0.25 mm ID × 0.25 micron film; J&W Scientific, Folsom CA, USA), which was programmed from 40ºC/5 min, then increased 5ºC/min to 280ºC, hold 5 min. The injector and transfer line temperatures were set to 280ºC, and injections were done in splitless mode. All spectra were taken in full scan mode with electron impact ionization (70 eV).

Samples from 2015-2016 were analyzed with an Agilent 78020A gas chromatograph fitted with an autosampler and coupled to an Agilent 5977E mass selective detector (Agilent Technologies, Santa Clara CA, USA). The GC was equipped with a DB-5MS column (30 m × 0.25 mm ID × 0.25 micron film; Agilent). The oven temperature was programmed from 40ºC/5 min, then increased

5ºC/min to 280ºC, final time 5 min; the injector and transfer line temperatures were set to 280ºC. One microliter aliquots of extracts were injected in splitless mode.

Chromatograms from insect aerations were checked against those from the western black, canyon live oak, white fir, and sugar water controls to locate insect-specific peaks. Compounds were identified by matching retention times and mass spectra with those of authentic standards of 2-methylbutanol, 3-hydroxyhexan-2-one, acetoin, and 2,3-hexanedione.

The absolute configuration of 2-methylbutanol and 3-hydroxyhexan-2-one was determined by analysis of extracts on a GC-FID fitted with a Cyclodex-B chiral stationary phase column (30 m × 0.25 mm ID × 0.25 micron film; J&W Scientific, Folsom CA, USA). Analyses were conducted using a temperature program of 50ºC/1 min, then 5ºC/min to 220ºC, held 20 min, with an injector temperature of 150ºC, and detector temp of 250ºC. Racemic and enantiomerically enriched standards of either compound were run under the same conditions, and ultimately co-injected with beetle samples to confirm the absolute configurations of the compounds.

Statistical Analyses For the pheromone blend field assay, the count data were heterogeneous for variances as tested by Bartlett's test. Thus, data were analyzed with a max-*t* test (Herberich et al. 2010; Hothorn et al. 2008; Zeilis 2004), preceded by an ANOVA model calculated for the max-*t* test. This is a multiple comparison

test that is robust to departures from assumptions of normality and homogeneity of variances. The multiple comparison only analyzed differences between each of the treatments versus the treatment that most closely represented the pheromone components produced by a given species – i.e., for *P. obliquus*, all treatments were compared for differences with the treatment that contained all three pheromone components (MB, AC, HH), which males of this species produce. For *P. grandis*, all comparisons were against the MB treatment, its sole pheromone component. For *B. gemmulatus*, males of which produce HH and may produce 2,3-hexanedione, treatment comparisons were made against the HH treatment because 2,3-hexanedione was not tested.

Replicates for each experiment were based on both spatial and temporal replication, with temporal replicates equalling the number of times the traps were checked. Temporal replicates where no beetles of a given species were trapped in any trap – usually due to inclement weather or being outside the species' flight period – were not included in analyses for that species.

The diel activity period data were assessed visually for overlap in activity between species, and separated by site to control for differences in micro-environmental factors which differed among the various sites (Table 3.1).

Results

Pheromone blend field assay In the experiment testing blends of racemic 2-methylbutanol, acetoin, and 3-hydroxyhexan-2-one, totals of 44 *P. obliquus*, 181 *P. grandis*, 99 *B. gemmulatus*, one *B. conspersus*, and two *X. albonotatus* were trapped.

Of the 99 *B. gemmulatus* attracted to lures, 53 were female and 46 male. Based on the max-*t* analysis there were no significant differences in either of the sexes' response between HH and the other compounds or blends (Fig. 3.1). The ANOVA for females did indicate a difference in response to the treatment blends ($F_{6,70}$ = 2.42, p = 0.035), but the appropriate assumptions were not met to run a stand-alone ANOVA, and the max-*t* did not detect any significant separations between treatments.

One-hundred four females and 77 male *P. grandis* were trapped in the experiment, with both sexes having significantly different response to MB versus the other pheromone blend treatments (females: $F_{6,189}$ = 7.76, p = 1.79^{e-07}; males: $F_{6,133}$ = 5.12, p = 9.24^{e-05}). Compared to the MB treatment, the AC, HH, and AC+HH were significantly less attractive to *P. grandis* females, whereas the MB+AC was marginally less attractive than MB alone (Fig. 3.2). For males, surprisingly the three-component blend of MB+AC+HH was significantly more attractive than the species' single known pheromone component, MB, but there were no other significant differences.

For *P. obliquus*, 28 females and 16 males were caught. There were significant differences among responses to the treatments for females but not males (females: $F_{6,63} = 4.37$, $p < 0.001$; males: $F_{6,35} = 2.00$, $p = 0.092$). Females were significantly more attracted to the three component blend of MB+AC+HH than to AC alone or MB+AC, and marginally more so than to MB alone, HH alone, or AC+HH (Fig. 3.3).

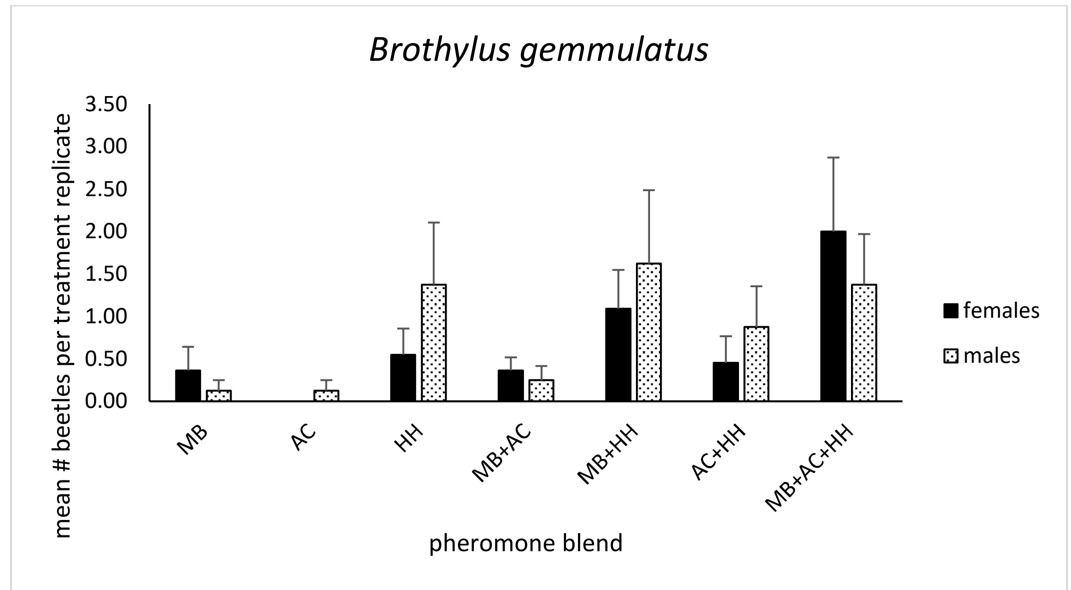

Fig. 3.1. Mean (± 1 SE) numbers of *B. gemmulatus* females and males caught in traps baited with single components, or binary, or ternary blends of racemic 2-methylbutan-1-ol (MB), acetoin (AC), and 3-hydroxyhexan-2-one (HH). There were no significant differences in responses to the HH treatment than any other treatment by either sex (max-*t* test, $p < 0.05$).

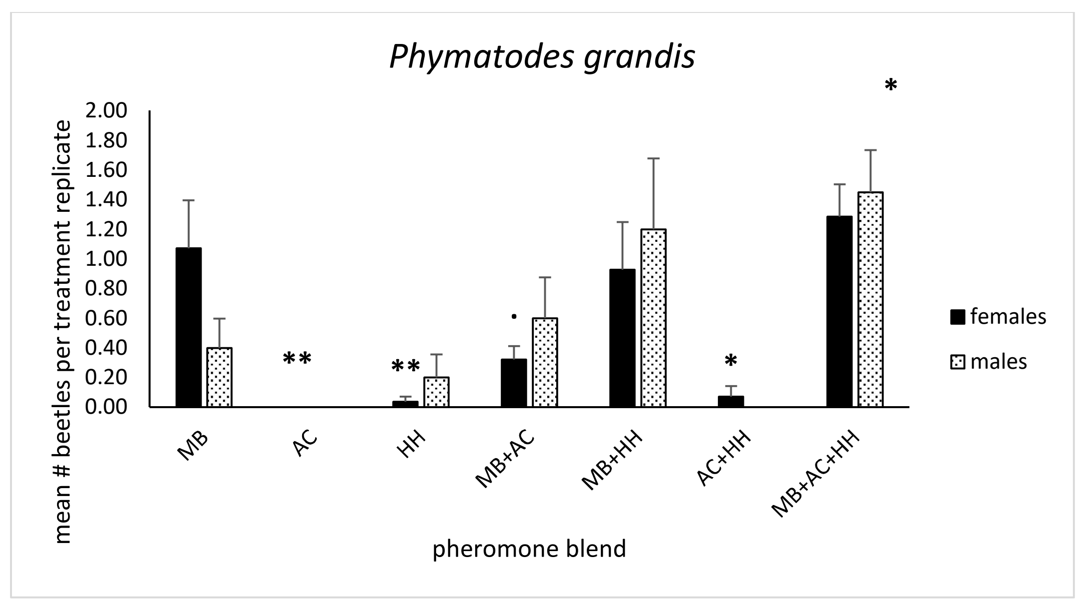

Fig. 3.2. Mean (± 1 SE) numbers of *P. grandis* females and males caught in traps baited with single components, or binary, or ternary blends of racemic 2-methylbutan-1-ol (MB), acetoin (AC), and 3-hydroxyhexan-2-one (HH). Double asterisks (**) indicate $p < 0.01$, single asterisk (*) $p < 0.05$, a period (.) $p < 0.1$, comparing pheromone blend treatments to the MB treatment (max-*t* test).

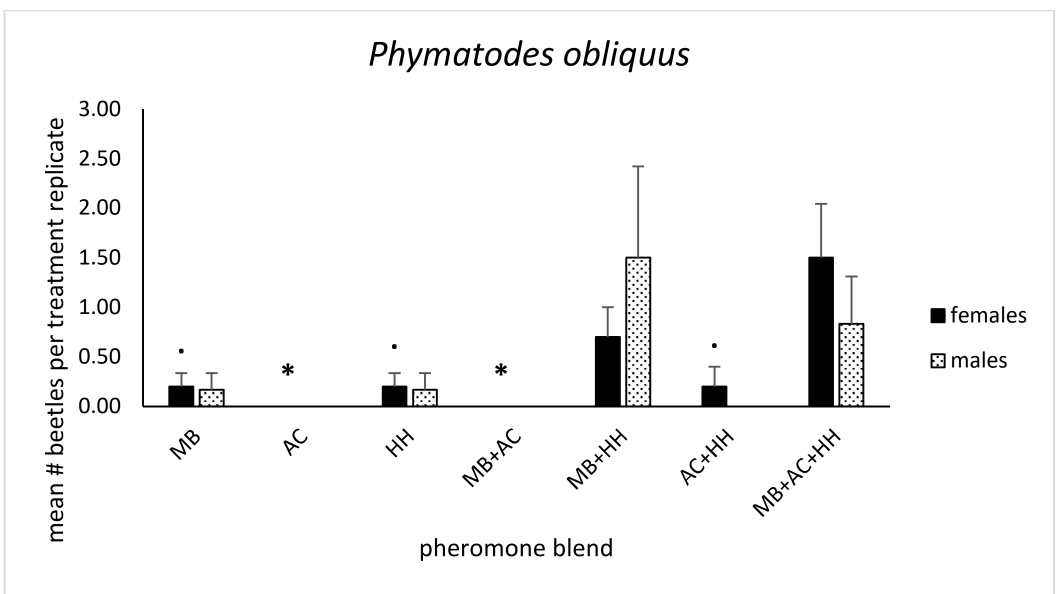

Fig. 3.3. Mean (± 1 SE) numbers of *P. obliquus* females and males caught in traps baited with single components, or binary, or tertiary blends of racemic 2-methylbutan-1-ol (MB), acetoin (AC), and 3-hydroxyhexan-2-one (HH). Double asterisks (**) indicate $p < 0.01$, single asterisk (*) $p < 0.05$, a period (.) $p < 0.1$, comparing pheromone blend treatments to the MB+AC+HH treatment (max-*t* test).

Diel partitioning assay Overall, the testing of possible diel partitioning provided evidence of at least some temporal separation among the test species (Table 3.1; Figs. 3.4-3.8). Thus, *P. obliquus* was strictly diurnal at all five sites, with beetles being caught from around 9am to 6pm, and peak activity around midday. In contrast, *P. grandis* exhibited primarily crepuscular activity, with beetles being caught as early as 3pm, and continuing until midnight. *Brothylus gemmulatus* also showed crepuscular activity, but with continued activity into the night, to as late as 3am. *Neoclytus modestus* was completely diurnal with trap catches beginning early in the day, between 6 to 9am. *Xylotrechus albonotatus* showed the most variable diel activity among sites, with trap catches occurring in the evening at the Santa Margarita site, but earlier in the day at the Emerson Oaks and Santa Rosa Plateau sites. Although only three *B. conspersus* were caught, all were caught between 3 and 6pm, suggesting peak activity in late afternoon.

Within sites, some overlap in diel activity among the various species was evident. For example, at Jenks Lake, there was substantial overlap between *P. obliquus* and *B. conspersus*, and between *P. grandis* and *B. gemmulatus*, as well as some overlap in the later part of the day among all species (Fig. 3.4). At Emerson Oaks, *P. grandis* was far more abundant than any of the other species, and overlapped with all of them at least somewhat. *Phymatodes grandis* also seemed to be active earlier at this site than others, with large numbers being caught as early as 3pm (Fig. 3.5). The situation was similar for Santa Rosa, with *P. grandis* being

most abundant, but trap catches were much more focused towards the evening, and so only minimally overlapped with the diurnal species, although it did overlap with *B. gemmulatus* (Fig. 3.6) at all sites at which the two species co-occurred. The James Reserve site showed the best diel separation between the diurnal *P. obliquus* and the crepuscular *P. grandis* and *B. gemmulatus* (Fig. 3.7). Finally, there was no evidence at the Santa Margarita site that the diurnal species *P. obliquus*, *N. modestus*, or *X. albonotatus*, were separating themselves via diel activity periods. However, *B. gemmulatus* trap catches appeared to be shifted more towards the night hours at this site than the others, perhaps due to the activity of *X. albonotatus* at this site late in the day (Fig. 3.8).

Table 3.1. Total numbers of beetles of each species trapped in experiments testing daily activity periods of males, at the sites listed.

Site	Years	Reps.	♀ *P. obliquus*	♂ *P. obliquus*	♀ *P. grandis*	♂ *P. grandis*	♀ *B. gemmulatus*	♂ *B. gemmulatus*
Emerson Oaks	2010, 2011	6	22	35	135	103	4	1
Santa Rosa Plateau	2010	4	2	1	49	67	1	1
James Reserve	2010	4	5	8	1	3	0	1
Santa Margarita	2011	1	10	3	0	0	1	0
Jenks Lake	2015, 2016	16	38	26	4	1	1	1

Site	Years	Reps.	♀ *B. conspersus*	♂ *B. conspersus*	♀ *N. modestus*	♂ *N. modestus*	♀ *X. albonotatus*	♂ *X. albonotatus*
Emerson Oaks	2010, 2011	6	0	0	13	15	8	21
Santa Rosa Plateau	2010	4	0	0	5	0	14	57
James Reserve	2010	4	0	0	0	0	0	0
Santa Margarita	2011	1	0	0	2	0	5	1
Jenks Lake	2015, 2016	16	2	1	0	0	0	0

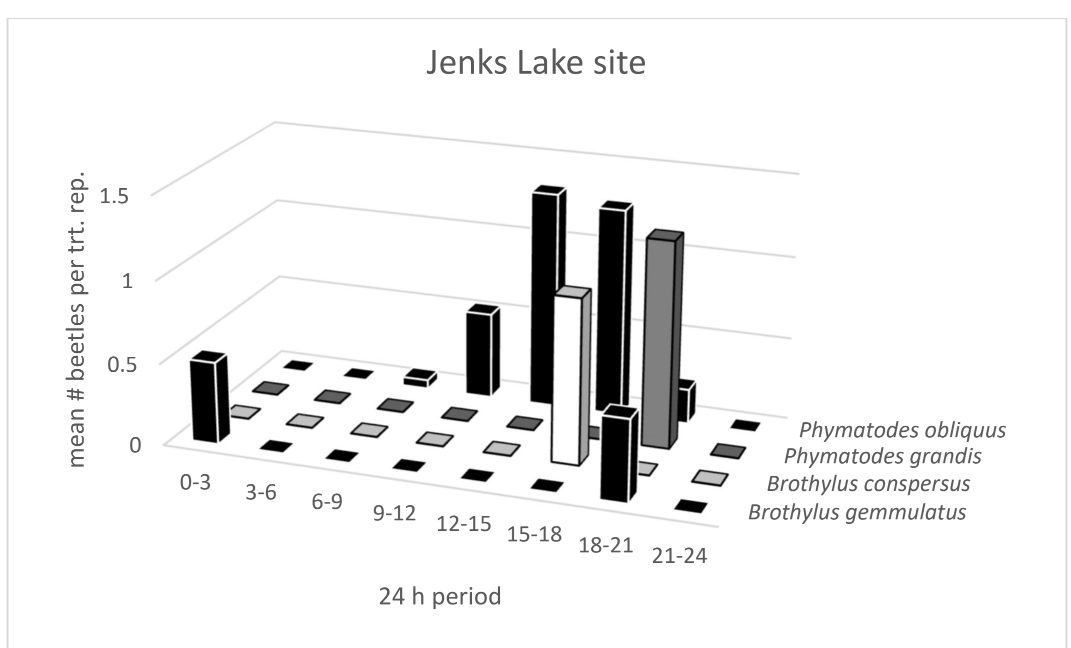

Fig. 3.4. Mean numbers of beetles trapped within 3 h time windows throughout the day at the Jenks Lake site in the San Bernardino National Forest.

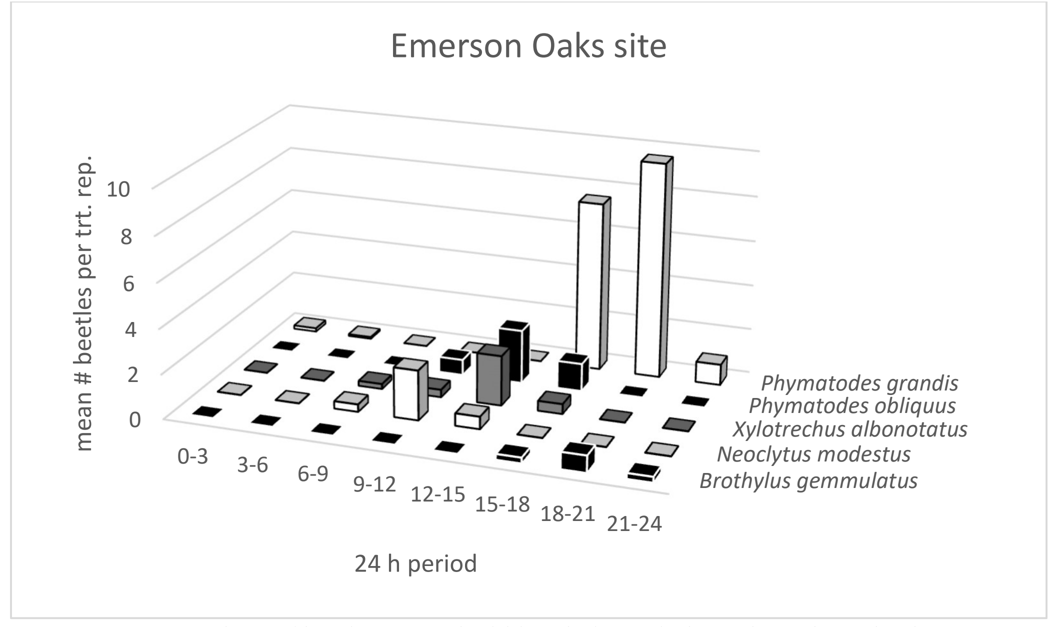

Fig. 3.5. Mean numbers of beetles trapped within 3 h time windows throughout the day at Emerson Oaks Reserve.

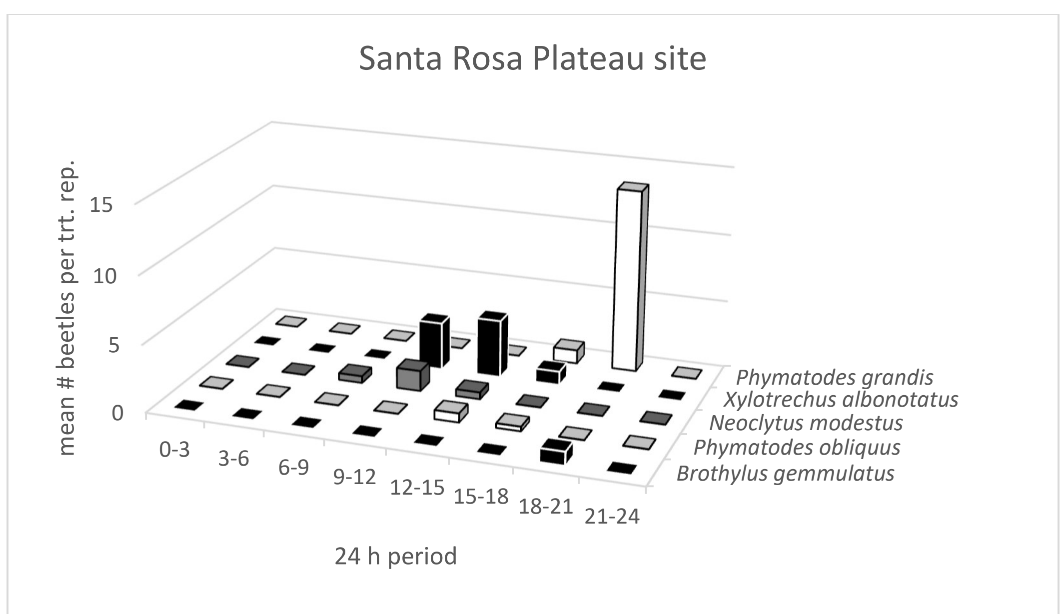

Fig. 3.6. Mean numbers of beetles trapped within 3 h time windows throughout the day at Santa Rosa Plateau Reserve.

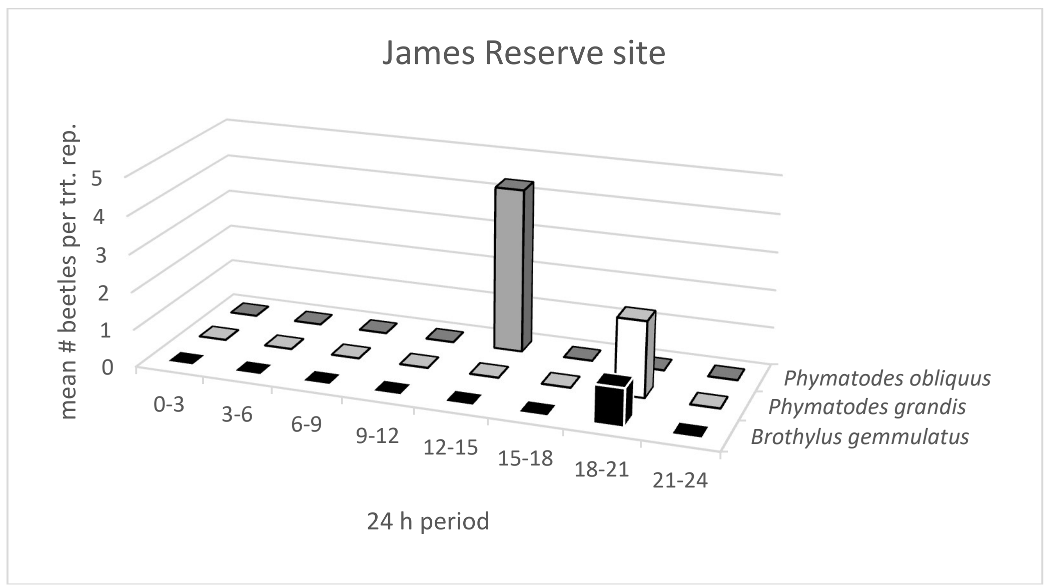

Fig. 3.7. Mean numbers of beetles trapped within 3 h time windows throughout the day at James Reserve.

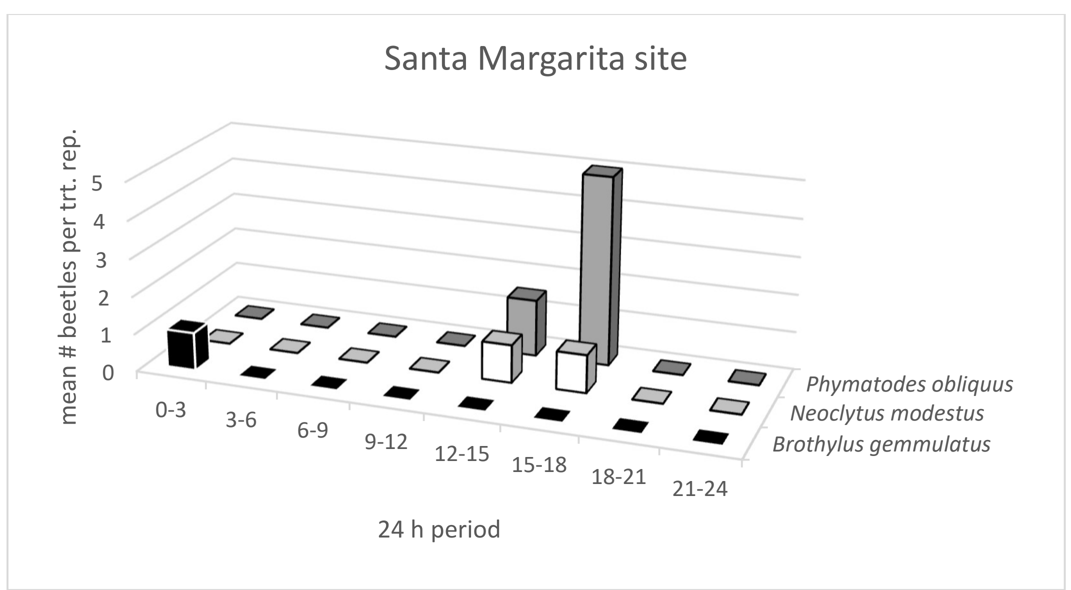

Fig. 3.8. Mean numbers of beetles trapped within 3 h time windows throughout the day at Santa Margarita Reserve.

Identification of beetle-produced compounds (*R*)-3-Hydroxyhexan-2-one and 2,3-hexanedione were detected in the headspace volatiles collected from males of *B. conspersus*, *B. gemmulatus*, and *X. albonotatus*. For *P. obliquus*, males produced (*R*)-2-methylbutanol, (*R*)-3-hydroxyhexan-2-one, and acetoin. None of these compounds were found in samples of headspace volatiles from females of the respective species, nor in the control samples from the host plants oak or white fir, or sugar water system blanks. For all species, the compounds were identified in at least five samples from males, except *X. albonotatus*, for which the compounds were found in two of a total of five samples from males of this species. No other insect-produced compounds were found in either single-sex aerations.

Discussion

The main intent of the research presented here was to probe two mechanisms (pheromone blends and temporal separation) by which sympatric wood-boring beetles which share pheromone components might minimize cross-attraction. I also confirmed the identities of several previously suspected male-produced pheromone compounds for *Phymatodes obliquus*, *Brothylus conspersus*, *Brothylus gemmulatus*, and *Xylotrechus albonotatus*, including acetoin, a potential novel pheromone motif for cerambycids.

Brothylus gemmulatus did not show a preference for any of the blends tested when compared with attraction to 3-hydroxyhexan-2-one alone. This species has not shown strong lure discrimination in previous experiments, with a high degree of variance in the treatment replicate means (Collignon et al. 2016). One possible explanation is that this species may use more of a random landing than primary attraction approach (Saint-Germain et al. 2007; Flaherty et al. 2013). Further testing will be required to determine why this species apparently seems so inexact in its response to lures. It is also possible that 2,3-hexanedione may be a pheromone component for this species, and not just a degradation product from 3-hydroxyhexan-2-one, and that a blend of the two compounds is required to obtain strong attraction.

In previous work, *P. grandis* had been shown to be significantly attracted to 3-hydroxyhexan-2-one even though this species does not appear to produce this

compound (Hanks et al. 2007). In the current study, females were significantly more attracted to 2-methylbutan-1-ol, which male beetles produce, than to acetoin, 3-hydroxyhexan-2-one, or acetoin + 3-hydroxyhexan-2-one. Combining 2-methylbutan-1-ol with 3-hydroxyhexan-2-one or 3-hydroxyhexan-2-one and acetoin did not increase attraction over 2-methylbutan-1-ol alone for females, indicating that 3-hydroxyhexan-2-one does not act additively or synergistically with 2-methylbutan-1-ol. However, males were more attracted to a blend of 2-methylbutan-1-ol + acetoin + 3-hydroxyhexan-2-one than to 2-methylbutan-1-ol alone. Thus, attraction of *P. grandis* males to the *P. obliquus* pheromone blend of 2-methylbutan-1-ol + acetoin + 3-hydroxyhexan-2-one may represent eavesdropping to find high quality hosts, particularly as the two species did exhibit partial temporal overlap at the Jenks Lake site. *Phymatodes obliquus* males are smaller than *P. grandis* males (~5-10 mm long and ~10-20 mm long, respectively), and so the latter may be able to outcompete the smaller species directly for host substrate. Although competitive interactions have not been shown between interspecific cerambycid males, male-to-male competition for host substrate does occur between conspecific males of other cerambycid species, and may occur between *P. grandis* and *P. obliquus* in a similar manner on host trees (Hughes and Hughes 1982; Hanks et al. 1996). It should also be noted that this was the only instance in this study in which possible increased attraction occurred due to acetoin being included in a lure.

For *P. obliquus*, the two treatments containing 2-methylbutan-1-ol and 3-hydroxyhexan-2-one were significantly more attractive to both sexes than any single compound, suggesting a synergistic interaction between the two compounds. Addition of acetoin neither increased nor decreased attraction, so it is not likely to be a pheromone component, even though it was present in male-produced volatiles.

Low numbers of *X. albonotatus* and *B. conspersus*, and no *N. modestus* were trapped during the pheromone blend experiment. This may be related to importance of HPVs in the response of *X. albonotatus* and *N. modestus* to pheromone, discussed below. For *B. conspersus*, comparing the results to another experiment run concurrently in 2014 (Collignon et al. 2016; Chapter 2), the data indicates that the species had an early phenology that year because the last specimen caught in the other experiment was a single male on 6 June 2014, the day the experiment reported here was deployed. However, data from other years shows that *B. conspersus* does sometimes have a later phenology that overlaps with the other species (Collignon et al. 2016; Chapters 2, 4).

In the experiment testing diel partitioning, there were two clear groups: the diurnal species, *P. obliquus*, *N. modestus*, and *X. albonotatus*; and the crepuscular/nocturnal species, *P. grandis* and *B. gemmulatus*. Too few specimens of *B. conspersus* were caught to properly determine its diel activity; the three trapped specimens could represent late-active diurnal individuals or early-active

crepuscular individuals. All that can be said is that this species does not appear to be nocturnal.

For the diurnal species, *P. obliquus* requires a blend of 3-hydroxyhexan-2-one plus 2-methyl-butanol, whereas *N. modestus* and *X. albonotatus* require only 3-hydroxyhexan-2-one, providing a mechanism for separation of the former from the latter. Host-plant volatiles (HPVs) are also likely to play a role in partitioning of pheromone use among these three species. It had previously been shown that *N. modestus* was most attracted to the pheromone blend plus ethanol, followed by attraction to an ethanol-only positive control (Collignon et al. 2016; Chapter 2). Thus, although *N. modestus* infests oaks, like *P. obliquus*, it seems likely that they specialize on a specific host condition associated with ethanol emission. For example, infection of coast live oak, a host of *N. modestus*, with the fungus *Phytophthora ramorum* (Pythiales: Pythiacaea), results in emission of increased levels of ethanol (Kelsey et al. 2013). Similarly, *X. albonotatus*, which infests conifers, may utilize HPVs in combination with pheromones. Although *X. albonotatus* were not significantly more attracted to pheromone plus HPVs than to pheromone plus non-host oak blend in my previous study, it is important to note that they were not attracted at all to traps baited with pheromone-only lures or pheromone plus oak blend (Collignon et al. 2016; Chapter 2). Thus, for the three diurnal species, *P. obliquus*, *N. modestus*, and *X. albonotatus*, the data suggests that HPVs help to partition these species. A better ecological understanding of the

preferred physiological condition of the hosts that these species infest, and the HPVs emitted by the preferred hosts and host-conditions in the field, would help to clarify the roles of HPVs in limiting cross attraction among these three species.

For the two crepuscular species, analyses of headspace samples of male *P. grandis* showed that they produce (*R*)-2-methylbutan-1-ol whereas male *B. gemmulatus* produce (*R*)-3-hydroxhexan-2-one, so the species do not have overlapping pheromone components. However, the situation is complicated by the fact that at least one site, *P. grandis* also appears to respond to (*R*)-3-hydroxhexan-2-one (Hanks et al. 2007), and the two species are synchronic, syntopic, and sympatric, so there is potential for competition. One hypothesis for why *P. grandis* are attracted to 3-hydroxyhexan-2-one on the Santa Rosa Plateau, but not in the San Bernardino Mountains, may be due to the presence of *B. gemmulatus* in the mountains, whereas the species is much less common on the plateau: only a handful of *B. gemmulatus* were trapped in the Hanks et al. (2007) study, and the timer trap at the same site in 2010 caught just two individuals over the course of the whole month the trap was deployed. By comparison, at the Jenks Lake site, dozens of *B. gemmulatus* were caught in just a few days with pheromone-baited traps. *Brothylus gemmulatus* is a larger beetle (~20-30 mm in length), and thus for the same reason that *P. grandis* males may respond well to the chemical blend emitted by *P. obliquus* – i.e., the larger *P. grandis* males can outcompete the smaller *P. obliquus* for the host substrate – *P. grandis* may be avoiding potential attraction to a host on

which the larger *B. gemmulatus* males are already calling. It is possible that the populations of *P. grandis* are altering their response to interspecific cues due to the varying competitive selective factors from heterospecifics at each site. Testing of the pheromones at both sites concurrently would clarify the response of *P. grandis* to 3-hydroxyhexan-2-one.

Overall, the findings from this study suggest that one or several mechanisms allow a number of sympatric species to share pheromone components while still maintaining reproductive isolation. These include temporal separation, as occurs with *P. obliquus* and *B. gemmulatus*, and use of HPVs and non-HPVs in concert with pheromones, as occurs for *N. modestus*, *X. albonotatus*, and *P. obliquus*. Further, there are ~100 cerambycid species in the San Bernardino Mountains (I. Swift, pers. comm.), many of which are in the subfamily Cerambycinae. It seems likely that some of these species utilize the pheromone compounds discussed in this research, but no other species were caught in significant numbers in our field trials. Further tests of the individual enantiomers or blends of enantiomers of 2-methylbutan-1-ol and 3-hydroxyhexan-2-one, as well as other common cerambycine chiral pheromones, such as the 2,3-hexanediols and 2-hydroxyhexan-3-ones, as possible antagonists or synergists may turn up more instances of cerambycine species using these types of compounds as pheromones (Hanks et al. 2012; Hanks and Millar 2013; Mitchell et al. 2015).

The field bioassays also elucidated an example of possible exploitation of the pheromone signal of *P. obliquus* by its congener, *P. grandis*. This ecological tactic may turn out to be relatively common among cerambycids, particularly as they often occur in guilds that jointly exploit a host resource. For example, a number of species can be reared out of a single fallen tree or cut log. Thus, cerambycid species that utilize 'generic' male-produced aggregation sex-pheromones run the risk of exploitation of their signals by conspecific mated/non-receptive and heterospecific females, conspecific males looking for females and/or host substrate from which to call, and heterospecific males also looking for host substrate. Whatever the case, the fitness benefit accruing from calling must outweigh the fitness cost of exploitation; otherwise, natural selection would rapidly select for mechanisms to reduce the cost of exploitation, such as shifts in temporal activity periods or pheromone blends, especially considering the selective pressure is not just on males, but on females and their progeny that would also both be experiencing direct competitive pressure. This suggests that in the context of cerambycid chemical ecology, pheromone partitioning may be a function of competition as opposed to or in addition to reproductive isolation (Cardé 1986). Other reproductive isolation mechanisms are also likely to be operative, such as close range utilization of species-specific cuticular hydrocarbons (Ginzel 2010) and/or trail pheromones (Hoover et al. 2014), partitioning via host species and condition, and segregation in different parts of host trees, from the roots to the upper

canopy (Vance et al. 2003, Graham et al. 2012). This plethora of possible isolating mechanisms may obviate the need for species-specific pheromone signals for many species. In fact, it suggests that there may be a continuum, with some species being promiscuous in their attraction to a given pheromone (/kairomone) blend, whereas others may be more selective. As the pheromones of additional species are identified, it should help to clarify the contexts and ecological conditions which have moulded the patterns of pheromone use within this large family of insects.

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Chapter 4: Possible optimal density strategies in use of aggregation pheromones by male longhorn beetles (Coleoptera: Cerambycidae).

Introduction

Aggregation by groups of insects can enhance opportunities for mating. However, under varying densities and ecological conditions this benefit can turn into a cost, by increasing competition and reducing per capita mating rates (Jones and Quinnell 2002; Wertheim et al. 2005; Bengtsson et al. 2008). Pheromones are often utilized by insects to assist in the formation of aggregations (Wertheim et al. 2005), and when males of a species are the sole producers of the species' aggregation pheromone, mate-finding is often the assumed primary function of the pheromone (Landolt 1997; Cardé 2014; Hanks and Millar 2016). This is borne out by examples from species such as the large grain beetle (*Prostephanus truncates*; Bostrichidae), males of which emit pheromones that are attractive to both females and males, but which cease calling upon the arrival of a female (Smith et al. 1996). Thus, male-produced pheromones (MPPs) are important signals for at least some insect species. However, male-produced pheromones constitute only a small fraction of the total number of insect pheromones that have been identified. A much larger proportion of species utilize female-produced pheromones for mate-finding, which attract only males (Landolt 1997). The majority of species known to use MPPs are coleopterans, and over the last decade, a large number of species within

the family Cerambycidae have been found to use these male-produced sex-aggregation pheromones (Hanks and Millar 2016). The structures of the pheromone components are frequently highly conserved, with a single compound or small group of compounds being utilized by a number of related species. Thus, fewer than twenty compounds (not including enantiomers) have been shown to attract females and males of around several hundred species in the subfamilies Cerambycinae, Lamiinae, and Spondylidinae.

The larvae of cerambycid beetles bore into and can cause extensive damage to woody plants. Cerambycids have the potential to be ecologically and economically damaging when they are introduced into new habitats as invasive species (Haack 2006; Parry and Teale 2011; Haack et al. 2010). Conversely, native cerambycids are important members of forest ecosystems because they play a major role in primary decomposition of woody material (Haack and Byler 1993; Ulyshen 2016; Chen and Forschler 2016). They also cull stressed and sickly trees within forests, maintaining baseline mortality and turnover of trees (Manion and Griffin 2001; Teale and Castello 2011).

There are a number of similarities in how beetles such as cerambycids use MPPs as sex-aggregation pheromones. For example, the presence of host-plant substrate and volatiles often stimulates pheromone production by males, as with the cerambycid *Hedypathes betulinus* (Lamiinae; Fonseca et al. 2010). Host-plant volatiles (HPVs) can also synergize and increase attraction to pheromones,

particularly for conifer-infesting species, but for others as well (Nakamuta et al. 1992; Silk et al. 2007; Teale et al. 2011; Fierke et al. 2012; Allison et al. 2012; Hanks et al. 2012; Hanks and Millar 2013; Collignon et al. 2016). Host-plant volatiles can act additively or synergistically with pheromones of other MPP-beetle species, including many curculionid beetles (Klimetzek et al. 1986; Dickens 1989; Raffa 2001; Erbilgin et al. 2003; Piñero and Prokopy 2003), *Carpophilus* species (Nitidulidae; Bartelt and James 1994), Colorado potato beetle (*Leptinotarsa decemlineata*; Chrysomelidae; Dickens 2006), and others (Landolt 1997). There is evidence that HPVs stimulate the production of pheromones by other MPP-species, such as *P. truncates* (Scholz et al. 1998), plum curculio (*Conotrachelus nenuphar*; Curculionidae; Hock et al. 2014), and the flat grain beetle (*Cryptolestes pusillus*; Cucujidae; Millar et al. 1985).

Another common characteristic of MPPs is their 'generic' use by multiple species. For example, the most common pheromones identified from cerambycids have been the C_6 and C_8 3-hydroxyalkan-2-ones and 2,3-alkanediols utilized by many species in Cerambycinae (Sakai et al. 1984; Iwabuchi et al. 1987; Fettkother et al. 1995; Leal et al. 1995; Lacey et al. 2004, 2008, 2009; Hanks et al. 2007; Ray et al. 2009, 2015; Wong et al. 2012; Imrei et al. 2013; Wickham et al. 2014; Narai et al. 2015; Hayes et al. 2016; Zou et al. 2016). Similarly, (*E*)-6,10-dimethyl-5,9-undecadien-2-ol – termed fuscumol after *Tetropium fuscum* from which it was first identified – and its ester (*E*)-6,10-dimethyl-5,9-undecadien-2-yl acetate (fuscumol

acetate) are emerging as common pheromone components for species in the cerambycid subfamilies Spondylidinae (Silk et al. 2007; Sweeney et al. 2010; Chapter 5) and Lamiinae (Fonseca et al. 2010; Mitchell et al. 2011; Hughes et al. 2016; Meier et al. 2016). In addition, for the lamiine genus *Monochamus* and some related genera, the compound 2-(undecyloxy)-ethanol, termed monochamol, is a demonstrated or likely pheromone component for at least 15 species (Pajares et al. 2010; Teale et al. 2011; Allison et al. 2012; Fierke et al. 2012; Ryall et al. 2014; Wickham et al. 2014). Other MPP beetle taxa also often 'share' pheromone components among related taxa and congeners. Examples include species of *Ips* bark beetles (Curculionidae: Scolytinae; Wood 1982), *Carpophilus* sap beetles (Williams et al. 1995), and *Tribolium* flour beetles (Tenebrionidae; Arnaud et al. 2002).

There is evidence of density- and dose-dependent effects on MPP pheromone emission and responses to pheromones, respectively (dose = release or emission rate). For example, males of several *Carpophilus* species produced less pheromone when in large groups than when held individually, the researchers' hypothesized reason being a negative feedback loop to reduce group sizes and conspecific competition (Bartelt and James 1994; Petroski et al. 1994; Bartelt et al. 1995). Regarding attractiveness of aggregations, small groups of *P. truncatus* males were more attractive to conspecifics than single males, large groups of ~60 beetles were not attractive (Scholz et al. 1998), and similarly, plum curculio males were

most attractive in groups of two rather than as individuals or in larger groups (Hock et al. 2014). Further, males of the bark beetles *Ips paraconfusus* and *Ips typographus* were less attracted to high rates of release of MPPs, likely because high emission rates indicate a fully exploited host (Byers 1983; Schlyter et al. 1987). It has also been suggested that density effects might span developmental stages, because male *I. typographus* offspring from lightly infested logs produced more pheromone than males from heavily infested logs (Anderbrant et al. 1985). Other scolytine species utilize various pheromonally-mediated mechanisms for density control (Raffa 2001). These findings suggest optimal density response strategies that vary with species and context, and which could be relevant to a better understanding of the ecology of cerambycid beetle species which use analogous MPP-based strategies.

Although MPP-cerambycid species and other MPP-beetle taxa share several key ecological characteristics as enumerated above, neither density-dependent production nor dose-dependent response to pheromones have been examined in depth in cerambycids. As part of the identification of semiochemicals, of *Neoclytus* species, pheromone candidates were tested at different dosages in field trials, with responses increasing with dose, but release rates were not measured (Lacey et al. 2004, 2007). Two studies of *T. fuscum* have tested the influence of pheromone release rate and influence of conspecific males on calling behavior. In the first, fuscumol released at an estimated 1, 4, or 32 mg/d did not result in large differences

in responses (Sweeney et al. 2010), in contrast to the *Neoclytus* studies mentioned above. In the latter study, holding males together in pairs elicited higher frequencies of pheromone-calling behavior than were observed with males held singly (Lemay et al. 2010). However, it is not known whether this behavioral density-response influences per capita pheromone emission by males. The occurrence of this behavior, and the ecological similarities of MPP-cerambycids with other MPP-beetle species suggests that density- and dose-response effects may be general characteristics of Coleoptera using MPP strategies. Confirming dose and density effects in pheromone use in cerambycids could have important implications for how native and invasive species are monitored with pheromone lures, as well as for the identification and testing of novel pheromones from cerambycids. Further, elucidation of the mechanisms of pheromone use may provide a better understanding of cerambycid ecology, for example, by demonstrating the role of MPPs in modulating cerambycid densities and reproductive behaviors.

Thus, the goal of the research described herein was to examine the dynamics of pheromone production, and possible dose-dependent responses to pheromones, for a group of cerambycine species in the forests of southern California that utilize a similar suite of pheromone components. My specific objectives were:

1. To determine whether pheromone release rate influences attraction of conspecifics, possibly in a non-linear manner.

2. To determine whether the presence of other males influences pheromone emission by conspecific males.

This research was undertaken as a first step in assessing whether use of male-produced aggregation pheromones by cerambycids may change in a context-dependent manner, which would support the hypothesis that MPP-species can modulate use of MPPs as part of broader optimal density strategies.

Methods

Dose-Dependent Responses to Cerambycid MPPs The goal of the first experiment was to determine the effect of release rates on the profile of responses of cerambycids – for example, whether responses increased linearly or exponentially with release rate, and whether they reached a plateau, or reached a maximum and then declined with further increases in release rate. I also wanted to assess whether females and males responded differentially to different release rates. This experiment was conducted at two sites approximately 500 m apart in the San Bernardino National Forest (SBNF) in San Bernardino Co., California, USA. The sites were near Jenks Lake (34°09'45.8"N 116°54'08.6"W) and are dominated by ponderosa pine (*Pinus ponderosa*; Pinales: Pinaceae) and white fir (*Abies concolor*; Pinaceae), with some western black oak (*Quercus kelloggii*; Fagales: Fagaceae), canyon live oak (*Quercus chrysolepis*), bigcone Douglas-fir (*Pseudotsuga macrocarpa*; Pinaceae), and incense cedar (*Calocedrus decurrens*; Pinales:

Cupressaceae). Black cross-vane intercept traps (Alpha Scents, Portland OR, USA) coated with Fluon® (Graham et al. 2010) were hung on 1.5 m tall, L-shaped stands made from PVC irrigation pipe mounted on sections of steel reinforcing bars driven into the ground. Traps were placed 50 m apart in transects to minimize potential interactions among treatments, with treatments initially assigned randomly to traps. Traps were checked twice weekly and their order was rerandomized at every checking. Treatments consisted of one blank control and four different release devices (see below). Two of the same devices were hung in the center of the intercept trap via wire, with one releasing neat, racemic 2-methylbutanol (MB; Aldrich Chemical Co., Milwaukee WI, USA), the other releasing racemic 3-hydroxyhexan-2-one (HH; Bedoukian Research, Danville CT, USA). 1% Butylated hydroxytoluene (BHT) was added to the neat HH to reduce oxidative degradation. Although 2,3-hexanedione was found in volatiles collected from target species, it was not included in baits because for numerous species in which it has been found, it appears to be a degradation product of HH rather than a pheromone component (Hanks and Millar 2016). The experiment was run from 12 May through 3 August 2016.

Beetles were trapped live so that they could be used for pheromone collection and in laboratory assays. A pouch (15 x 15 cm, aluminium window screening) filled with ~200 ml of activated charcoal (6-14 mesh; Fisher Scientific, Pittsburgh PA, USA) was placed in the collection jar to adsorb any pheromone that

captured beetles might emit while in the collection jar. Excess beetles were released at least 200 m from the field sites. Voucher specimens of all species have been deposited in the Entomology Museum at UC Riverside.

Quantification of 2-Methylbutanol and 3-Hydroxyhexan-2-one Release Rates

To quantify the amount of pheromone being released from the release devices and from beetles in the density-response experiment (see below), authentic standards of the compounds were serially diluted in hexane in a range spanning five orders of magnitude. These dilutions then were mixed 1:1 with a solution of 3-octanol (100 ng/ml in hexane) as an internal standard. One microliter aliquots of the resulting solutions were analysed with an Agilent 78020A gas chromatograph coupled to an Agilent 5977E mass selective detector (GC-MS; Agilent Technologies, Santa Clara CA, USA) fitted with a DB-5MS column (30 m × 0.25 mm ID × 0.25 micron film; Agilent). The temperature program used had an initial 5 min hold at 40ºC to allow separation of MB from the solvent peak, and then increased at 10 ºC per min, up to 280 ºC with no final hold. Samples were run in splitless mode with an injector temperature of 280 ºC.

From the serial dilutions, relative response factors of 0.56 for MB and 0.50 HH versus 3-octanol were calculated, and used to correct subsequent quantitations. All samples with unknown quantities of the two pheromones (for release device development and testing for density-dependent pheromone emission) were mixed

1:1 with the same internal standard solution. Pheromone quantification was calculated as: amount of analyte = ((amount of internal standard injected / response factor) x (peak area of analyte / peak area of internal standard)). The response factor was calculated as an average from the serial dilutions of the pheromones with internal standard, and equals ((peak area of analyte/peak area of internal standard) x (amount of internal standard injected/amount of analyte injected)).

Release Device Development and Quantification of Release Rates The release devices for the dose-response field experiment were developed by measuring the release rates of the two compounds from various carriers and containers, using headspace collections of MB and HH from the various devices. Headspace collections were conducted as opposed to gravimetric quantification of release because HH appeared to be very hygroscopic, based on increasing rather than decreasing mass measurements over time in open containers. The volatile collections were conducted for 3 h in 500 ml glass jars fitted with Swagelok (Swagelok, Solon OH, USA) inlets and outlets, with a flow of 500 ml/min provided by connecting the outlet to house vacuum. Incoming air was filtered through activated charcoal (6-14 mesh; Fisher Scientific), and volatiles were collected on 200 mg of thermally-desorbed activated charcoal (50-200 mesh; Fisher Scientific) held between glass wool plugs in a short piece of glass tubing. Volatiles were eluted from the charcoal with 1 ml dichloromethane (DCM), and quantified as above.

Headspace sampling was conducted in an environmental chamber at a constant temperature of 25ºC.

The most consistent emission profiles found were from neat material released from clear glass vials. The devices used were as follows: Device A was a 2 ml glass vial (35 mm tall, 10 mm wide, 5 mm opening, 8 mm neck) with a pinhole bored into the polyurethane cap with a heated needle, which released 37.3 ± 7.2 (mean ± S.E) µg/hr of MB and 18.8 ± 4.0 µg/hr of HH (eight sampling replicates). Device B was also a 2 ml vial, with a 2 mm hole in the cap, which released 141 ± 12 µg/hr MB and 73 ± 10 µg/hr of HH (eight replicates). Device C was an 8 ml glass vial with no cap (90 mm tall, 16 mm wide, 10 mm opening, 8 mm neck), and released 565 ± 135 µg/hr MB and 359 ± 164 µg/hr of HH (12 replicates). Device D was a 20 ml glass scintillation vial, again with no cap, and released 1,446 ± 257 µg/hr MB and 719 ± 118 µg/hr of HH (13 replicates). Each release device was made up of two of the same type of vials, each loaded with a given amount of one of the two pheromones. Devices A and B were both initially loaded with 1 ml of compound per vial, Device C was loaded with 4 ml per vial, and Device D with 7 ml per vial. For all these devices, maintaining the vials between one quarter and half full of neat compound resulted in relatively consistent release rates (Fig. 4.1-4.4; Weatherston et al. 1985). In the field, release devices were wrapped in duct tape to reduce light-induced degradation.

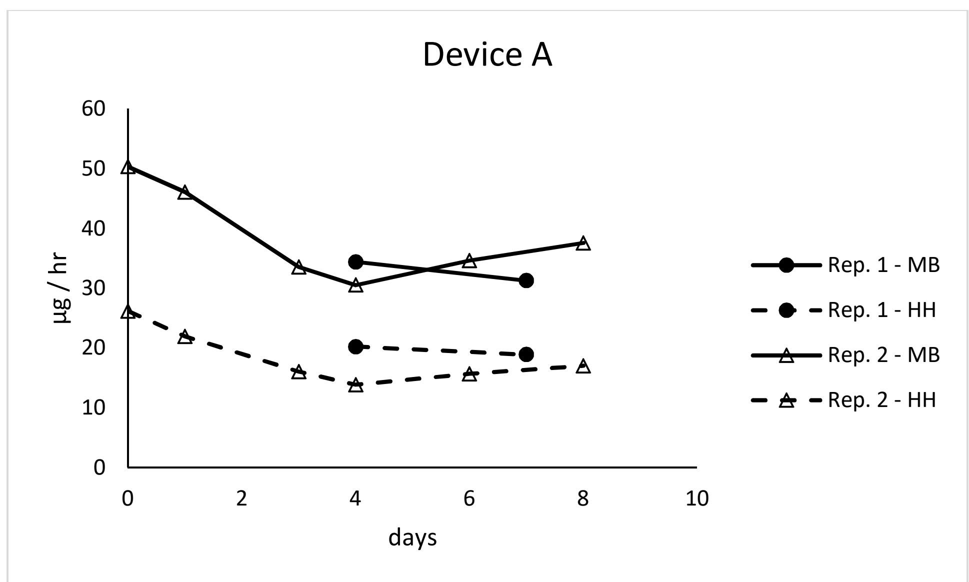

Fig. 4.1. Replicated release rates of 2-methylbutanol (MB) and 3-hydroxyhexan-2-one (HH) from a 2 ml glass vial with a pinhole in the cap (Device A), sampled in an environmental chamber at 25ºC.

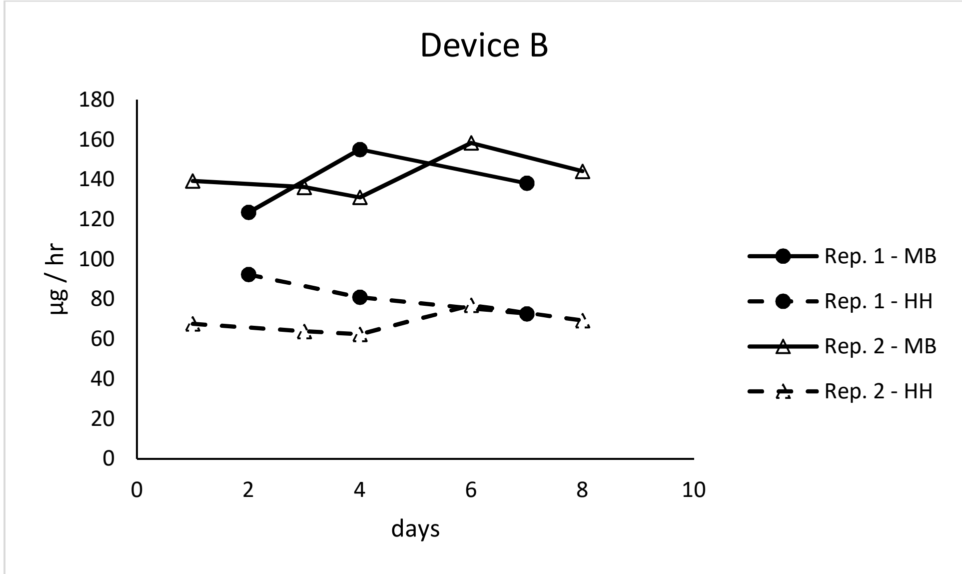

Fig. 4.2. Replicated release rates of 2-methylbutanol (MB) and 3-hydroxyhexan-2-one (HH) from a 2 ml vial with a 2mm hole in the cap (Device B), sampled in an environmental chamber at 25ºC.

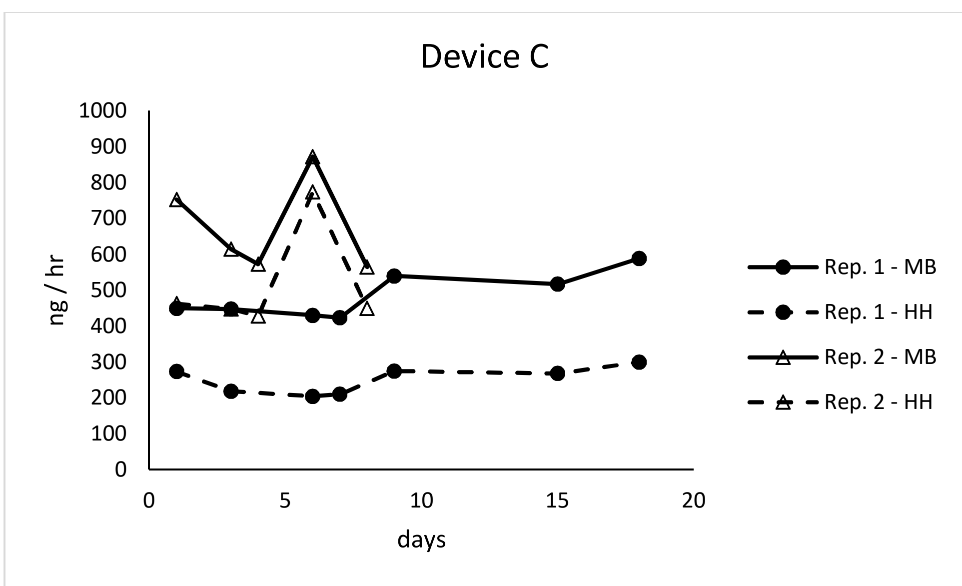

Fig. 4.3. Replicated release rates of 2-methylbutanol (MB) and 3-hydroxyhexan-2-one (HH) from an 8 ml glass vial with no cap (Device C), sampled in an environmental chamber at 25ºC.

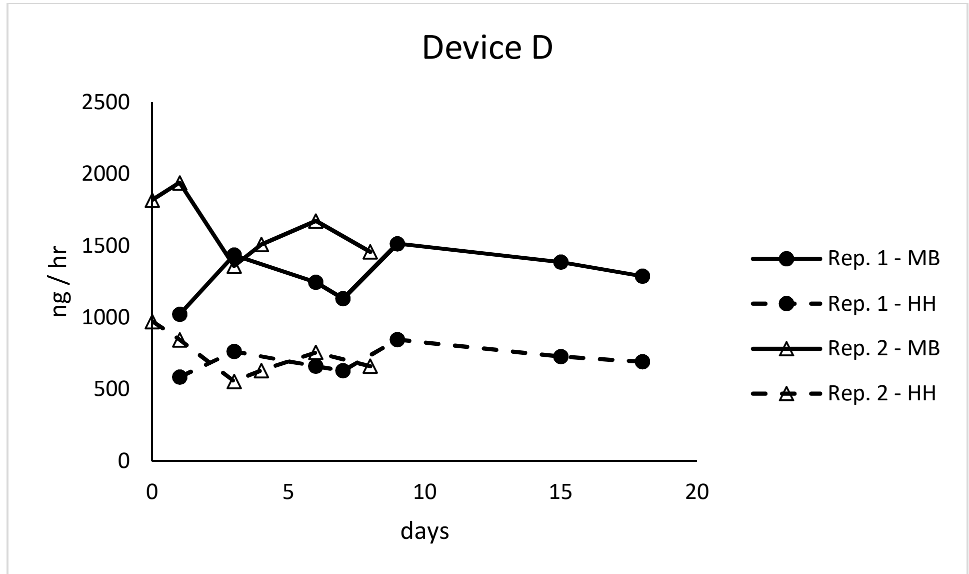

Fig. 4.4. Replicated release rates of 2-methylbutanol (MB) and 3-hydroxyhexan-2-one (HH) from a 20 ml glass scintillation vial with no cap (Device D), sampled in an environmental chamber at 25ºC.

Density-Dependent Pheromone Emission Assay The purpose of this experiment was to determine if male *Phymatodes grandis* beetles emitted differential rates of pheromone per capita when held in groups of varying sizes. Males were aerated individually (22 males), or in groups of two (31 pairs), three (15 groups), or four males (21 groups) respectively, for 24 h in 500 ml translucent Teflon bottles (Fisher Scientific). Headspace volatiles were collected on 50 mg of activated charcoal and extracted with 0.5 ml DCM; all other aspects of the headspace collection setup and air flow were identical to the release device sampling methods described above. Aerations were conducted in an environmental chamber under ReptiSun 10.0 UVB lights (Zoo Med Laboratories Inc., San Luis Obispo CA, USA) with an L:D cycle of 16:8 h, and a constant temperature of 25ºC. Sampled males were held in a holding jar with other males and access to 10% sugar water for 24-72 h prior to testing. Individuals were obtained from infested logs and reared out in an emergence chamber, or were collected live from the field experiment. Samples were analyzed as above for the lure devices. Samples with no detectable amount of pheromone in the GC-MS chromatograms were reanalyzed on an HP 58090 Series II GC in splitless mode with a flame-ionization detector (GC-FID) to obtain higher sensitivity. Headspace samples were collected from 4 May through 16 July 2016.

Statistical Analyses For the field bioassay testing responses of beetles to different release rates, females and males were counted separately for all species

(*Phymatodes obliquus*, *P. grandis*, *Brothylus conspersus*, and *Brothylus gemmulatus*), except *Xylotrechus albonotatus* due to a lack of confidence in accurately sexing individuals from this species. Replicates for each experiment were based on both spatial and temporal replicates, with temporal replicates equalling the number of times the traps were checked. Temporal replicates where no beetles of a given species were trapped in any treatment – usually due to inclement weather or deployment outside a species' flight period – were removed from analyses. Because almost no beetles were caught in the blank controls (only three female *P. grandis* of 235 total trapped), this treatment was removed from the analyses. Many of the count response variables did not have homogenous variances among treatments, as tested by Bartlett's test. Thus, data were analyzed with a max-*t* test (Herberich et al.; 2010; Hothorn et al. 2008; Zeilis 2006), preceded by an ANOVA model calculated for the max-*t* test. This test is robust to departures from assumptions of normality and homogeneity of variances, a common occurrence with biological data.

For the density-response pheromone emission experiment, the amounts of pheromone emitted by groups of male *P. grandis* were converted into a per capita value. Ranked transformed data were homogeneous for variances based on Bartlett's Test, so Kruskal-Wallis chi-squared analysis was used. Untransformed data had heterogeneous variances and had unequal replicate numbers amongst the treatment groups, so a max-*t* test (Herberich et al. 2010) was run to determine if

number of males influenced pheromone emission rates compared to single males. The treatments were also analyzed with Pearson's chi-squared analysis to determine if there was a difference in the frequency of pheromone emission among the groups. All analyses were conducted with R (3.3.1; The R Foundation; 2016).

Results

Dose (=Release-rate) Dependent Responses to Cerambycid MPPs Five cerambycine species were caught in the blocks of traps baited with devices emitting four different release rates of the cerambycid pheromone components MB and HH. These included *Brothylus conspersus* (12 treatment replicates), *Brothylus gemmulatus* (24 replicates), *Phymatodes grandis* (36 replicates), *Phymatodes obliquus* (35 replicates), and *Xylotrechus albonotatus* (18 replicates). The pheromone of *Phymatodes grandis* was previously reported as (*R*)-2-methylbutanol (Hanks et al. 2007), and all others were reported for the first time in Chapter 3. Briefly, *B. conspersus, B. gemmulatus*, and *X. albonotatus* males produce (*R*)-3-hydroxyhexan-2-one and 2,3-hexanedione, whereas *P. obliquus* males produce (*R*)-2-methylbutanol, (*R*)-3-hydroxyhexan-2-one, and acetoin.

For *B. conspersus*, only data from females was analyzed due to the small number of males trapped (23 females, 3 males). Female *B. conspersus* had significantly different responses to release rate (ANOVA: $F_{3,44} = 10.91$, p = 1.76^{e-05}). Females were significantly more attracted to the highest release rate (Device D)

than to any of the other release devices (Fig. 4.6), whereas none of the other devices differed in their attractiveness. *Brothylus conspersus* females showed an exponential response to increasing release rates of racemic 3-hydroxyhexan-2-one (Fig. 4.7).

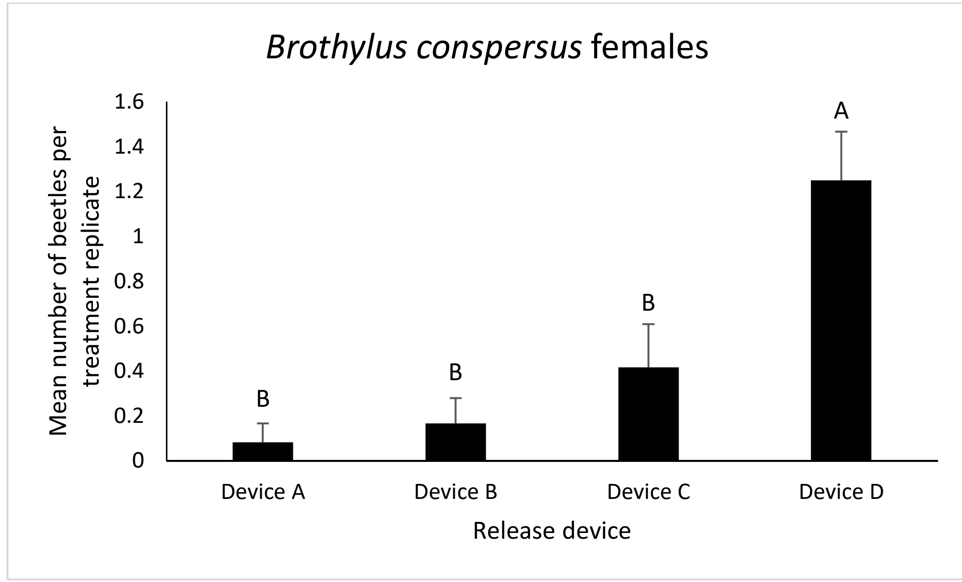

Fig. 4.6. Mean (± 1 SE) numbers of *B. conspersus* females caught in traps baited with four release rates of racemic 2-methylbutanol and racemic 3-hydroxyhexan-2-one. Means with different letters are significantly different (max-*t* test, $p < 0.05$).

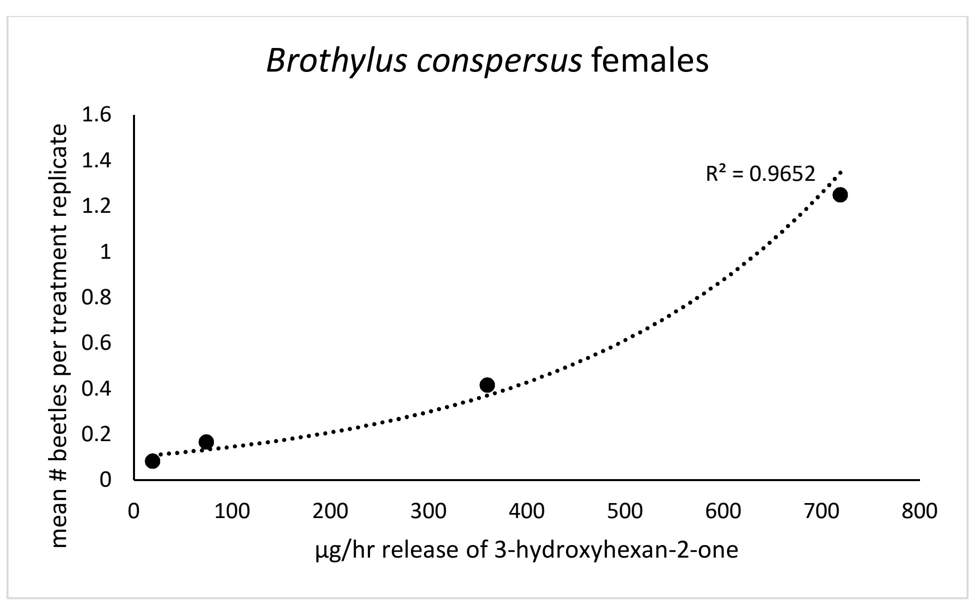

Fig. 4.7. Mean numbers of *B. conspersus* females caught in traps plotted against the measured release rates of racemic 3-hydroxyhexan-2-one from the four release devices. An exponential trendline fit the data best (compared to linear or logarithmic trendlines).

For *B. gemmulatus*, 117 females and 114 males were trapped, with significant differences in responses among the release rates for both sexes (females: $F_{3,92} = 5.24$, p = 0.0022; males: $F_{3,92} = 11.68$, p = 1.49^{e-06}). For females, Device D was the most attractive by mean number of beetles caught (= 2.66 per treatment replicate), but the variance was high in relation to the mean (SE 0.59), and thus, Device D was statistically only more attractive than Device A (Fig. 4.8). Device C, with less variance (SE 0.26), was significantly more attractive than Devices A and B. For males, both Device C and D were significantly more attractive than Devices A or B, but were not different from one another. Both sexes had a strongly linear response to increasing release rate of 3-hydroxyhexan-2-one (Fig. 4.9).

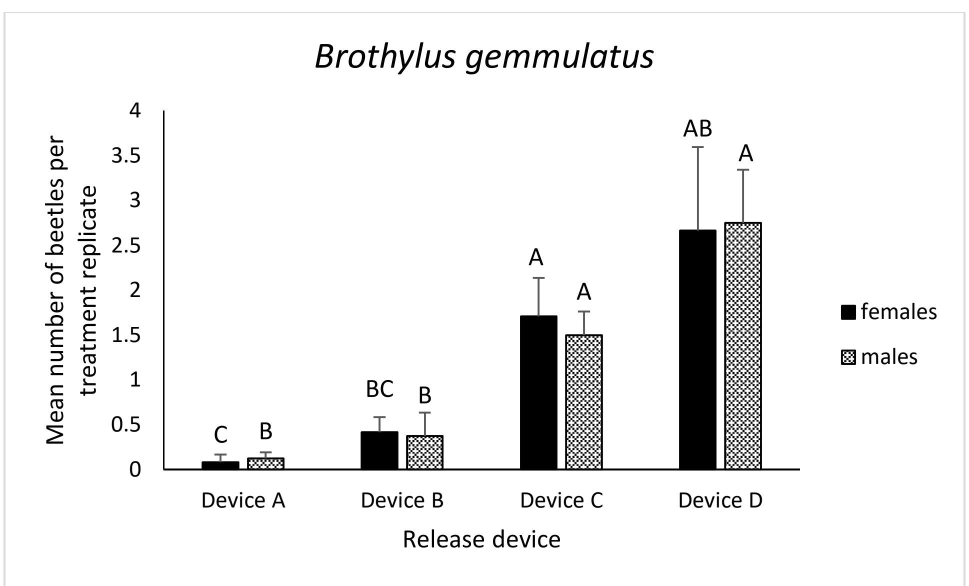

Fig. 4.8. Mean (± 1 SE) numbers of *B. gemmulatus* females and males caught in traps baited with four release rates of racemic 2-methylbutanol and racemic 3-hydroxyhexan-2-one. For each sex, means with different letters are significantly different (max-*t* test, $p < 0.05$).

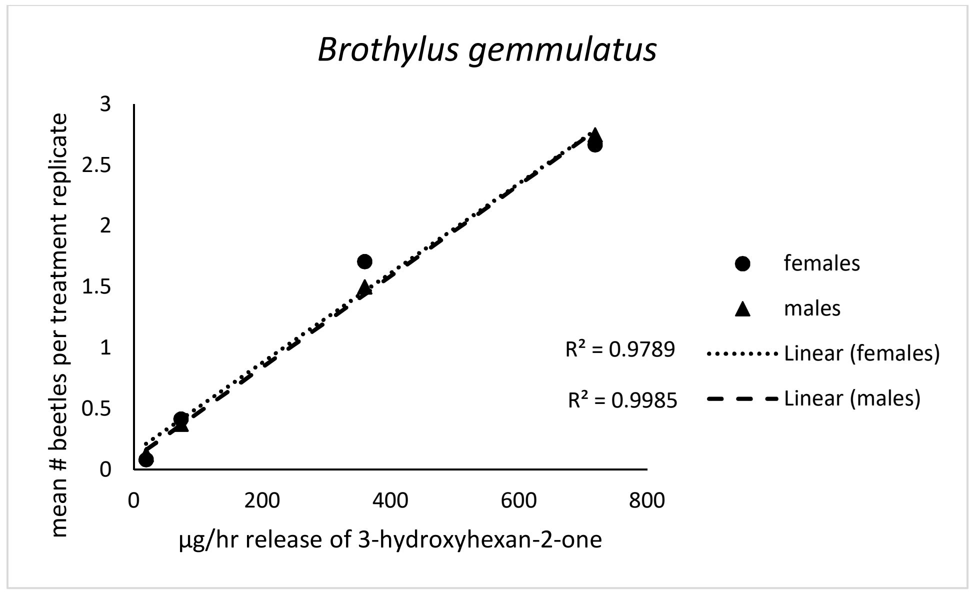

Fig. 4.9. Mean numbers of *B. gemmulatus* females and males caught in traps plotted against the measured release rates of racemic 3-hydroxyhexan-2-one from the four release devices; a linear trendline fit both data sets best (compared to exponential or logarithmic trendlines).

For *P. grandis*, 232 females and 122 males were trapped, and responses among treatments were significantly different (females: $F_{3,140} = 7.20$, p = 0.0002; males: $F_{3,140} = 3.58$, p = 0.016). Females showed increased attraction to increasing release rate, with Devices B-D being more attractive than Device A. However, Devices B-D were not statistically different from each other. Males of this species only showed a significant preference for Device D, and only when compared with the lowest release rate from Device A (Fig. 4.10). The responses of both sexes as a function of release rate were best fit by a linear relationship, as with *B. gemmulatus* (Fig. 4.11).

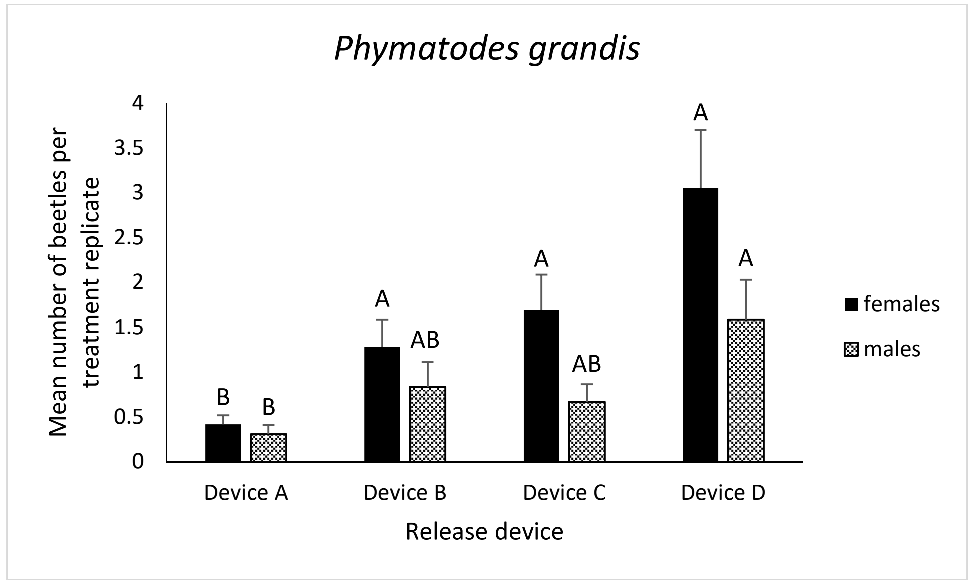

Fig. 4.10. Mean (± 1 SE) numbers of *P. grandis* females and males caught in traps baited with four release rates of racemic 2-methylbutanol and racemic 3-hydroxyhexan-2-one. For each sex, means with different letters are significantly different (max-*t* test, $p < 0.05$).

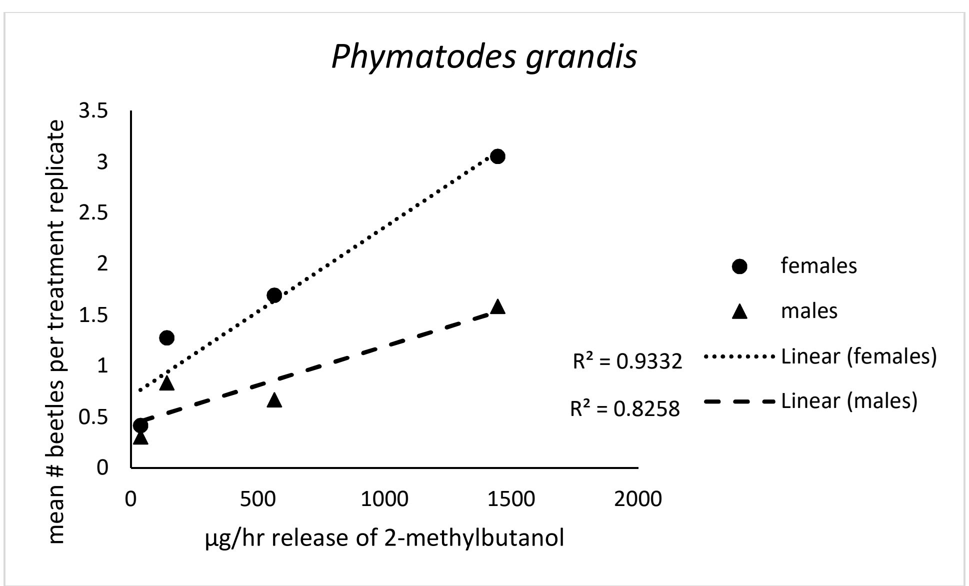

Fig. 4.11. Mean numbers of *P. grandis* females and males caught in traps plotted against the measured release rates of racemic 2-methylbutanol from the four release devices; a linear trendline fit both data sets best (compared to exponential or logarithmic trendlines).

The ANOVA model that was used by the max-*t* test found that whereas females responded significantly differently to the four release rates ($F_{3,136} = 6.31$, p = 0.0005), males did not ($F_{3,136} = 1.85$, p = 0.14). However, although the ANOVA model is the omnibus basis of the max-*t* test, it is not robust to non-normal data or to heterogeneous variances, whereas the max-*t* test is and so the max-*t* test was still utilized for analyzing the *P. obliquus* male count. Females were significantly more attracted to Device D than to Devices A or B, but Devices C and D were equivalent (Fig. 4.12). Males were only more attracted to Device D than to Device A. Although Device B caught a higher mean number of beetles (= 0.77 per replicate), it also had higher variance (SE 0.33) than Device D (mean 0.6; SE 0.15). *Phymatodes obliquus* is the only species in this study that produces both test compounds. Plotting the

mean number of females or males against the laboratory-determined release rates of the devices for the two different pheromones showed that both sexes responded logarithmically to both pheromones' increasing release rate, although the trendline for males was rather weak ($R^2 = 0.29$, Fig. 4.13; $R^2 = 0.46$, Fig. 4.14).

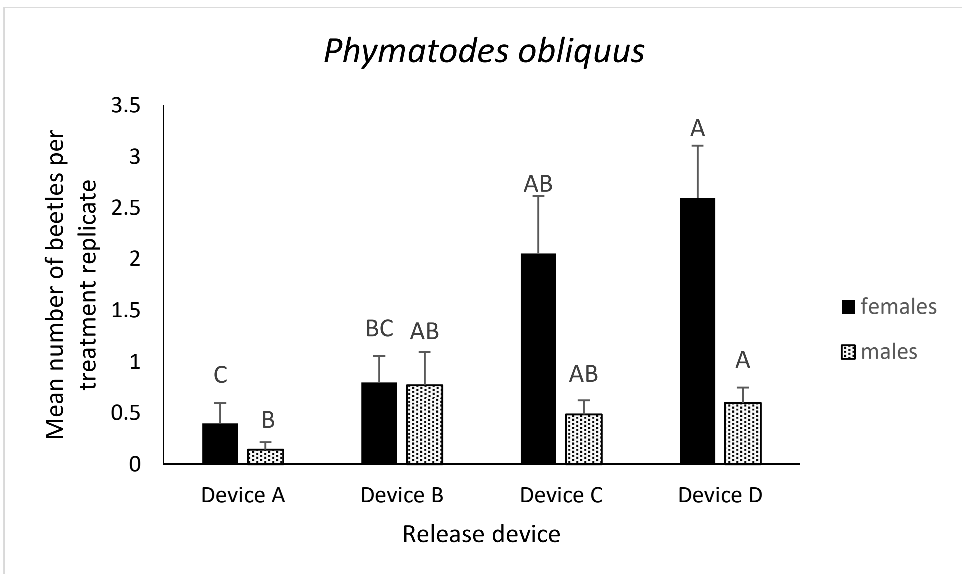

Fig. 4.12. Mean (± 1 SE) numbers of *P. obliquus* females and males caught in traps baited with four release rates of racemic 2-methylbutanol and racemic 3-hydroxyhexan-2-one. For each sex, means with different letters are significantly different (max-*t* test, $p < 0.05$).

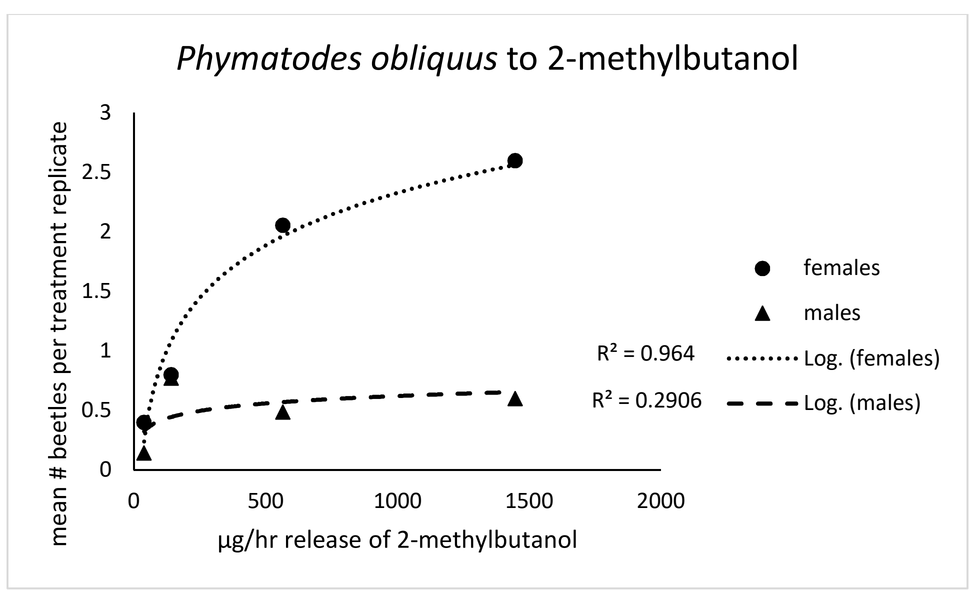

Fig. 4.13. Mean numbers of *P. obliquus* females and males caught in traps plotted against the measured release rates of racemic 2-methylbutanol from the four release devices; a logarithmic trendline fit both data best (compared to linear or exponential trendlines), though the R^2 value for males was rather low.

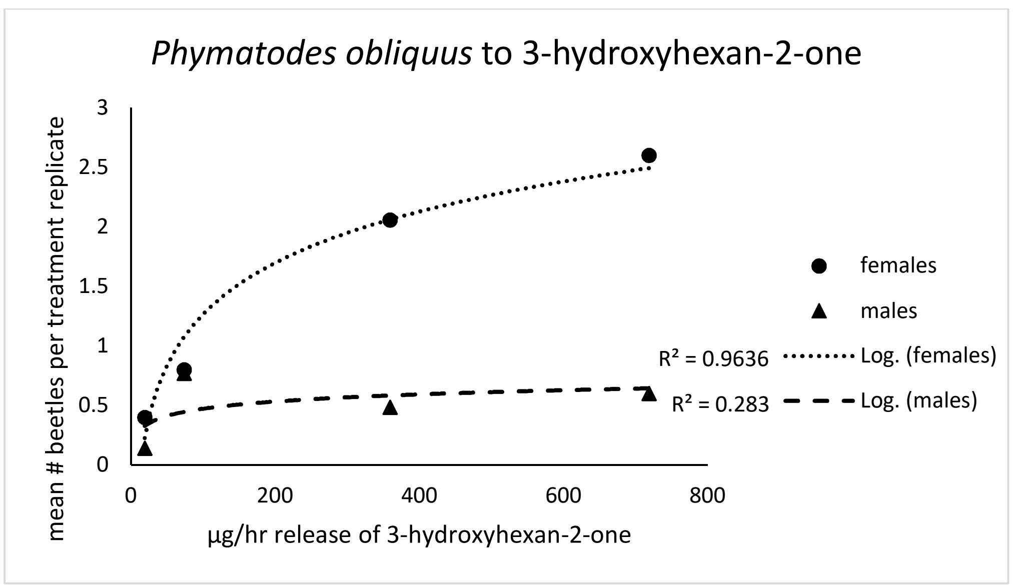

Fig. 4.14. Mean numbers of *P. obliquus* females and males caught in traps plotted against the measured release rates of racemic 3-hydroxyhexan-2-one from the four release devices; a logarithmic trendline fit both data best (compared to linear or exponential trendlines), though the R^2 value for males was rather low.

For *X. albonotatus*, which were not separately analyzed by sex, 40 beetles were trapped. Responses were significantly different to the four release rates ($F_{3,68}$ = 10.91, p = 6.13^{e-06}), with beetles only being caught in traps baited with the two highest release rates, Devices C and D (Fig. 4.15). *Xylotrechus albonotatus* individuals responded linearly to increasing release rates of 3-hydroxyhexan-2-one (Fig. 4.16).

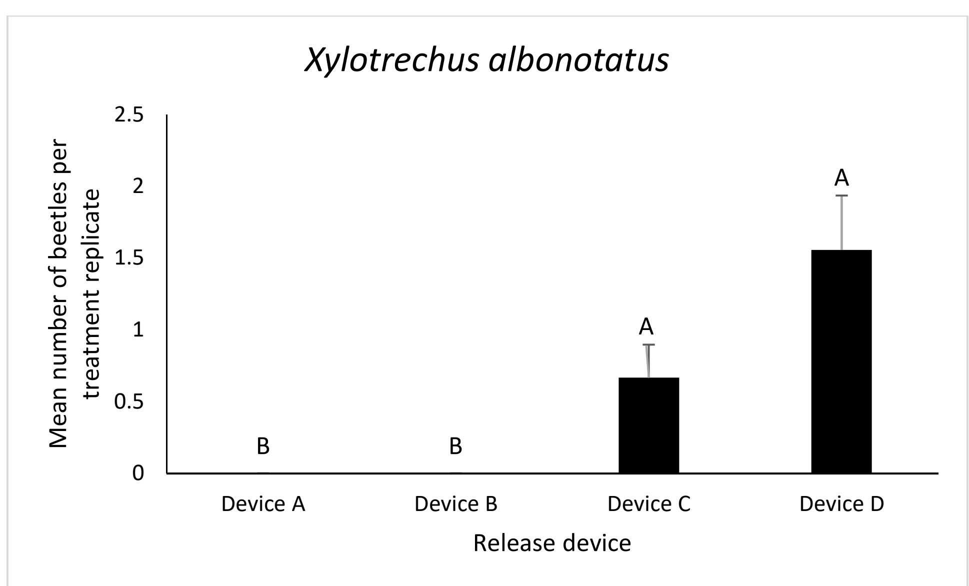

Fig. 4.15. Mean (± 1 SE) numbers of *X. albonotatus* individuals caught in traps baited with four release rates of racemic 2-methylbutanol and racemic 3-hydroxyhexan-2-one. Females and males were pooled for analysis. Means with different letters are significantly different (max-*t* test, $p < 0.05$).

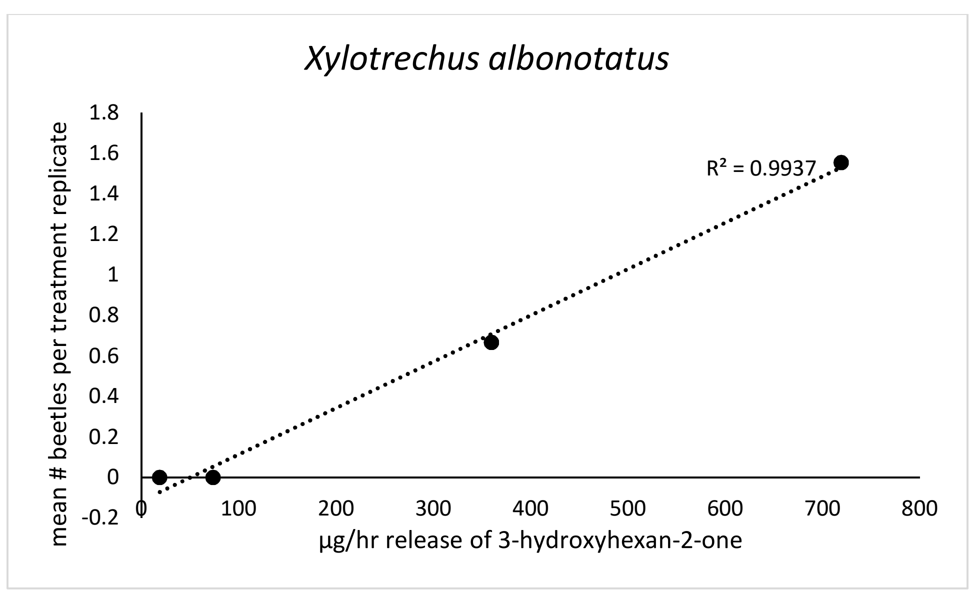

Fig. 4.16. Mean numbers of *X. albonotatus* caught in traps plotted against the measured release rates of racemic 3-hydroxyhexan-2-one from the four release devices; a linear trendline fit the data best (compared to exponential or logarithmic trendlines).

Density-Dependent Pheromone Emission Assay Of the four treatment groups used, 11 of the 22 individual males produced detectable amounts of pheromone, 15 of the 31 pairs produced pheromone, 12 of the 15 groups of three males produced pheromone, and 12 of the 21 quartets of males produced pheromone. From the samples with detectable amounts of pheromone, individual males produced 149 ± 51 (mean ± S.E.) ng/hr, and per capita, pairs produced 44 ± 16 ng/hr, three males produced 27 ± 11 ng/hr, and four males 14 ± 5 ng/hr. There was no difference in the frequency of presence or absence of detectable pheromone among any of treatments (chi-squared = 4.57, df = 3, p = 0.21). Because there was not a significant effect of density on frequency of pheromone emission, the data were analyzed without the samples that contained no detectable pheromone. A Kruskal-Wallis test

indicated a significant treatment response (p = 0.038; Fig. 4.17). The max-*t* test comparing the per capita pheromone emission of multiple-male treatment groups to the single male treatment detected several pairwise significant differences. Single male samples differed from those with three or four males, whereas there was no significant difference between samples for single and two males.

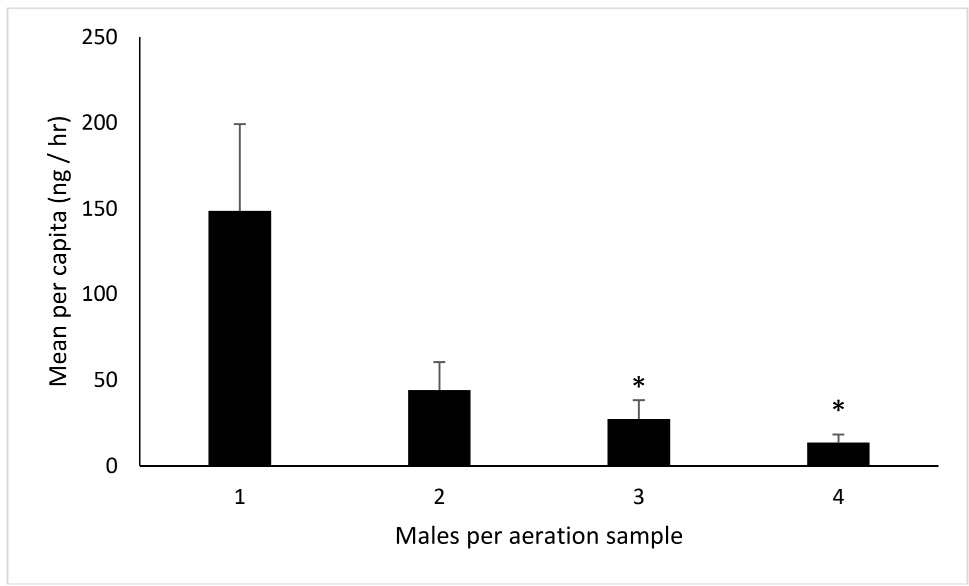

Fig. 4.17. Mean (± 1 SE) amount of (*R*)-2-methylbutanol (ng/hr) produced per capita by different sized groups of male *P. grandis*, with samples in which no pheromone was detected removed. Means with an asterisk are significantly different from the single male treatment (max-*t* test, p < 0.05).

Discussion

This research was designed to test two aspects of male aggregation pheromone use for cerambycids that also are shared by several other species of MPP-beetles. Specifically, we wanted to determine how cerambycid species' attraction to pheromone – as measured by trap catch – was affected by release rate

(i.e., do cerambycids respond in a dose-dependent manner to pheromones), and secondly, whether the production of pheromone was influenced by the presence of conspecific males (i.e., is pheromone emission by males density-dependent). The working hypothesis was that cerambycids utilize one or both of these pheromone tactics in optimal density strategies. Whereas these experiments do not test this hypothesis directly, they do provide baseline information with which to formulate more intricate ecological and evolutionary hypotheses.

The first experiment provided limited evidence that response to pheromone release rate is being utilized as a tactic in maintaining optimal densities of beetles. Three of the five test species, *B. gemmulatus*, *P. grandis*, and *X. albonotatus*, showed a linear response to increasing release rates of their pheromones – with neither a downturn or plateau effect at higher release rates – and females of the fourth species, *B. conspersus,* showed an exponential response to increasing release rates of pheromone. This might suggest that the tested release rates were not high enough to elicit an antagonistic effect, but this seems unlikely because the highest release rates measured from Device D averaged 1.45 mg/hr for 2-methylbutanol, whereas the highest mean hourly release of the compound from a *P. grandis* male in the second experiment was 456 ng/hr. Even assuming that beetles only produced pheromone for one or a few hours out of the 24 h aeration period, the release rate from Device D was still several orders of magnitude higher than that from the most productive *P. grandis* male that was measured for 2-methylbutanol emission.

Furthermore, the data from the second experiment suggested that the amount released per male may decrease with increasing numbers of males, and so a release rate of 1,445 μg/hr would probably represent the output from several hundred males at least. Aggregations containing 100 or more of these beetles have never been reported. Thus, it does not seem likely that this species uses high pheromone release rates as a signal of a potentially overexploited resource. The linear response of these three species does not support, but also does not rule out an optimal density strategy being used, because there are other potential mechanisms to modulate density, including density-dependent pheromone emission by males (second experiment) and female ovipositional behaviours (see refs. below).

The finding that beetles were not repelled by unnaturally high release rates of pheromone – tens of milligrams per day – has important implications for pest management or conservation applications. This finding indicates that to increase efficacy and attractiveness of a lure for cerambycids, the release rate need only be increased – at least for the five species in this study, but likely for other cerambycids as well. Although release rates were not specifically measured, results for *Neoclytus* species from Lacey at al. (2004, 2007) support this, at least for cerambycine species. Study of three species of *Tetropium* in Nova Scotia and Poland did not find a significant difference in trap catches among three release rates with a 32-fold difference between the lowest and highest release rates (Sweeney et al. 2010). This range of release rates is similar to that in the study reported here (e.g., Device A

released ~895 μg/d of 2-methylbutanol, and Device D released ~35 mg/d). It may be that the subfamilies of MPP-cerambycids utilize emission rates differently due to different ecological contexts.

For the one conifer-infesting species in this study, *X. albonotatus*, individuals were only attracted to the two highest release rates in this study, whereas the other four species were attracted to all treatments in a graded response. This result could be because *X. albonotatus* has a higher threshold response to pheromone in the absence of host-plant volatiles, as seen with other conifer-infesting species (reviewed in Collignon et al. 2016); although *X. albonotatus* were not statistically significantly more attracted to pheromone plus HPVs in my previous study, it is important to note that they were nonetheless *not* caught in traps baited with pheromone-only lures. This could indicate that HPVs work in part by decreasing the response threshold to pheromones for species that appear to be attracted largely or exclusively to blends of pheromones with HPVs (e.g., *Tetropium* spp.). In sum, these results suggest that for at least some species, attraction to pheromone lures can be increased by increasing the release rate, or by releasing the pheromone in combination with HPVs, or both.

It also should be noted that the release rates measured in the laboratory were likely very conservative in comparison to the rates of release under field conditions. For example, the measured release rate of Device D was approximately 242 mg per week of MB, and 120 mg per week for HH. In the field, however, it was necessary

to add ~1-2 g per week to the MB Device D, and about ~1-2 g every other week to the HH Device D, to maintain the level of pheromone in the vials relatively constant (between half and one quarter full). The situation was similar for Device C, but Devices A and B did not require refilling throughout the season due to their much lower release rates overall. Possible reasons for these discrepancies between the measured and observed release rates were likely due to a combination of factors, including higher wind velocities over the openings of the devices in the field, and the increased vapor pressure of the pheromones at the altitude of the field sites (~2000 m vs 260 m at the laboratory, resulting in a difference of ~2.8 psi). However, even though the actual release rates of the devices in the field was higher than the rates measured in the laboratory, the determination of the relative release rates among the four devices should be robust because they were all measured under standardized conditions. Consequently, the overall interpretation of the experiment should also be robust.

In contrast to the pheromone response results, the experiment testing whether males altered their pheromone production in the presence of other males did provide some support for an optimal density strategy. Thus, pheromone emission rates per capita were inversely correlated with group size for male *P. grandis*. This observed decrease and other known aspects of cerambycid ecology and behavior (reviewed in Hanks and Millar 2016 and Introduction) suggest a potential host-/mate-finding scenario for *P. grandis*. Larvae of the species infest

dying and recently dead *Quercus* and other angiosperm tree species, and assuming males call from such resources – an important assumption requiring confirmation from future research – then males are offering two possible benefits to females: A) the direct benefit of an adequate host substrate for oviposition; and, B) the indirect benefit of a male that can find hosts and is virile, overall indicative of 'good genes' (Wyatt 2004). In this scenario, there could be male-to-male competition for mates, with larger males dominating often scarce and patchily distributed hosts, and consequently mating with the most females (Hughes and Hughes 1982; Hanks et al. 1996a). Conversely, contact with conspecific males may simply lower likelihood of pheromone emission in males regardless of dominance. Nevertheless, smaller, non-calling satellite males could still successfully mate by searching for females via trail pheromones in scramble competition with larger males (Aldrich et al. 1984; Hanks et al. 1996b; Hoover et al. 2014), and thus would still glean benefit from responding to MPPs. Reports that pheromone calling behavior in *T. fuscum* induces calling in conspecific males (Lemay et al. 2010) does not preclude the above, firstly, because a decrease in per capita emission was only seen with *P. grandis* in group sizes larger than two. Secondly, it could allow females to directly choose among the calling males (Fisherian sexual selection), possibly assessing the fitness of males in the aggregation by their pheromone emission rates. This type of sexual selection has been noted for female tobacco/cacao moths (*Ephestia elutella*; Lepidoptera: Pyralidae), which prefer large males that produce more pheromone

(Phelan and Baker 1986). Finally, it is entirely possible that *T. fuscum* males would be less likely to call in the presence of densities higher than just two males. Either way, because more males calling from a host should increase attraction of females (based on results from the first experiment), males of various cerambycid species may tolerate other males up to a point, beyond which, male-to-male encounters and competition would increase in cost, outweighing the benefits of the increased apparency of aggregations to female conspecifics (Raffa 2001; Jones and Quinnell 2002; Greenfield and Schul 2008). This could lead to a density 'tipping point' for males, beyond which the threshold for further pheromone calling is increased to the point where most males remain 'silent', or it could simply increase the likelihood that males will leave a densely occupied host substrate in search of an unoccupied one (Byers 1983).

Another mechanism that would support an optimal density strategy, which was not tested here, is female behavior to density indicators on the host substrate, as has been shown for *Monochamus alternatus* (Anbutsu and Togashi 1997, 2001, 2002). Females of other species likely utilize similar oviposition deterrents on overcrowded hosts, because larvae from eggs which are laid on a host which is already infested are less likely to survive, due to being cannibalized by the older, larger larvae (Ware and Stephen 2006).

The first experiment, showing increasing responses with increasing release rates, even to unnaturally high release rates, would seem to be in contrast to the

context dose-dependent responses demonstrated by other MPP-beetle species such as grain beetles or bark beetles, in which aggregation responses are inhibited by high pheromone release rates. However, although the ecological details and contexts are different, there is nonetheless a common thread in how MPPs are utilized. The ecological and evolutionary advantages of MPP-usage are hypothesized to be related to the host substrate from which many pheromone-producing males call (Landolt 1997). Modelling of mate-attraction indicates that males will take the role of signal sender or receiver depending on which increases the odds of mate-finding, because males' fitness is limited by the total number of females with which they mate (Wyatt 2004; Yoshida and Iwasa 2013). Many MPP-species utilize scarce and ephemeral resources. Searching for such resources requires energy and also exposes the searcher to greater biotic and abiotic threats. Thus, if males call preferentially from host substrates and/or females respond preferentially to MPPs in the presence of HPVs – both scenarios common in cerambycid chemical ecology – MPPs would serve the dual purpose of increasing mating opportunities while simultaneously attracting unmated females to a suitable host for oviposition, increasing the fitness of both partners. This ecological scenario is likely shared by many MPP-species, and the variety of responses males and females can have to various indicators of density or pheromone release rate (including female-produced oviposition deterrents), support the theme of MPP

utilization being associated with HPVs, ephemeral resources, and optimal density tactics.

In summary, this study is the first to show that *pheromone emission* in a male-pheromone-producing cerambycid species can be influenced by density of conspecific males (Lemay et al. [2010] previously found a connection between density and calling behavior). The finding that *P. grandis* males decrease pheromone emission with increasing density supports the hypothesis that cerambycid populations utilize an optimal density strategy, at least in the context of pheromone emission. From a practical standpoint, this study also suggests that lure efficacy for monitoring MPP-cerambycids can be increased by simply increasing the release rate of the pheromones, because even unnaturally high release rates did not result in any indication of decreased trap captures. Future research efforts should prioritize examining MPP-cerambycids ethologically, specifically, to confirm whether males call from host substrates, how they distribute themselves on the host, the behavior of males and females once they have arrived on the host, and finally, female post-mating ovipositional behavior. By deepening our understanding of a single MPP-cerambycid's adult ecology, we can start to formulate more specific evolutionary hypotheses to test whether the main function of male-produced aggregation pheromones is for mate-finding, and more specific ecological hypotheses testing whether species using MPPs do indeed utilize optimal density strategies.

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Chapter 5: Fuscumol and fuscumol acetate as pheromone components of longhorn beetles (Coleoptera: Cerambycidae) of southern California in the subfamily Spondylidinae

Introduction

Cerambycid beetles are wood-boring insects that predominantly feed on woody plants as larvae. They have the potential to be ecologically and economically damaging when they are introduced into new habitats as invasive species (Haack 2006; Parry and Teale 2011; Haack et al. 2010). Conversely, native cerambycids are crucial components of forest ecosystems because they play a major role in the primary decomposition of woody material (Haack and Byler 1993; Grove 2002; Ulyshen 2016; Chen and Forschler 2016). Cerambycids also cull stressed and sickly trees in forest systems, maintaining baseline mortality and tree turnover (Manion and Griffin 2001; Teale and Castello 2011). Although not as speciose as other cerambycid subfamilies (Bezark 2016), beetles in the subfamily Spondylidinae are distributed globally and are integral components of many coniferous forest systems (e.g., Majka and Ogden 2010; Swift et al. 2010; Kundu 2015; Özbek et al. 2015; Monné 2016). However, there are several examples of ecologically disruptive invasive spondylidines, such as the brown spruce longhorn beetle *Tetropium fuscum* (F.), which was accidentally introduced into eastern Canada from Europe (Smith and Hurley 2000; Dearborn et al. 2016), and several invasive *Arhopalus*

species across Australasia (Wang and Leschen 2012). Spondylidines are also possible vectors of the nematode *Bursephelencus xylophilus* (Linit et al. 1983), the causative agent of the devastating pine wilt disease, as well as congeneric nematodes (Robertson et al. 2008) and other plant pathogens (Jacobs et al. 2003).

Relatively little is known of the chemical ecology of spondylidines. The first reported pheromone from the subfamily was produced by males and attracted both sexes of the invasive European *T. fuscum* and *Tetropium castaneum* (L.) and the native North American *Tetropium cinnamopterum* Kirby (Silk et al. 2007; Sweeney et al. 2010). The compound, (2*S*,5*E*)-6,10-dimethyl-5,9-undecadien-2-ol, was given the common name fuscumol, (Fig. 5.1). To date, this is the only confirmed pheromone identified from the subfamily. It has been shown to be potentially attractive to eight other spondylidine species (Hanks and Millar 2013; Sweeney et al. 2014; Collignon et al. 2016), and it has been field tested as a potential cerambycid pheromone in numerous other field trials in North America (Mitchell et al. 2011; Hanks et al. 2012), China (Wickham et al. 2012), and Australia (Hayes et al. 2016). Most spondylidine species infest conifers, and it has been shown that at least some species are attracted to lures based on terpenes and other host plant volatiles (HPVs; Chénier and Philogène, 1989; Sweeney et al. 2004; Miller 2006). Terpenes also have been shown to synergize attraction of some species to fuscumol, particularly when the terpenes are released at high rates (Sweeney et al. 2010; Collignon et al. 2016).

Fuscumol and its corresponding acetate [(*E*)-6,10-dimethyl-5,9-undecadien-2-yl acetate] were subsequently found to form a pheromone motif that is shared by a number of species in the subfamily Lamiinae (Mitchell et al. 2011; Hanks et al. 2012; Hanks and Millar 2013; Wickham et al. 2014; Wong et al. 2012; Hayes et al. 2016; Hughes et al. 2016; Meier et al. 2016). Fuscumol acetate was first found in volatiles from males of the South American lamiine *Hedypathes betulinus*, which produces (*R*)-fuscumol acetate, (*R*)- and (*S*)-fuscumol, and geranylacetone (Fonseca et al. 2010; Vidal et al. 2010). However, to date, field trials to determine which blend or subset of these components might be attractive have not been successful (P. Zarbin, pers. comm.).

Field screening trials in southern California's San Bernardino National Forest (SBNF) of a generic cerambycid pheromone blend that included racemic fuscumol, fuscumol acetate, 3-hydroxyhexan-2-one, 2-methylbutanol, and monochamol demonstrated its efficacy in attracting a variety of cerambycid species. Traps baited with this mixture of pheromones in combination with high release rate terpene lures caught six spondylidine and three lamiine species (Collignon et al. 2016 and RMC, unpublished data). Of these, three spondylidine and one lamiine species – *Monochamus clamator* (Leconte), which was likely attracted to the monochamol in the blend – were attracted in numbers that were significantly higher than controls (Collignon et al. 2016). Thus, the goal of the research described here was to follow up on these field screening results and identify the actual pheromones

of the three spondylidine species. The specific objectives of this research were to determine: 1) if fuscumol is necessary and sufficient for attraction of the three spondylidine species; 2) if fuscumol acetate is utilized by any of the attracted spondylidines, either alone or in combination with fuscumol; 3) if additional spondylidine and lamiine species would be attracted to lures containing fuscumol, fuscumol acetate, or a blend of the two.

Methods and Materials

Field assays testing fuscumol and fuscumol acetate Field bioassays were conducted at two sites approximately 0.5 km apart in the SBNF in San Bernardino Co., California, USA. The sites were near Jenks Lake (34°09'45.8"N 116°54'08.6"W) and are dominated by Ponderosa pine (*Pinus ponderosa* Douglas) and white fir (*Abies concolor* [Gordon]) (Pinales: Pinaceae), with some western black oak (*Quercus kelloggii* Newbury), canyon live oak (*Quercus chrysolepis* Liebm.) (Fagales: Fagaceae), big-cone Douglas-fir (*Pseudotsuga macrocarpa* [Vasey]) (Pinales: Pinaceae), and incense cedar (*Calocedrus decurrens* Torr.) (Pinales: Cupressaceae). Black cross-vane intercept traps (Alpha Scents, Portland OR, USA) coated with Fluon® (Graham et al. 2010) were hung on 1.5 m tall, L-shaped stands made from PVC pipe. Traps were placed 10-15 m apart in transects, with treatments initially assigned randomly to traps. Traps were checked twice weekly and their order was rerandomized at every check. Beetles were live trapped

so that they could be used for pheromone collection. Excess beetles were released at least 200 m from the field sites. Voucher specimens of all species have been deposited in the Entomology Museum at UC Riverside.

Two similar and sequential assays were run. For the first, the treatments consisted of a host plant volatiles blend (HPV) + ethanol as a positive control, and HPV and ethanol with racemic fuscumol, racemic fuscumol acetate, or racemic fuscumol + fuscumol acetate. The pheromones (Bedoukian Research, Danville CT, USA; 1 ml of a 50 mg/ml solution in isopropanol) were dispensed from 5 × 7.5 cm low-density polyethylene resealable bags (2 mil wall thickness; Fisher Scientific, Pittsburgh PA, USA). The HPV lure was a previously developed synthetic conifer volatiles blend (Table 5.1; Collignon et al. 2016) and was released from open-topped 25 ml glass jars (4.3 cm tall × 4.3 cm outer diameter × 3.1 cm opening), reloaded with 10 ml of conifer blend twice per week. Ethanol was released from 10 × 15 cm low-density polyethylene resealable bags (2 mil wall thickness; Fisher Scientific) loaded with 50 ml ethanol. Traps were deployed from 12 May through 24 June 2016 to target *Neospondylis upiformis* (Mannerheim). For the second assay, the conifer blend and ethanol lures were changed to a 2:1 (*S*):(*R*) α-pinene lure (same release device as for the conifer blend) without ethanol from 24 June through 3 August 2016 to target *Asemum nitidum* [L.]. These two HPV blends were most attractive to the respective species in previous research (Collignon et al.

2016). All lures were deployed at the center of the trap, so all components would be collocated in the same volatile plume.

Table 5.1. Synthetic blend composition for the host volatiles blend, with each compound's contribution expressed as milliliters per 100 ml. For chiral compounds, ratios of enantiomers are listed.

Compound	Enantiomeric Ratio	ml/100 ml	Source
α-pinene	2:1 (*S*:*R*)	17.2	Sigma-Aldrich
camphene	racemic	1	Sigma-Aldrich
β-pinene	pure (*S*)	17.2	Alfa-Aesar
myrcene	-	17.2	Acros Organics
3-carene	racemic	17.2	Sigma-Aldrich
limonene	2:1 (*R*:*S*)	17.2	Alfa-Aesar
1,8-cineole	-	4	Alfa-Aesar
borneol	pure (*S*)	2	Alfa-Aesar
camphor	2:1 (*S*:*R*)	2	Alfa-Aesar
4-allylanisole	-	1	Alfa-Aesar
(-)-*trans*-β-caryophyllene	-	4	Acros Organics

Identification of beetle-emitted volatiles Live beetles were sampled for pheromone emission individually or in groups of up to five beetles (Table 5.2). A total of seven aerations of *N. upiformis* with ponderosa pine sprigs were made, including some with 10% sugar water provided. Beetles were aerated in groups of one to five individuals of unknown sex. Two *Tetropium abietis* aerations were made, one with a single unsexed individual with a white fir sprig, and the other with two males and a single female, with wire mesh perches and 10% sugar water provided. Five *A. nitidum* with white fir; three of these samples were with individuals

identified as males, the other two with unsexed individuals. Aerations were conducted for as long as individuals were alive; no beetles were re-sampled. Most beetles were aerated until they died. The host substrates used are the preferred hosts of these species' populations in the SBNF (Linsley and Chemsak 1997). Host plant twigs were aerated under the same conditions as controls. Volatile collections were conducted in wide-mouth 500 ml Teflon containers (Thermo Scientific, Fisher Scientific; #24030250), with the screw-cap lids fitted with Swagelok bulkhead unions (Swagelok, Solon OH, USA) to connect inlet and outlet tubes. Air was pulled through the system by vacuum at 500 ml/min. Incoming air was cleaned by passage through granulated activated charcoal (14-16 mesh; Fisher Scientific). Volatiles were collected onto ~50 mg of thermally-desorbed activated charcoal (50-200 mesh; Fisher Scientific) held between glass wool plugs in a short piece of glass tubing. Aerations were conducted under ReptiSun 10.0 UVB lights (Zoo Med Laboratories Inc., San Luis Obispo CA, USA) with a 16:8 h L:D cycle. Volatiles were eluted from the charcoal with 1 ml dichloromethane (DCM).

Table 5.2. Aerations of spondylidine species. Individuals with unspecified sex were not sexed.

Species	# inds.	Host Substrate	Sugar Water	Pheromone Detected
N. upiformis	1	ponderosa pine	no	no
N. upiformis	1	ponderosa pine	no	no
N. upiformis	1	ponderosa pine	no	no
N. upiformis	4	ponderosa pine	no	no
N. upiformis	4	ponderosa pine	no	no
N. upiformis	5	ponderosa pine	no	no
N. upiformis	4	ponderosa pine	yes	no
A. nitidum	1♂	white fir	no	no
A. nitidum	1♂	white fir	no	(*S*)-fuscumol, geranylacetone
A. nitidum	1♂	white fir	no	no
A. nitidum	2	white fir	no	(*S*)-fuscumol, geranylacetone
A. nitidum	2	white fir	yes	no
Asemum caseyi	1♀	white fir	no	no
T. abietis	1	white fir	no	no
T. abietis	2♂,1♀	n/a (wire mesh perch)	yes	(S)-fuscumol
Arhopalus aspiratus	1	ponderosa pine	no	no
A. aspiratus	1♀	white fir	no	no

Extracts were analysed by coupled gas chromatography-mass spectrometry (GC-MS) with an Agilent 78020A gas chromatograph fitted with an autosampler and coupled to an Agilent 5977E mass selective detector (Agilent Technologies, Santa Clara CA, USA). The GC was equipped with a DB-5MS column (30 m × 0.25 mm ID × 0.25 micron film; Agilent). The oven temperature was programed from 40ºC/1 min, then increased 5ºC/min to 280ºC, final time 5 min; the injector and transfer line temperatures were set to 280ºC. One microliter aliquots of extracts were injected in splitless mode.

Chromatograms from insect aerations were checked against those from the ponderosa pine and white fir controls to locate insect-specific peaks. Compounds were identified by matching retention times and mass spectra with those of authentic standards of fuscumol, fuscumol acetate, and geranylacetone.

The absolute configuration of fuscumol acetate was determined by analysis of extracts on a GC-FID fitted with a Cyclodex-B chiral stationary phase column (30 m × 0.25 mm ID × 0.25 micron film; J&W Scientific, Folsom CA, USA). Analyses were conducted using a temperature program of 50ºC/1 min, then 5ºC/min to 220ºC, hold 20 min, with injector and detector temperatures of 150ºC and 250ºC, respectively. Racemic and enantiomerically enriched standards of fuscumol acetate were run under the same conditions. The (*S*)-enantiomer eluted first, and the two enantiomers were separated to baseline. The enantiomers of fuscumol do not resolve on this column, and so extracts containing fuscumol were first derivatized to the corresponding acetates before analysis. Samples were derivatized by adding 10 µl of 10% pyridine + 1% 4-dimethylaminopyridine solution in DCM to 100 µl of an aeration extract, followed by addition of 20 µl of 4.5% acetyl chloride in DCM, followed by vortexing, then stirring overnight. A standard of racemic fuscumol was derivatized as a control. The reaction was quenched by addition of 5 µl ethanol and stirring for 3 h, after which 0.5 ml of pentane was added, followed by addition of ~50 µl saturated aqueous sodium bicarbonate solution and vortexing. The top, organic layer was then transferred to a clean vial containing 5-10 mg

anhydrous sodium sulfate, vortexed and stirred for 1 h, and then transferred to a new vial. The esterified samples were run on the Cyclodex-B column as above to determine chirality.

Statistical Analyses For the field assays, none of the count data were homogeneous for variances as tested by Bartlett's test. Thus, data were analyzed with a max-*t* test (Herberich et al. 2010; Hothorn et al. 2008; Zeileis 2004), preceded by an ANOVA model calculated for the max-*t* test. This test is robust to departures from assumptions of normality and homogeneity of variances, a common occurrence with biological data.

Replicates for each experiment were based on both spatial and temporal replication, with temporal replicates equalling the number of times the traps were checked. Temporal replicates where no beetles of a given species were trapped in any trap – usually due to inclement weather or being outside the species' flight period – were not included in analyses for that species.

Results

Field assay testing racemic fuscumol and fuscumol acetate Five spondylidine species were trapped during the course of this research. Thirty-three *N. upiformis* (8 treatment replicates), 22 *T. abietis* (13 replicates), 4 *Asemum caseyi* [L.], and 1 *Asemum striatum* [L.] were trapped during the first half of the experiment, testing

the pheromones in combination with conifer blend volatiles and ethanol (Table 5.1; Collignon et al. 2016). Forty-seven *A. nitidum* (14 treatment replicates) were trapped during the second half of the experiment, testing pheromones in combination with a 2:1 blend of (*S*):(*R*) α-pinene. No lamiine species were trapped during the experiment.

There were no significant differences in the numbers of *N. upiformis* responding to any of the tested treatments (ANOVA: $F_{3,28} = 1.59$, $p = 0.21$; Fig. 5.1), but differences among treatments may have been obscured by the relatively low numbers of beetles caught. Based on my previous findings that *N. upiformis* responded strongly to high release conifer blend and a generic blend of pheromones (Collignon et al. 2016) and the trend of the data, it seems likely that there was a preference for the pheromone plus HPV treatments over the HPV-only positive control.

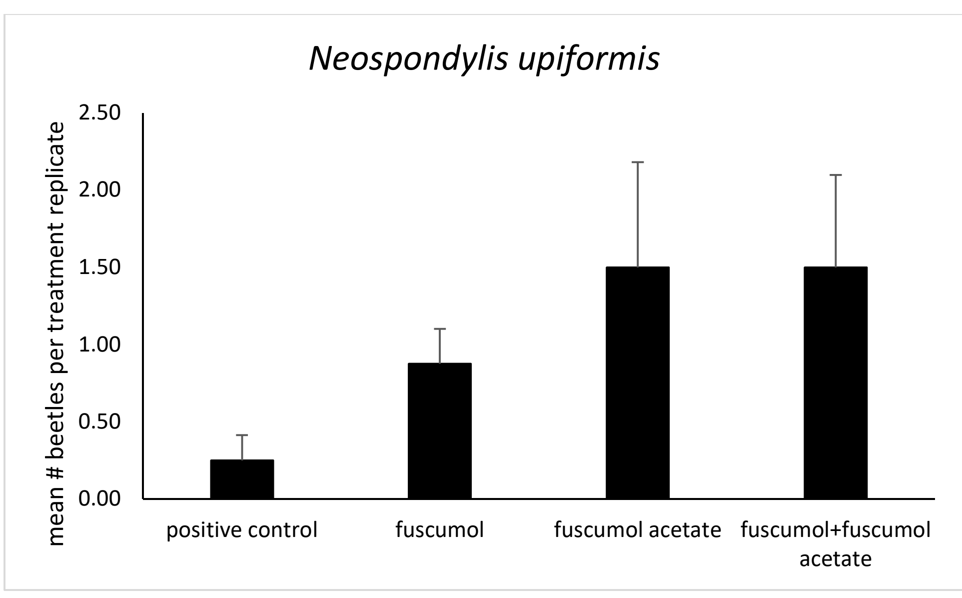

Fig. 5.1. Mean (± 1 SE) numbers of *N. upiformis* caught in traps baited with host plant volatiles (see Table 5.1) and ethanol, alone (positive control) or in combination with fuscumol, fuscumol acetate, or fuscumol + fuscumol acetate. There were no significant differences among treatments (max-*t* test, $p < 0.05$).

All *T. abietis* trapped during the experiment were caught during the first half of the experiment, and there were differences among the treatments ($F_{3,48} = 7.87$, $p = 0.0002$; Fig. 5.2). Beetles were attracted equally to the fuscumol and fuscumol + fuscumol acetate treatments, indicating that fuscumol was a likely pheromone component, and that fuscumol acetate was not inhibitory. Neither fuscumol acetate alone nor the host plant volatiles controls attracted any beetles.

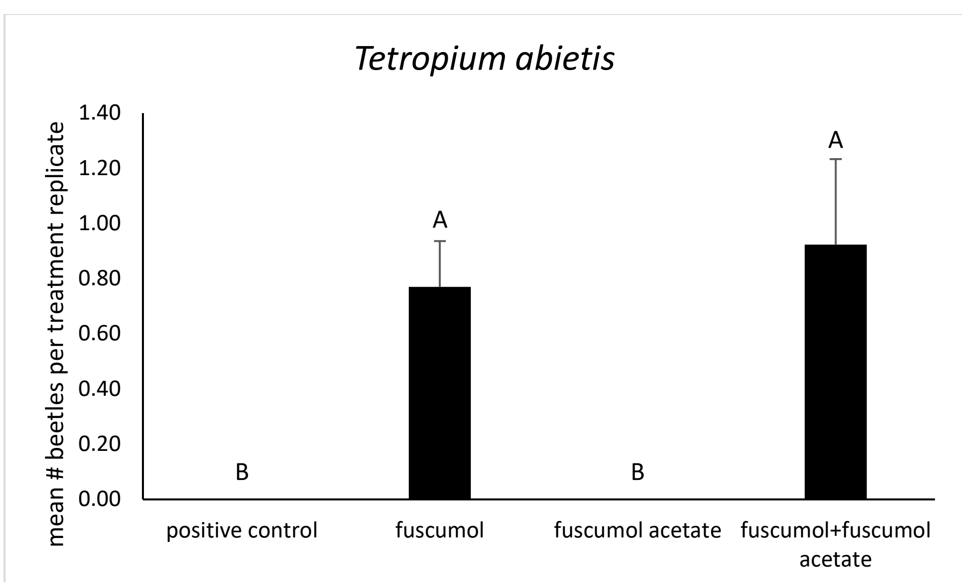

Fig. 5.2. Mean (± 1 SE) numbers of *T. abietis* caught in traps baited with host plant volatiles (see Table 5.1) and ethanol, alone (positive control) or in combination with fuscumol, fuscumol acetate, or fuscumol + fuscumol acetate. Means with different letters are significantly different (max-*t* test, $p < 0.05$).

During the second half of the experiment, using a 2:1 (*S*):(*R*) α-pinene blend as the host plant volatiles, there were significant differences among the responses of *A. nitidum* individuals to the different treatments ($F_{3,52} = 3.79$, $p = 0.016$; Fig. 5.3). However, the pattern of responses was unclear, in part because no treatments were significantly more attractive than the HPV control alone.

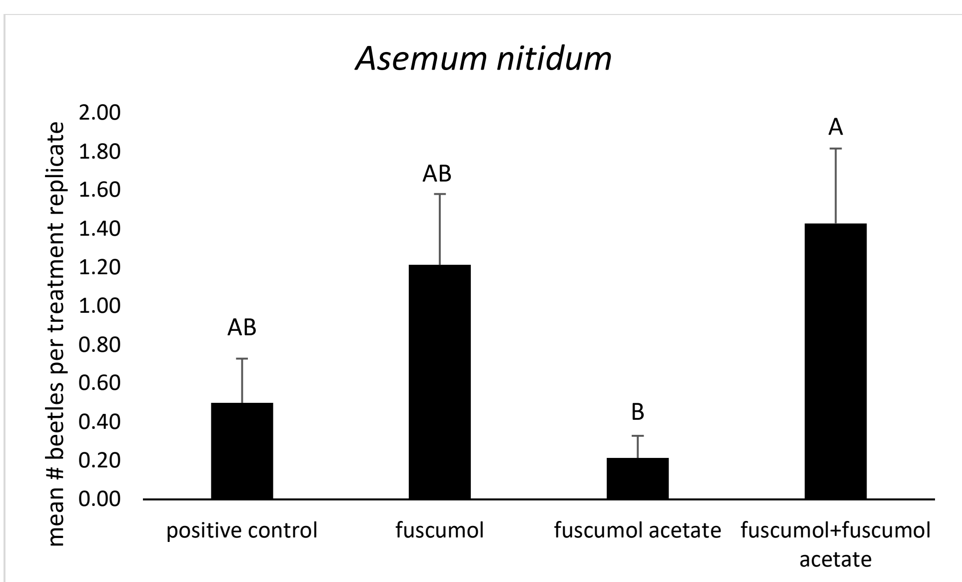

Fig. 5.3. Mean (± 1 SE) numbers of *A. nitidum* caught in traps baited with 2:1 (*S*):(*R*) α-pinene alone (positive control) or in combination with fuscumol, fuscumol acetate, or fuscumol + fuscumol acetate. Means with different letters are significantly different (max-*t* test, $p < 0.05$).

Identification of possible pheromone components In the analyses of aeration extracts from live beetles, two *A. nitidum* samples were found to contain (*S*)-fuscumol and geranylacetone – one from an aeration of a single male with white fir, and one from an individual of undetermined sex with white fir. One *T. abietis* sample from a mixed-sex group of beetles contained (*S*)-fuscumol. Fuscumol of undetermined chirality had previously been detected in an aeration of a male *T. abietis* (J. Millar and A. Ray, unpublished data). No fuscumol was detected in ponderosa pine or white fir aerations. No *N. upiformis* aerations contained compounds that were not also found in ponderosa pine aerations.

Discussion

Individuals of the three main responding species in this study – *N. upiformis*, *T. abietis*, and *A. nitidum* – had been previously caught in field bioassays testing host plant volatiles in combination with a generic blend of cerambycid pheromones which included fuscumol, fuscumol acetate, monochamol, 3-hydroxyhexan-2-one, and 2-methylbutanol (Collignon et al. 2016). The work described here was intended to clarify which components of the generic bait might actually be pheromone components for these three species. For *N. upiformis*, no clarification was possible because relatively low numbers of beetles were caught, and there were no significant differences among any of the lures tested. In addition, for reasons unknown, the survivorship of these beetles was poor, with many beetles dying in the few hours between collecting them from traps and setting them up in aeration chambers, and the majority dying within 24 h of collection of headspace volatiles. Consequently, none of the seven aeration extracts prepared from these beetles had detectable quantities of any insect-produced compounds.

For *T. abietis*, the field bioassays and the analytical results provided support for (*S*)-fuscumol being the major and possibly only pheromone component for this species, with fuscumol being identified from a mixed-sex aeration, and in a sample from a male that was collected and analyzed several years ago. Based on these data and precedents from other members of the genus (*T. fuscum*, *T. cinnamopterum*, and *T. castaneum*; Silk et al. 2007; Sweeney et al. 2010), it seems likely that (*S*)-

fuscumol is male-produced. The field bioassay data support this conclusion, with fuscumol in combination with HPVs being significantly attractive, whereas fuscumol acetate with HPVs, or HPVs alone, were completely unattractive. It is also noteworthy that fuscumol acetate was neither synergistic nor inhibitory, possibly because fuscumol acetate may not be a pheromone component for related spondylidine species. That is, to date, there is no evidence that spondylidine species produce or are attracted to this compound (reviewed in Hanks and Millar 2016).

For *A. nitidum*, aeration samples containing detectable amounts of insect-produced compounds were obtained only from one male and one specimen of undetermined sex. Geranylacetone and (*S*)-fuscumol were detected in both aerations. Thus, as with *T. abietis*, it is likely that fuscumol is male-produced, but it is impossible to say conclusively until replicated aerations from males and females can be compared directly. Furthermore, the field bioassays provided no further information to help clarify the pheromone because there were no significant differences between the fuscumol-containing treatments and the host plant volatiles control.

The role of geranylacetone as a possible pheromone component for *A. nitidum* is unclear. Geranylacetone has been shown to be a biosynthetic precursor to fuscumol in both lamiines (*H. betulinus*; Zarbin et al. 2013) and spondylidines (*T. abietis*; Mayo et al. 2013), and it has been detected in aeration samples of males of the spondylidine *Arhopalus productus* (J. Millar, unpublished data) and the

lamiine *Moneilema semipunctata* (Appendix). Further testing will be required to determine if it is simply a pheromone precursor, or if it is indeed a functional pheromone component in species that produce it.

We had expected that fuscumol and fuscumol acetate, either individually or as a blend, and released with host plant volatiles, would be more widely attractive to cerambycids in the San Bernardino National Forest, particularly to lamiine species. One possible explanation for the general lack of response may be that the bioassays used racemic fuscumol and fuscumol acetate, whereas recent results suggest that lamiine species may use individual enantiomers, or even nonracemic blends of enantiomers of these two compounds in their pheromone blends, with off-ratios sometimes being antagonistic (Hughes et al. 2016; Meier et al. 2016). Additional testing of the pure enantiomers of fuscumol, or nonracemic blends, may help to clarify the pheromone chemistry of the three species in this study, as well as possibly attracting additional lamiine and spondylidine species.

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Chapter 6: Conclusion

The past decade and a half has been a very productive period for elucidating cerambycid chemical ecology, with a number of pheromones and pheromone motifs identified that attract ~200 or more species in this large coleopteran family. This expansion of knowledge has provided tools for monitoring and detecting the species already shown to be attracted, but also probably for hundreds more in different areas of the world which have not yet been sampled. We have also begun to understand some of the means by which cerambycids utilize host plant volatiles alone or in combination with pheromones for host- and mate-finding, and how species may partition or exploit pheromone signals. The work described in my dissertation has contributed to this understanding, while also suggesting numerous new ideas and questions for follow-up research.

My initial goal was to explore how host-plant volatiles might contribute to attraction of cerambycids to semiochemical lures (Chapter 2). My results generally supported scattered results from the literature which suggested that conifer-infesting cerambycid species are usually most strongly attracted to pheromones when co-released with conifer volatiles. Interestingly, there was a difference in response between species that preferentially infest pines versus species that prefer white fir: *Neospondylis upiformis* and *Monochamus clamator* are both pine-infesting species and both were most attracted to pheromones co-released with a complex synthetic conifer blend, whereas *Asemum nitidum* and *Asemum striatum*

are white fir-infesting species and were most strongly attracted to pheromones plus the single host compound α-pinene. It is likely that the *Asemum* species are more attracted to α-pinene than to the complex conifer blend due to α-pinene's correlation with stress for many conifer species (Matson and Haack 1987), because similar results were also found by previous researchers for *A. striatum* (Chénier and Philogène 1989; Sweeney et al. 2004). The results of these studies suggested that attraction might be increased by α-pinene plus ethanol, another stress cue emitted by trees, further indication that these species preferentially attack stressed conifers. However, my study did not show this trend of increased attraction with co-released ethanol.

Results for the angiosperm-infesting species were less informative. In retrospect, this is possibly due to how I developed the synthetic oak volatiles blend, by sampling volatiles from chipped branches, with the resulting ground wood and foliage being richer in volatiles – both quantitatively and qualitatively – than either the whole twig pieces initially used in aeration samples with the beetles, or in comparison to a pilot study of oak trees sampled in the field (unpub. data). Thus, the synthetic oak volatiles blend developed for the research in Chapter 2 may not have represented the correct physiological host condition (Niinemets 2010) for oak-infesting species. A systematic, replicated volatile sampling of experimentally stressed versus unstressed canyon live oak trees, followed by harvesting of the tree, and rearing out the cerambycid species attracted to the two different physiological

host states could be very informative. Such an experiment would help define host condition preferences for many of the species studied here, while also better defining the volatiles associated with stress in oaks and subsequent likelihood of attack. If this experiment were taken a step further, different regions of the tree could be separated for rearing, to determine if species are partitioning space on the host as a possible means of reducing interspecific competition, especially among species with overlapping pheromone components, preferred hosts, and similar seasonal activity periods.

In the second portion of my dissertation research, I explored means other than segregation on the host by which cerambycid beetles using overlapping pheromone components might partition their use. I specifically tested diel and pheromone blend partitioning (Chapter 3). Diel partitioning effectively isolated at least one species – the crepuscular/nocturnal *Brothylus gemmulatus* – from three diurnal species – *Phymatodes obliquus*, *Neoclytus modestus*, and *Xylotrechus albonotatus* – that utilize the same pheromone component, (*R*)-3-hydroxyhexan-2-one. Host plant volatiles may play a role in partitioning these three diurnal species, as I discuss in that chapter.

Phymatodes grandis, which had previously been shown to be attracted to 3-hydroxyhexan-2-one but not produce it (Hanks et al. 2007), was not attracted to 3-hydroxyhexan-2-one in my study. Males of the species, however, were attracted to a full blend of the emitted compounds released by the smaller *P. obliquus*. I

hypothesize this difference may be due to differing competitive factors at the San Bernardino Mountains site versus the Santa Rosa Plateau site used by Hanks et al. (2007). A concurrent testing of blends of 3-hydroxyhexan-2-one by itself and in blends with the other tested pheromones at both sites would help clarify whether *P. grandis* is in fact responding differentially to heterospecific kairomones between the two sites, by not only replicating the species attraction patterns to the blends, but also by replicating the confirmed presence or absence of potential competitors – *P. obliquus* and *B. gemmulatus* – at both sites. Further, it may be worthwhile to test 2,3-hexanedione in this experiment as well, because this compound may actually be produced by some species, rather than being just an artefact due to oxidation of 3-hydroxyhexan-2-one during collection or analysis of extracts. In addition, experiments using live males as pheromone sources could help to clarify how strongly the various species are co-attracted to one another. The number of live males being used as lures could be important, however, as indicated by my results on the effect of density on per capita pheromone emission (Chapter 4).

The last major portion of my dissertation research examined whether the density and dose (= release or emission rate) effects seen in emission and response to other beetle taxa that use male-produced aggregation sex-pheromones were being utilized by cerambycids as well, to specifically begin to determine if cerambycid species are employing optimal density strategies (Chapter 4). Response

of four species – *Brothylus conspersus*, *B. gemmulatus*, *P. grandis*, and *X. albonotatus* – increased with increasing release rates of two cerambycine pheromones – even well past biologically relevant release rates of these pheromones; a plateau or saturation effect was only seen for a fifth species, *P. obliquus*. This agrees with what has been reported previously for *Neoclytus* species (Lacey et al. 2004, 2007), but not with spondylidine *Tetropium* species, where increasing release rates of their pheromone did not have a significant effect on attraction (Sweeney et al. 2010). This may relate to the different ecological contexts under which these species are working. More ethological studies to determine how these species interact with conspecifics and heterospecifics at host trees would be useful to place these results into a better ecological framework from which to ask further questions regarding potential optimal density strategies of cerambycids.

As discussed and hypothesized in Chapter 4, host plant volatiles may lower threshold responses to pheromones, as exemplified by the conifer-infesting *X. albonotatus* which was only trapped with the highest two release rates (~359 μg/h and ~719 μg/h of racemic 3-hydroxyhexan-2-one), even though in previous experiments (Chapter 2), the species was never trapped with pheromone baits alone and was most attracted to pheromone lures co-deployed with high-release conifer volatiles. If it is the case that host plant volatiles lower thresholds for pheromone responses – especially amongst conifer-infesting cerambycid species, where the role of host volatiles is more firmly established – this could explain why Sweeney

et al. (2010) did not find a difference among pheromone release rates, because the co-released synthetic spruce blend may have ameliorated any graded response that the *Tetropium* species might have exhibited to the different pheromone release rates. This is highly speculative, but testing varying release rates of known attractive host plant blends and pheromones would shed some clarity on the apparently different responses of cerambycine and spondylidine species to increasing pheromone release rate.

This hypothesis also may explain results from my final set of experiments, attempting to identify the pheromones used by the spondylidine species of the San Bernardino Mountains (Chapter 5). (*S*)-Fuscumol was tentatively identified as a male-produced pheromone for the spondylidine species *Asemum nitidum* and *Tetropium abietis*, based on collection of headspace volatiles from live beetles and field responses to various blends of racemic fuscumol and fuscumol acetate in combination with host plant volatiles. However, the pheromone chemistry of the related *Neospondylis upiformis* remains unclear, in part because of a lack of strong discrimination among various pheromone blend treatments. From the experiments in Chapter 2 and 5, we know that *N. upiformis* is not strongly attracted to the synthetic conifer blend by itself, but responds well to conifer volatiles co-released with pheromones. Perhaps the racemic fuscumol and fuscumol acetate are inexact pheromone mimics if the species utilizes specific enantiomers of these compounds

as its pheromone, and the conifer volatiles are masking nuanced attraction or repellence to non-pheromone enantiomers.

Overall, one obvious extension of the work described here would be to systematically test the enantiomers of the male-produced pheromones for the subfamilies Cerambycinae, Spondylidinae, and Lamiinae, for those pheromones which are chiral. There are an estimated 13 species of Spondylidinae, 90 species of Cerambycinae, and 21 species of Lamiinae in the San Bernardino National Forest (I. Swift, pers. comm.). Whereas not all of these species occur at the Jenks Lake site where most of this testing was done, and whereas there are innumerable other factors that may influence attraction to pheromone-baited traps – such as vertical segregation of species from the ground to the canopy top, trap design and floral volatiles attractive to floral-feeding cerambycines – it nonetheless seems likely that we are missing some species by testing only racemic pheromones. So far, four spondylidines, seven cerambycines, and a single lamiine species have been attracted in significant numbers to specific lures of defined composition, indicative of actual attraction and not simply by-catch. However, now that it has been demonstrated that non-pheromone stereoisomers for a given species may strongly antagonize attraction (Hanks et al. 2012; Hanks and Millar 2013; Mitchell et al. 2015; Hughes et al. 2016; Meier et al. 2016), it seems likely that at least some novel species may be utilizing these pheromone motifs, but which have been repelled by non-pheromonal enantiomers in the racemic materials used in lures to date. Such

field trials could include the pure stereoisomers of cerambycine-produced compounds such as 2,3-alkanediols, 3-hydroxyalkan-2-one, 2-hydroxyalkan-3-one, and 2-methylbutan-1-ol. They could also include the non-enantiomeric 2,3-hexanedione and further tests of chiral acetoin. Further, testing the enantiomers of the spondylidine and lamiine pheromones fuscumol and fuscumol acetate, and the possible pheromone component geranylacetone could prove fruitful, especially in light of the lack of attraction of lamiines to baits with racemic fuscumol and fuscumol acetate, despite other studies finding these compounds to be highly attractive to lamiine species (e.g., Mitchell et al. 2011). Ideally, these pheromones and the various blends of them would be tested alone and in combinations with host plant volatiles. These trials would serve to clarify and further elaborate many of the results reported in this thesis, and more broadly, provide a better understanding of the interplay of pheromones and host plant volatiles in the chemical ecology of the Cerambycidae.

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Appendix 1: Partially completed pheromone identifications for several longhorn beetle species.

The purpose of this appendix is to provide information on progress made on the identification of the pheromones of five species of cerambycids, but for which the pheromone candidates are either not yet fully identified or have not been fully tested for biological activity.

***Moneilema semipunctatum* Leconte and *Coenopoeus palmeri* (Leconte)**

Both *Moneilema semipunctatum* and *Coenopoeus palmeri* are flightless cerambycids in the subfamily Lamiinae. Both species feed as adults on cholla (*Cylindropuntia*; Caryophyllales: Cactaceae), and larvae feed internally in the base and roots of the plant. The adults of *M. semipunctatum* are nocturnal.

Methods

Insects and field site All *M. semipunctatum* and a single *C. palmeri* were hand-collected in the summers of 2011 and 2012 from cholla in Anza-Borrego State Park in San Diego County, an hour or so after sundown, when beetles become active. The area is dominated by cholla, desert agave (*Agave deserti*; Asparagales: Asparagaceae), and catclaw acacia (*Senegalia greggii*; Fabales: Fabaceae). Both female and male *M. semipunctatum* beetles can be found at the tips of cholla after dark. Infrequently, beetles were found walking between cholla plants. Mating pairs were always found on the top of cholla. Very few (less than five) beetles were found during two visits in the summer of 2013.

Collection and identification of possible pheromones Beetles were held individually in glass jars with cholla for approximately 12 h between field collection of the specimens and starting headspace collections from them. Volatiles were sampled by aerating single female or male beetles in 250 ml wide-mouth glass

canning jars with the lid replaced with a 1/16” thick Teflon sheet and fitted with Swagelok bulkhead unions (Swagelok, Solon OH, USA) to connect inlet and outlet tubes. Beetles were provided cholla as a perch and food source during sampling, and cholla only controls were taken as well. Aerations were conducted under ReptiSun 10.0 UVB lights (Zoo Med Labs. Inc., San Luis Obispo CA, USA) with a 16:8 h L:D cycle. Air was pulled through the aeration chambers by vacuum at 250 ml/min. Incoming air was cleaned by passage through granulated activated charcoal (14-16 mesh, Fisher Scientific). Volatiles were collected on ~50 mg of thermally-desorbed activated charcoal (50-200 mesh; Fisher Scientific) held between glass wool plugs in a short piece of glass tubing. Volatiles were eluted from the charcoal with ~1 ml dichloromethane (DCM).

One microliter sample aliquots were analysed with an HP 6890 gas chromatograph (GC) coupled to an HP 5973 mass selective detector (GC-MS; Hewlett-Packard, now Agilent, Santa Clara CA, USA). The GC was fitted with a DB-17 column (30 m × 0.25 mm ID × 0.25 micron film; J&W Scientific, Folsom CA, USA), which was programmed from 40ºC/1 min, then increased 5ºC/min to 280ºC, hold 5 min. The injector and transfer line temperatures were set to 280ºC, and injections were done in splitless mode. All spectra were taken in full scan mode with electron impact ionization (70 eV).

Chromatograms from insect aerations were checked against those from the cholla controls to locate insect-specific peaks. Compounds were identified by

matching retention times and mass spectra with those of authentic standards of fuscumol (= (*E*)-6,10-dimethyl-5-9-undecadien-2-ol), fuscumol acetate, and geranylacetone.

The absolute configuration of fuscumol acetate was determined by analysis of extracts on a GC with a flame ionization detector (FID) fitted with a Cyclodex-B chiral stationary phase column (30 m × 0.25 mm ID × 0.25 micron film; J&W Scientific, Folsom CA, USA). Analyses were conducted using a temperature program of 50ºC/1 min, then 5ºC/min to 220ºC, hold 20 min, with injector and detector temperatures of 150ºC and 250ºC, respectively. Racemic and enantiomerically enriched standards of fuscumol acetate were run under the same conditions. The (*S*)-enantiomer eluted first, and the two enantiomers were separated to baseline. The enantiomers of fuscumol do not resolve on this column, and so extracts containing fuscumol were first converted to the corresponding acetates before analysis. Samples were derivatized by adding 10 µl of 10% pyridine solution in DCM to 100 µl of an aeration extract, followed by addition of 20 µl of 4.5% acetyl chloride in DCM, followed by vortexing, then stirring overnight. A standard of racemic fuscumol was derivatized as a control. The reaction was quenched by addition of 5 µl ethanol and stirring for 3 h, after which 0.5 ml of pentane was added, followed by addition of ~50 µl saturated aqueous sodium bicarbonate solution and vortexing. The top, organic layer was then transferred to a clean vial containing 5-10 mg anhydrous sodium sulfate, vortexed and stirred for 1 h, and then

transferred to a new vial. The esterified samples were run on the Cyclodex-B column as above.

Gas chromatography-electroantennogram detection assays (GC-EAD) GC-EAD analyzes were conducted on an HP 5890 Series II GC fitted with a DB-17 column (30 m × 0.25 mm ID × 0.25 micron film; J&W Scientific) programmed from 50°C/1 min, then increased 5°C/min to 250°C, hold 10 min. The injector temperature was 250°C, and injections were made in splitless mode. A glass X-cross split the effluent equally between the FID and the EAD, with helium being added through the fourth arm of the cross as makeup gas. The column effluent was directed into a humidified air stream that then was directed over an antennal preparation, which was made by excising the distal three to five segments of a live beetle's antenna and then removing the tip of the antenna with a razor blade to provide electrical contact. The antennal section was suspended between two saline-filled (7.5 g NaCl, 0.21 g $CaCl_2$, 0.35 g KCl, and 0.20 g $NaHCO_3$ in 1 L Milli-Q purified water) glass capillary electrodes, with 0.2 mm diameter gold wire connections between the electrodes and a custom-built electroantennogram amplifier. The signals from the GC and the EAD were recorded simultaneously using Peak-Simple software (SRI International, Menlo Park, CA, USA).

Trapping bioassays Trapping was conducted at the site at Anza-Borrego where the beetles had been collected. Traps consisted of a Lindgren trap funnel with a one liter container affixed to the bottom. Traps were deployed by burying them up to the edge of the funnel, so it was flush with the ground. Two experiments were run. The first tested *Moneilema-* versus *Coenopoeus*-produced compounds, and a second tested the three possible combinations of the two *Moneilema*-produced compounds. Compounds tested included racemic fuscumol, racemic fuscumol acetate, and geranylacetone (Bedoukian Research, Danville CT, USA) dissolved in 2-propanol at a concentration of 50 mg/ml for the two racemic compounds, and 25 mg/ml for geranylacetone. The pheromones were dispensed from 5 × 7.5 cm low-density polyethylene resealable bags (2 mil wall thickness; Fisher Scientific, Pittsburgh PA, USA), with all pheromones being tested for a given treatment dissolved in the same solution, and 1.5 ml of the solution being used in each lure.

The first experiment had three treatments: 1) fuscumol + geranylacetone; 2) fuscumol acetate; and, 3) blank. The lures were draped in the funnel for the first experiment, rather than being suspended above it. Four replicates were deployed in a transect on 6 August 2012, with the traps checked on 19 August 2012.

The second experiment used lures suspended above the funnel on wire hangers and had the following treatments: 1) fuscumol; 2) geranylacetone; 3) fuscumol + geranylacetone; and, 4) blank control. Five spatial replicates were

deployed in a transect on 19 August 2012, and traps were checked 26 August and 6 September 2012.

Results

Identification of likely pheromone compounds Volatile samples were collected from female (n = 3) and male (n = 9) *M. semipunctatum* held with cholla substrates. Of these, 7 aerations from males contained (*S*)-fuscumol and geranylacetone, whereas samples of volatiles from female *M. semipunctatum* or cholla controls did not contain these compounds.

Antennae from male *M. semipunctatum* responded to racemic fuscumol and geranylacetone in GC-EAD tests (n = 2). Antennae of females responded predominantly to racemic fuscumol, but fuscumol and geranylacetone elute close to each other, and the small antennal response to geranylacetone appeared as a shoulder on the response to fuscumol in EAD analyses (n = 3).

A single sample of volatiles from a male *Coenopoeus palmeri* was taken and found to contain (*S*)-fuscumol acetate, based on matching its retention time and mass spectrum with those of a known standard on a DB-17 column, and its retention time with that of a known standard on a Cyclodex-B chiral stationary phase GC column.

Trapping bioassays A total of seven *M. semipunctatum* were trapped in the first experiment (Table A1), and five were trapped in the second experiment (Table A2).

Table A1. Total number of *Moneilema semipunctata* trapped in pheromone baited pitfall traps in the first field experiment deployed August 6-19, 2012.

Treatment	female	male	sex not determined
fuscumol + geranylacetone	1	1	2
fuscumol acetate	0	0	2
blank control	0	1	0

Table A2. Total number of *Moneilema semipunctatum* trapped in pheromone- baited pitfall traps in the second experiment deployed August 19-September 6, 2012. The number before the slash represents the beetles trapped at the first trap check, and the number after the slash the beetles trapped during the second trapping period.

Treatment	female	male	sex not determined
fuscumol	0 / 0	0 / 3	1 / 0
geranylacetone	0 / 0	0 / 0	0 / 0
fuscumol + geranylacetone	1 / 0	0 / 0	0 / 0
blank control	0 / 0	0 / 0	0 / 0

Discussion

The likely pheromone compounds of these two species were readily identified because a number of other species in the subfamily Lamiinae produce and/or are known to be attracted to fuscumol, fuscumol acetate, and geranylacetone (reviewed in Hanks and Millar 2016). The pilot assay suggested that fuscumol and/or geranylacetone are pheromone components of *M. semipuntatum*, with only

one of 13 beetles trapped in an unbaited control trap. However, overall trap catches were low, and so further testing of the compounds and the enantiomeric forms of fuscumol will be required to properly elucidate the pheromone blend. If the male-produced compounds of either of these two species are verified to be attractant pheromones, this will be the first evidence of volatile pheromone use by a flightless cerambycid species. Y-olfactometer assays may be useful to initially assess activity, because beetles must be orienting to pheromones by walking in natural settings. Field bioassays should consider whether pitfall traps are the best trap design, because there was considerable by-catch in the traps (such as *Eleodes* spp. stink beetles (Coleoptera: Tenebrionidae), tarantulas, scorpions, lizards, and a mouse). Traps deployed on the stalks of cholla would probably be more species specific, and would also assess whether the pheromone attracts beetles up into the cholla. Trail pheromones (Hoover et al. 2014) might also be utilized. Due to the structural complexity of cholla, and the flightless nature of the beetles, it seems likely that the species would use shorter-range, less volatile compounds for mate attraction, as well as the volatile long-range fuscumol-based compounds.

***Centrodera spurca* (Leconte)**

Adult *Centrodera spurca* (subfamily Lepturinae) are nocturnal and thought to feed on pine pollen, whereas the larvae feed in the roots of dying or dead angiosperm and conifer species, and pupate in earthen cocoons underground (Leech 1963).

Methods

Insects and field site Beetles were collected in black cross-vane intercept traps (Alpha Scents, Portland OR, USA) baited with ethanol or red wine + vinegar. Traps were coated with Fluon® (Graham et al. 2010) and hung on 1.5 m tall, L-shaped stands made from PVC pipe. Beetles were also collected by nocturnal attraction to an ultraviolet light. Beetles were collected near Jenks Lake in the San Bernardino National Forest (34°09'45.8"N 116°54'08.6"W; San Bernardino Co., CA). The site is dominated by Ponderosa pine (*Pinus ponderosa* Douglas) and white fir (*Abies concolor* [Gordon]) (Pinales: Pinaceae), with some western black oak (*Quercus kelloggii* Newbury), canyon live oak (*Quercus chrysolepis* Liebm.) (Fagales: Fagaceae), big-cone Douglas-fir (*Pseudotsuga macrocarpa* [Vasey]) (Pinales: Pinaceae), and incense cedar (*Calocedrus decurrens* Torr.) (Pinales: Cupressaceae).

Preparation and analysis of extracts Volatiles were collected by aerating single female or male beetles in 250 ml wide-mouth glass canning jars as described above. Females and males were separately aerated on both white fir and canyon live oak,

because the larval host is not well-defined. Later aerations included ad libitum access to 10% sugar water and a piece of wire mesh to perch on, because presence of host material did not seem to influence likelihood of pheromone emission, and the sugar water improved survivorship of aerated beetles. Extracts were analysed by GC-EAD and GC-MS as described above.

In an attempt to obtain a mass spectrum of a female-produced compound that elicited a consistent response from antennae of males in GC-EAD analyses, five extracts from females were pooled for liquid chromatography fractionation. The methylene chloride solvent was first replaced with a nonpolar hydrocarbon solvent by concentrating the pooled extract under a stream of nitrogen almost to dryness, reconstituting with ~100 µl pentane, blowing down again, and reconstituting with ~100 µl hexanes. The resulting hexane solution was fractionated on 100 mg of pentane-wetted silica gel in a Pasteur pipette, eluting with 125 µl aliquots of 100% pentane, 95:5, 90:10, 80:20, and 50:50 pentane:ether, and 100% ether. The resulting fractions were concentrated to a few microliters, and reanalyzed by GC-EAD and GC-MS to determine which fraction(s) contained the active compound.

Results

A trace compound found exclusively in samples from *C. spurca* females elicited strong antennal responses from males (Fig. A1); this compound was present in

samples of volatiles from females regardless of the host substrate or access to sugar water. The Kovat's index of the compound was 2005 on DB-17, and 2166 on DB-WAX, indicating that it was of relatively low polarity. Antennae of females did not respond to this compound.

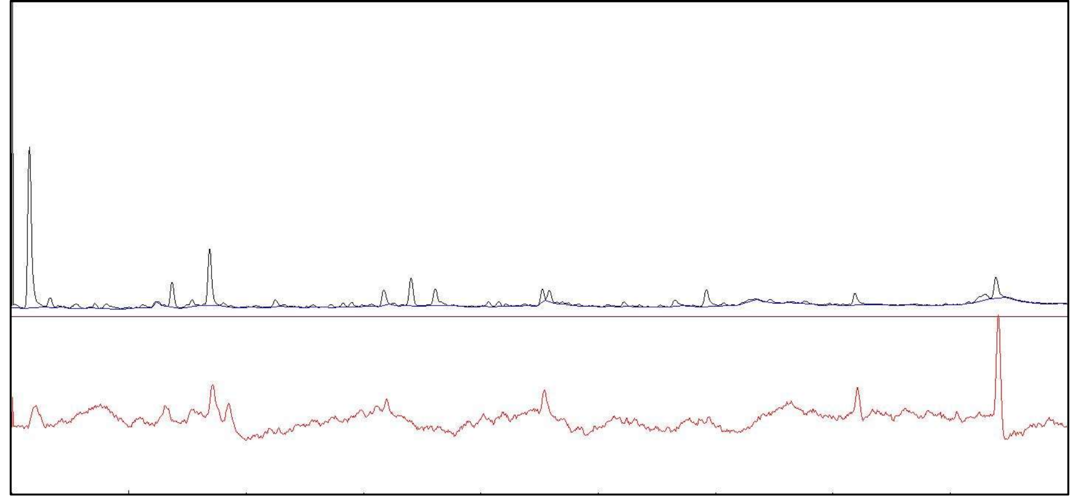

Fig. A1. GC-EAD analysis of headspace volatiles collected from a female *Centrodera spurca* tested with antenna from a male. The top trace is the gas chromatogram, and the bottom trace shows the response from the antenna of a male beetle, indicating one large response. The smaller responses were to unidentified compounds that were not consistently found in samples of volatiles from females.

The compound which elicited responses from the antennae of male beetles was too low in abundance to provide useable mass spectra, and was only detectable by GC-EAD with antennae of males. Pooling and fractionating several headspace samples from females still did not result in a detectable GC-MS peak. The active compound eluted in the 80:20 and 50:50 pentane:ether liquid chromatography fractions, indicating that it was of moderate polarity, as previously suggested by the relatively small difference in its Kovat's indices on medium polarity and polar GC

columns. Due to the very small amounts present, it was not possible to obtain any further information about the active compound.

***Paranoplium gracile* (= *imbele* (Leconte)) and *Eudistenia costipennis* Fall**

Both of these cerambycine species are in the tribe Oemini and are known to infest California live oaks (Linsley 1962). *Paranoplium gracile* is present in the lowland areas around Riverside, whereas *E. costipennis* is present in the San Bernardino Mountains.

Methods

Insects and field site Adult *P. gracile* were caught in a compact fluorescent bulb light trap deployed in a copse of Eucalyptus trees (*Eucalyptus* spp.; Myrtales: Myrtaceae) at the UC Riverside Agricultural Operations fields; two individuals were also hand-collected at lights at nearby residences. Beetles were caught between 23 July and 10 August in 2015, and throughout July and August in 2016. *Eudistenia costipennis* adults were trapped as by-catch in black cross-vane intercept traps set up for other experiments near Jenks Lake in the San Bernardino National Forest (see description above for *Centrodera spurca*); there was no obvious pattern of attraction to any of the lures being used in those experiments.

Preparation and analyses of extracts Beetles were aerated individually, in pairs, or in groups of three to four, with a wire mesh perch and sugar water; sugar water controls were also aerated. Beetles were sexed based on my previous experience sexing cerambycine individuals, whereby light squeezing of the abdomen causes the bifurcated ovipositor of the female to be extruded, and by sexual dimorphism in antennal length. The sexes were aerated separately. Beetles were aerated using the conditions described above, with the exception that beetles were held in wide-mouth 500 ml Teflon containers (Thermo-Fisher Scientific; #24030250), with the screw-cap lids fitted with Swagelok bulkhead unions (Swagelok, Solon OH, USA) to connect inlet and outlet tubes, rather than in glass chambers. Air was pulled through the chambers by vacuum at 500 ml/min. All other conditions were as described previously.

One microliter aliquots of the samples were analysed on an Agilent 78020A GC coupled to an Agilent 5977E MSD (Agilent Technologies, Santa Clara CA, USA) fitted with a DB-5MS column (30 m × 0.25 mm ID × 0.25 micron film; Agilent). The temperature program was 40ºC for 5 min, increased 5ºC per min to 280ºC and then stop. The injector temperature was set to 150ºC. Compounds were tentatively identified by matches with database spectra in the National Institute of Standards (NIST11) mass spectral library, with five identifications confirmed with authentic standards, as follows: α-terpinene (Aldrich Chemical, Milwaukee WI), *p*-cymene (Aldrich Chemical), dehydro-*p*-cymene (Bedoukian Research, Danbury

CT), *p*,α,α-trimethylbenzyl alcohol (Aldrich Chemical), and *p*-mentha-1,3-dien-8-ol (synthesized as described by Roy et al. (1995)).

To help confirm that the main compounds in the extracts shared the same carbon skeleton, an aliquot of an extract (~50 μl) was hydrogenated to remove all nonaromatic C=C bonds. Thus, the aliquot was diluted with ~100 μl pentane, ~1-2 mg of 5% Pd on carbon was added, and the mixture was stirred under hydrogen atmosphere for 1 h. The mixture was then filtered through a plug of celite, rinsing with pentane, and the filtrate was concentrated and analyzed by GC-MS. Standards of ~1 μl of α-terpinene and α-terpineol in hexane were reduced under the same conditions to provide standards of hydrocarbons and alcohols respectively, with the same 1-isopropyl-4-methylcyclohexyl carbon skeletons as the insect-produced compounds.

In addition, the six aeration samples with the most material were combined and concentrated to ~10 μl, and the concentrated sample was fractionated by preparative GC on a Megabore column (DB-5, 30 m x 0.53 mm ID, 5 μ film thickness; J&W Scientific), with an injector temperature of 250ºC, and an oven program of 40ºC for 0 min, then 20ºC per min to 90ºC, hold for 40 min, then 20º per min to 250ºC for 10 min. The column effluent was split ~28:1, with the majority going to a heated outlet port (200ºC). Eluting compounds were collected in dry-ice cooled glass capillaries, which, after warming to room temperature, were rinsed into conical vials with ~20 μl of deuterated methylene chloride. The resulting

solutions were then transferred to 1 mm diameter tubes for microbore NMR analyses, which were carried out on a Bruker Avance spectrometer at 600 MHz.

Field bioassays In the eucalyptus copse at UCR Agricultural Operations, black cross-vane intercept traps (Alpha Scents, Portland OR, USA) coated with Fluon® (Graham et al. 2010) were hung on 1.5 m tall, inverted L-shaped stands made from PVC pipe. Traps were placed 10-15 m apart in transects, with treatments initially assigned randomly to traps. Traps were checked twice per week at minimum and rerandomized at every check. Beetles were live trapped so that they could be used for pheromone collection.

From 12 July to 3 August 2016, α-terpinene, *p*-cymene, dehydro-*p*-cymene, and *p*,α,α-trimethylbenzyl alcohol were tested as a blend versus traps baited with live males, along with blank controls. The compounds were each dissolved in 2-propanol at 25 mg/ml, and 1 ml was dispensed from 5 × 7.5 cm low-density polyethylene resealable bags (2 mil wall thickness; Fisher Scientific, Pittsburgh PA, USA). The live males were placed in cylinders of wire mesh (5 × 15 cm), capped with plastic petri dishes, and wedged into the lure opening at the center of the traps. One to three males were deployed in the traps as available. Two replicates were used.

From 3 to 18 August 2016, synthesized *p*-mentha-1,3-dien-8-ol was tested as a single component (25 mg/ml in 2-propanol) deployed in 1 ml aliquots from the

small plastic bags described above. This treatment was only tested against a blank control, with six spatial replicates; four were deployed in a copse of eucalyptus trees at UCR Agricultural Operations, one replicate was deployed at a private residence in Riverside backing onto a gulley with mixed trees and vegetation, and another replicate was deployed at a second private residence which faces the Box Springs Nature Reserve in Riverside. Traps were checked twice per week at minimum and rerandomized at every check.

Results

Pheromone candidate identification The profiles of volatiles emitted by males of both species were very similar, with the same major compounds (compounds A-H; Figs. A2, A3; Table A3), none of which were detected in aerations of *P. gracile* females or sugar water controls. Furthermore, no novel compounds were found in aerations from female *P. gracile*. For *E. costipennis*, no females were obtained, and so no comparisons between volatiles of females and males were possible. In GC-EAD analyses, antennae of female *P. gracile* responded to compounds G and H (Fig. A4; $n = 3$), whereas antennae of males responded only to compound G (Fig. A5; $n = 2$).

Four of the nine major compounds were tentatively identified by matching their mass spectra with those from the NIST mass spectral database as α-terpinene (compound A; Table A3), *p*-cymene (compound B), dehydro-*p*-cymene (compound

C), and *p*,α,α-trimethylbenzyl alcohol (compound H). The identifications were verified by matching the mass spectra and GC retention times of the compounds with those of authentic standards. All of these compounds share the same carbon skeleton, and because the mass spectra of the remaining four major compounds were similar, it seemed likely that they might also have the same basic carbon skeleton. Compounds D, E, and F had apparent molecular ions at *m/z* 134, suggesting that they were isomeric monoterpene hydrocarbons with one more double bond than a typical monoterpene, whereas compounds H and I had molecular ions of *m/z* 152, suggesting that they were oxygenated monoterpenes.

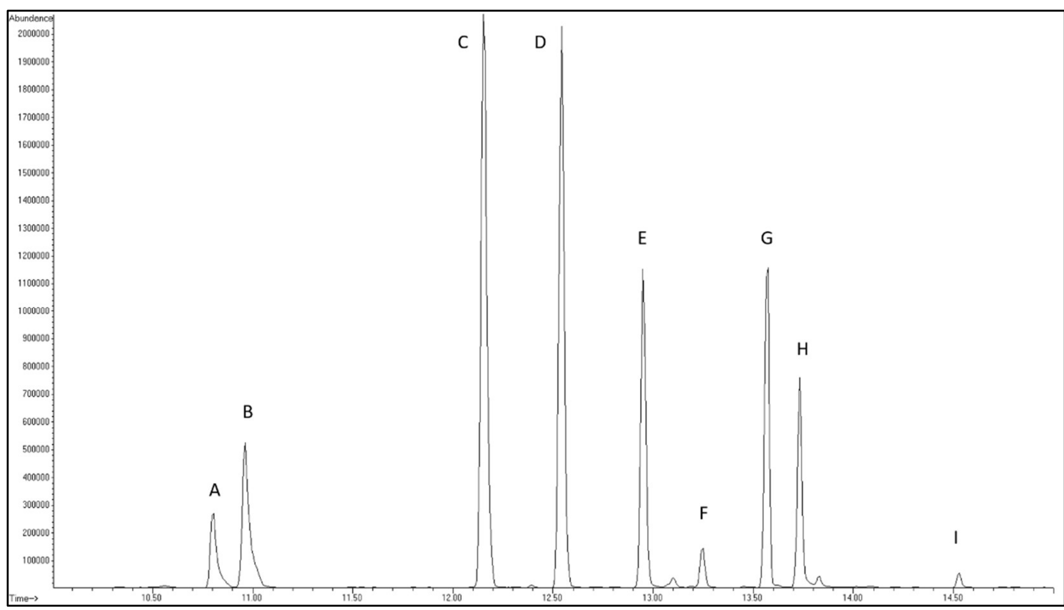

Fig. A2. GC chromatogram of male *Paranoplium gracile* headspace volatile sample. Labelled peaks correspond to Table A3.

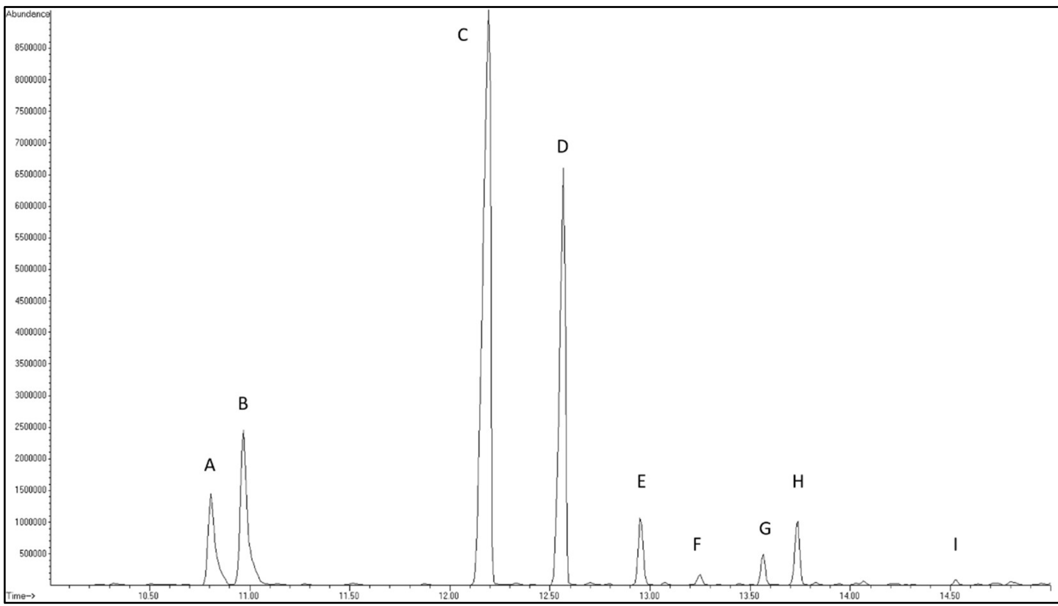

Fig. A3. GC chromatogram of male *Eudestinia costipennis* headspace volatile sample. Labelled peaks correspond to Table A3.

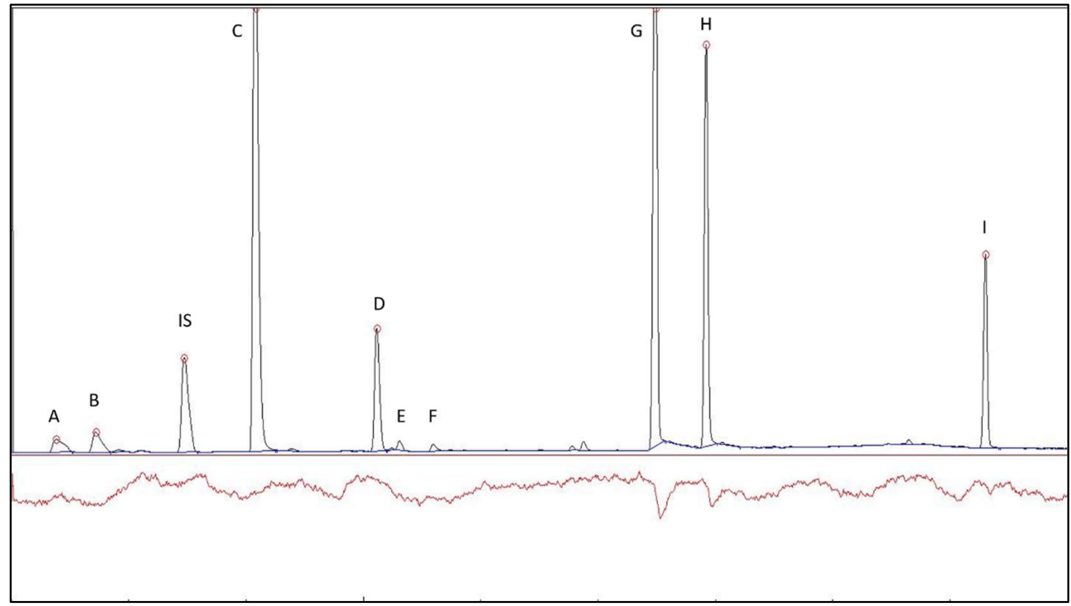

Fig. A4. GC-EAD analysis of headspace volatiles from male *Paranoplium gracile* tested with antenna from a female. Upper trace: FID chromatogram, lower trace, responses from the antennae of a female *P. gracile*. Labelled peaks correspond to Table A3.

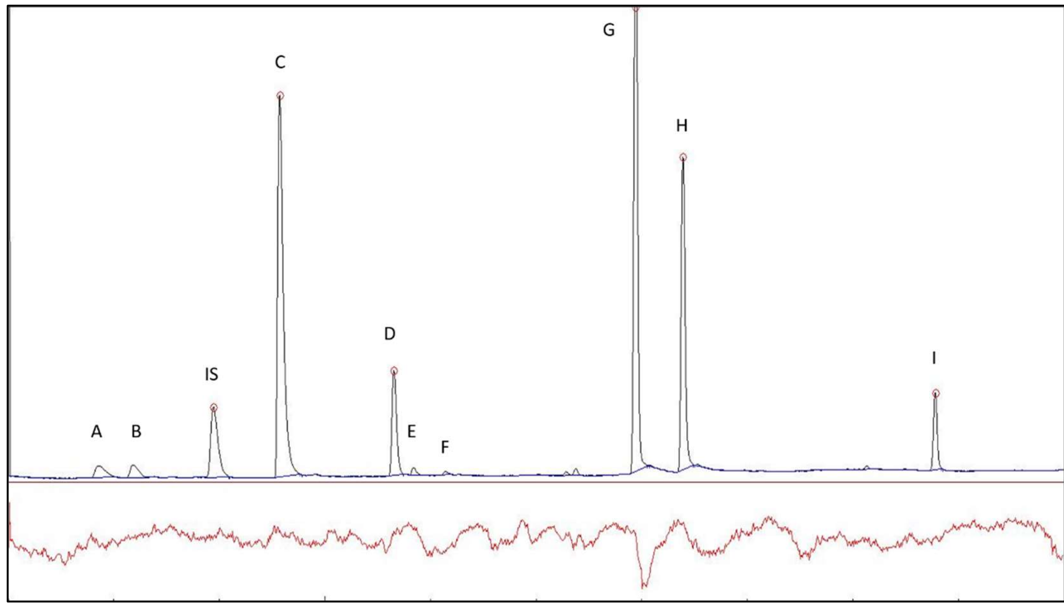

Fig. A5. GC-EAD analysis of headspace volatiles from male *Paranoplium gracile* tested with antenna from a male. Upper trace: FID chromatogram, lower trace, responses from the antennae of a male *P. gracile*. Labelled peaks correspond to Table A3.

Table A3. Major compounds found in headspace samples of male *Paranoplium gracile*. The same compounds were also found in headspace samples from male *Eudistenia costipennis*.

Cmpd	Confirmed structures in bold; tentative structures in normal font	Diagnostic ions, final ion indicating molecular ion	Average abundance ± SE	Structure
A	**α-terpinene**	93,121,136	5.2±1.6	
B	***p*-cymene**	91,119,134	22.4±5.9	
C	**dehydro-*p*-cymene**	91,117,132	8.2±2.4	
D	**1,3,8-*p*-menthatriene**	91,105,119,134	4.5±1.0	
E	Isomer of D	91,105,119,134	3.0±1.6	
F	Isomer of D	91,119,134	0.9±0.7	
G	***p*-mentha-1,3-dien-8-ol**	43,91,109,119,134,137,152	37.8±4.9	OH
H	***p*,α,α-trimethylbenzyl alcohol**	43,91,117,135,150	14.4±3.3	OH
I	Isomer of G	91,109,119,134,137, 152	3.0±1.8	

That all the compounds had the same carbon skeleton was confirmed by catalytic hydrogenation of an aliquot of the extract with palladium on carbon under a hydrogen atmosphere, and then comparing the products to those derived from catalytic reduction of terpinolene (which on reduction yields 1-isopropyl-4-methylcyclohexane stereoisomers) and α-terpineol (which yields stereoisomers of 2-(4-methylcyclohexyl)propan-2-ol)). Reduction of the extract resulted in the disappearance of compounds A, C, D, E, F, G, and I, and the appearance of 1-isopropyl-4-methylcyclohexane and 2-(4-methylcyclohexyl)propan-2-ol isomers, whereas *p*-cymene (compound B) and *p*,α,α-trimethylbenzyl alcohol (compound H) were still present, as expected, because they would not be expected to be reduced under the conditions used.

Compounds D and G were isolated by preparative GC and conclusively identified by microprobe proton NMR. Thus, compound D had alkene protons at 5.98 ppm (d, 1H, J ~ 6 Hz), 5.75 ppm (m, 1H), 5.05 ppm (s, 1H), and 4.90 ppm (s, 1H), two broadened triplets from pairs of allylic protons at 2.40 (t, 2H, J ~ 9.5 Hz) and 2.17 ppm (t, 2H, J~9.5 Hz), and two 3-proton singlets from allylic methyl groups at 1.92 and 1.82 ppm. In addition, there were ^{1}H-^{1}H COSY cross-peaks linking the methyl singlet at 1.92 ppm to the alkene proton at 4.90 ppm, between the methyl at 1.82 ppm and the alkene proton at 5.75 ppm, between the two alkene protons at 5.98 and 5.75 ppm, and between the allylic triplets at 2.40 and 2.17 ppm. Given that D was known to have a carbon skeleton that produced 1-isopropyl-4-

methylcyclohexane upon hydrogenation, the NMR data unequivocally identified D as 1-methyl-4-(1-methylethenyl)-1,3-cyclohexadiene (= *p*-mentha-1,3,8-triene).

The proton NMR spectrum of G had similarities to that of compound D. Thus, there were alkene protons at 5.86 (d, 1H, J = 5.5 Hz) and 5.67 ppm (m, 1H), two pairs of allylic protons at 2.20 (2H, t, J ~9 Hz) and 2.09 ppm (2H, t, J ~9 Hz), an allylic methyl singlet at 1.78 ppm, and a six-proton singlet at 1.31 ppm from an isopropoxy group. There were ^{1}H-^{1}H COSY cross-peaks between the alkene proton at 5.67 and the methyl at 1.78 ppm, between the two alkene protons, and between the allylic triplets at 2.20 and 2.09 ppm. These data, in combination with the mass spectral data, unequivocally identified compound G as *p*-mentha-1,3-dien-8-ol (= α,α,4-trimethyl-1,3-cyclohexadiene-1-methanol). The identification was confirmed by synthesis of an authentic standard as described by Roy et al. (1995).

The remaining three compounds E, F, and I have not been conclusively identified, but it is clear that E and F are isomers of D, and I is an isomer of G. In both cases, given the fact that all nine compounds share the same carbon skeleton, this restricts the structural possibilities to only a few compounds.

Field bioassays A total of six female and 12 male *P. gracile* were attracted to traps in 2016. In the first trial, traps baited with a blend of *p*-cymene, dehydro-*p*-cymene, α-terpinene, and *p*,α,α-trimethylbenzyl alcohol or with live males both caught a total of 8 beetles, whereas no beetles were caught in unbaited control traps (Table

A4). In the second trial, testing responses to crude synthesized *p*-mentha-1,3-dien-8-ol, 4 beetles were caught in baited traps versus none in controls (Table A5).

Table A4. Response of *P. gracile* to a blend of *p*-cymene, dehydro-*p*-cymene, α-terpinene, and *p*,α,α-trimethylbenzyl alcohol, to live males, or blank controls. Numbers before the slash are the number of females, and numbers after the slash are the numbers of males.

Date (2016)	Blend	Live males	Blank
13 July	0 / 1	0 / 0	0 / 0
24 July	0 / 1	2 / 0	0 / 0
27 July	1 / 1	0 / 2	0 / 0
29 July	0 / 0	1 / 2	0 / 0
1 August	0 / 4	0 / 1	0 / 0
Totals	1 / 7	3 / 5	0 / 0

Table A5. Response of *P. gracile* to synthesized *p*-mentha-1,3-dien-8-ol versus blank controls. Numbers before the slash are the numbers of females, and numbers after the slash are numbers of males.

Date (2016)	Pheromone candidate	Blank
4 August	1 / 0	0 / 0
8 August	1 / 0	0 / 0
17 August	0 / 1	0 / 0
18 August	0 / 1	0 / 0
Totals	2 / 2	0 / 0

Discussion

It seems likely that at least some of the compounds seen in the GC-MS traces of the insect extracts are artefacts because the major compound, *p*-mentha-1,3-dien-8-ol, with a tertiary allylic alcohol, is not very stable and can readily lose water to give 1,3,8-*p*-menthatriene (D), or its isomers E and F (such changes were empirically observed in older samples and samples injected using a higher injection

port temperature, 250ºC vs. 150ºC). Similarly, compound H could arise from dehydrogenation of G to form *p*,α,α-trimethylbenzyl alcohol H, which in turn, by ready loss of water from the resulting tertiary benzylic alcohol, could form dehydro-*p*-cymene (C). Thus, it seems likely that the male beetles actually produce a much simpler blend than is shown in the GC-MS traces. This is also suggested by the fact that in GC-EAD analyses, strong antennal responses were only elicited by *p*-mentha-1,3-dien-8-ol (F) and *p*,α,α-trimethylbenzyl alcohol (G). Thus, further analytical chemistry experiments need to be performed, using compounds purified by e.g., liquid chromatography, and with their purities confirmed by NMR, to verify whether *p*-mentha-1,3-dien-8-ol (G) does indeed produce some of the other compounds seen under the GC-MS conditions used in the analyses of extracts. We know, for example, that authentic standards of *p*,α,α-trimethylbenzyl alcohol (G), when diluted in DCM and run on GC-MS include a small peak of the dehydrated isomer, dehydro-*p*-cymene (C).

In both the field trials, low numbers of *P. gracile* of both sexes were attracted to both of the lures tested, versus no beetles trapped in controls. Whereas these results are suggestive, more highly replicated field trials need to be carried out to verify that one or more of the identified compounds are indeed pheromone components that are significantly attractive either alone or in blends. Analogous field trials need to be carried out with *E. costipennis*, in which the profiles of headspace volatiles released by males were very similar to the volatiles profiles of

male *P. gracile*. Because we do not know how extensively some of these compounds lose water and/or aromatize under the collection and analysis conditions, these possible degradative changes must be taken into account when developing pheromone blends for testing, as well as in how the pheromones are deployed for field testing.

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