PHEROMONE CHEMISTRY AND REPRODUCTIVE ISOLATION IN THE SUBFAMILY LAMIINAE (COLEOPTERA: CERAMBYCIDAE)

BY

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DISSERTATION

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ABSTRACT

Research on the chemical ecology of cerambycid beetles has revealed that the pheromone chemistry of related species is often highly conserved. Sympatric species often share pheromone components, even having identical attractant pheromones. For example, many cerambycines native to different continents use pheromones composed of hydroxyalkanones and related alkanediols. Avoidance mechanisms of interspecific attraction in cerambycines that share pheromone components include segregation by seasonal and diel phenology and synergism and antagonism by minor pheromone components. Less is known about the pheromone chemistry of cerambycid species in the largest subfamily, the Lamiinae. As with the cerambycines, all known pheromones of lamiines are male-produced aggregation-sex pheromones, and pheromone chemistry has been conserved across continents. Lamiine pheromones identified to date are based on either hydroxyethers or terpenoids.

The purpose of my dissertation research is to broaden the current understanding of pheromone chemistry in lamiines and to elucidate chemical mechanisms of reproductive isolation among sympatric species that share pheromone components. In Chapter 1, I summarize what is known about the pheromone chemistry of cerambycids, with emphasis on the subfamily Lamiinae. In Chapter 2, I identify the pheromone composition for two species of lamiines common in East-Central Illinois. I demonstrate that reproductive isolation is achieved by through differences in stereochemistry, as well as the phenomenon of enantiomeric synergism, which is rare among insects. In Chapter 3, I present data confirming the pheromone composition for four species, as well as a third pheromone component for a species for which a pheromone was already identified, and tentative pheromone identifications for one additional species. In Chapter 4, I identify pheromones for two species and a tentative pheromone for one additional

species, thereby providing evidence for a third class of pheromone compounds in lamiines that is based on sulcatone.

TABLE OF CONTENTS

CHAPTER 1: PHEROMONES OF THE CERAMBYCIDAE: CURRENT UNDERSTANDING AND PRACTICAL APPLICATIONS 1

CHAPTER 2: SYNERGISM BETWEEN ENANTIOMERS CREATES SPECIES-SPECIFIC PHEROMONE BLENDS AND MINIMIZES CROSS-ATTRACTION FOR TWO SPECIES OF CERAMBYCID BEETLES.....32

CHAPTER 3: PHEROMONE COMPOSITION OF FIVE SPECIES PROVIDES INSIGHT INTO CHEMICAL COMMUNICATION AND AVOIDANCE OF INTERSPECIFIC ATTRACTION IN LAMIINE CERAMBYCID BEETLES 67

CHAPTER 4: SULCATONE AND SULCATOL ARE PHEROMONE COMPONENTS FOR TWO SPECIES OF CERAMBYCID BEETLES IN THE SUBFAMILY LAMIINAE 105

APPENDIX: SUPPLEMENTARY INFORMATION 135

CHAPTER 1: PHEROMONES OF THE CERAMBYCIDAE: CURRENT UNDERSTANDING AND PRACTICAL APPLICATIONS

Introduction

The family Cerambycidae is one of the largest families of insects, with about 36,300 described species (Monné et al. 2017). Most species are endophytic as larvae, developing in woody plant tissues (Haack 2016). These insects play important roles in forest ecosystems as nutrient recyclers, initiating the breakdown of dead woody plants (Linsley 1959) and can be used as indicators of ecosystem health (Maeto et al. 2002). However, many cerambycids are serious pests in their native regions, especially when the larvae bore within dead wood destined for lumber or kill living trees (Reddy 2007). Because the larvae can be long-lived and feed concealed in woody tissues, they are readily transported by international commerce and so are among the insects most commonly intercepted in international quarantine (e.g., Brockerhoff et al. 2006; Haack 2006; Liebhold et al. 1995; Nowak et al. 2001).

When introduced to new areas, exotic species of cerambycids can become devastating pests. Monitoring and survey programs are critical tools in stemming the invasion of exotic species, especially as rates of introduction rise with the growth of international trade (Aukema et al. 2010). Long-range volatile pheromones are widespread in the Cerambycidae and play instrumental roles in cerambycid reproduction (Millar and Hanks 2017). As such, effective pheromone-baited traps are an indispensable component of cerambycid monitoring, eradication, and management programs. Here, I review existing knowledge about the pheromones of cerambycid beetles by subfamily, with an emphasis on the Lamiinae, and applications for semiochemicals in the management of pest species.

Pheromones of the Cerambycidae

Of the approximately 115 cerambycid species for which pheromones have been definitively or tentatively identified, most are in the two largest subfamilies, the Cerambycinae (~48.5% of species) and Lamiinae (30%), with fewer in the smaller subfamilies Prioninae (12.5%), Lepturinae (5%), and Spondylidinae (4%; from Millar and Hanks 2017). These represent five of the eight described subfamilies in the Cerambycidae (Švácha and Lawrence 2014), with no pheromones yet identified for species in the remaining subfamilies Dorcasominae, Necydalinae, or Parandrinae. Pheromones of cerambycids apparently fall into two main types: male-produced aggregation-sex pheromones that attract both sexes (Cerambycinae, Lamiinae, Spondylidinae), and female-produced sex pheromones that attract only males (Prioninae, Lepturinae).

Research on pheromones of cerambycid beetles over the last 15 years has revealed that certain structures are commonly shared among sympatric species, and even among species that are native to different continents (Hanks and Millar 2016). These common pheromone components include the hydroxyketones of cerambycines, monochamol of lamiines, and prionic acid of prionines. The sharing of pheromone components among sympatric species of cerambycids, and at least partial attraction to traps baited with dominant components, results in simultaneous capture of multiple species, as has been demonstrated with independent field bioassays of the same compounds in Asia, Europe, Africa, Australia, South America, and North America (Fan et al. 2018; Hayes et al. 2016; Imrei et al. 2013; Millar et al. 2018; Silva et al. 2017; Sweeney et al. 2014; Wickham et al. 2014).

Cerambycinae

Pheromone structures within this group appear to span the range between chemical conservation and diversity. Among cerambycines, hydroxyketones of 6, 8, and 10 carbons and the corresponding 2,3-alkanediols are common pheromone components of several species native to different parts of the world (Hanks and Millar 2016). For certain species, the 2,3-alkanediols are found in the headspace volatiles of males, but it seems that they are only an analytical artifact, as there is no evidence that they serve as attractants or synergists for these species (Hanks and Millar 2016). In 2015, Zou et al. reported variations of the hydroxyketone structure ([2*S*,4*E*]-2-hydroxyoct-4-en-3-one and [3*R*,4*E*]-3-hydroxyoct-4-en-2-one, i.e., unsaturated analogs of hydroxyoctanone) in the headspace volatiles of *Tylonotus bimaculatus* Haldeman.

Despite this overall conservation in pheromone structures among the Cerambycinae, there is an increasing body of evidence suggesting that pheromones in this group are quite diverse in structure, ranging from simple compounds such as 2-methylbutanol in the species *Phymatodes amoenus* (Say), to terpenoids such as neral in species of *Megacyllene* (Lacey et al. 2008), to the novel compound (2*E*,6*Z*,9*Z*)-2,6,9-pentadecatrienal produced by *Elaphidion mucronatum* (Say) (Millar et al. 2017). The pheromones of the cerambycine *Rosalia funebris* Motschulsky are similar in structure to pheromones of some species of hemipterans (Ray et al. 2009). Based on general trends, it is likely that these unique pheromones are also pheromone components for other species and represent conserved structures (Millar and Hanks 2017).

Spondylidinae

Like cerambycines and lamiines, species in the Spondylidinae produce male-produced aggregation-sex pheromones. (*E*)-Dimethylundeca-5,9-dien-2-ol, or “fuscumol” was first

identified from the species *Tetropium fuscum* (F.) and *T. c. cinnamopterum* Kirby (Silk et al. 2007, Sweeney et al. 2010), and later from *T. castaneum* (L.). For all three species, the pheromone itself is a weak attractant, but it is strongly synergized by volatiles associated with the spruce trees that are hosts of the larvae (Sweeney et al. 2010). In field trials, fuscumol and fuscumol acetate also attracted the congener *T. schwarzianum* Casey (Hanks and Millar 2013), further evidence that these compounds represent a conserved structural class of pheromones of spondylidines, as apparently is the case among lamiines.

Prioninae

In contrast to the subfamilies previously discussed, pheromones identified for prionines are female-produced sex pheromones that attract only males (Millar and Hanks 2017). Two classes of pheromone structures have been identified. First, females of *Prionus californicus* Motschulsky, native to the western USA, were found to produce 3,5-dimethyldodecanoic acid (hereafter "prionic acid") (Rodstein et al. 2009), which is the likely pheromone of another six species of *Prionus* native to North America and one European congener (Barbour et al. 2011). The same compound is also known to attract males of the Asian *Dorysthenes granulosus* (Thomson) in the same tribe, Prionini (Wickham et al. 2016a). Thus, this compound has been conserved, at least among species of the Prionini, which are distributed across multiple continents.

Members of the second structural class of prionine pheromones are biosynthetically unrelated to prionic acid, 2,3-alkanediols, but they also are female-produced sex pheromones. The same compounds also serve as pheromone components of many species of the Cerambycinae (Hanks and Millar 2016). Females of the North American species *Tragosoma*

soror Laplante (tribe Meroscelisini) produce (2*R*,3*R*)-2,3-hexanediol, while (2*S*,3*R*)-2,3-hexanediol is the likely pheromone of the congeners *T. harrisii* LeConte and *T. pilosicorne* Casey based on evidence from field bioassays and electroantennography (Ray et al. 2012; see Laplante 2017 for current taxonomy of the genus). The pheromone of Asian prionine *Nepiodes c. costipennis* White (tribe Callipogonini) is the eight-carbon analog, (2*R*,3*S*)-2,3-octanediol (Wickham et al. 2016b).

Lepturinae

Pheromones have been verified for only two species of lepturines. As in the Prioninae, both are female-produced sex pheromones. *Ortholeptura valida* (LeConte), a North American species, produces *cis*-vaccenyl acetate (Ray et al. 2011), which is also a male-produced aggregation pheromone for flies in the genus *Drosophila* (Bartelt et al. 1985). Females of *Desmocerus californicus californicus* Horn produce a pheromone of different structure, (4*R*,9*Z*)-hexadec-9-en-4-olide ("desmolactone"; Ray et al. 2012), which is probably biosynthetically related to *cis*-vaccenyl acetate. Desmolactone has also been shown to attract several other species of *Desmocerus* in North America, evidence that it is at least a component of their pheromones (Ray et al. 2014).

Lamiinae

Pheromones of lamiines identified fall into two structural classes: hydroxyethers and compounds based on the sesquiterpene degradation product geranylacetone, (*E*)-dimethylundeca-5,9-dien-2-one. As with the Cerambycinae, some pheromone components of lamiines are shared among species that are native to different continents. For example, *Anoplophora glabripennis*

(Motschulsky), the Asian longhorned beetle, produces two compounds of the hydroxyether class; 4-(heptyloxy)butanol and its corresponding aldehyde, 4-(heptyloxy)butanal (Table 1; Zhang et al. 2002). Males of the sympatric congener *A. chinensis* (Forster) produce the aldehyde as the dominant pheromone component (Hansen et al. 2015). Males of *Monochamus galloprovincialis* (Olivier) produce 2-(undecyloxy)ethanol (hereafter "monochamol"; Pajares et al. 2010), which is now known to be shared among at least five congeners native to North America, Europe, and Asia (Table 1; Millar and Hanks 2017). In Asia, monochamol has been identified as a candidate pheromone for lamiine species in four other genera in the same tribe as *Monochamus*, the Monochamini (Wickham et al. 2014).

The second structural class of compounds that have been identified as pheromone components of lamiines is comprised of geranylacetone and related structures, including the corresponding alcohol termed fuscumol, (*E*)-dimethylundeca-5,9-dien-2-ol, and its acetate ester termed fuscumol acetate, (*E*)-dimethylundeca-5,9-dien-2-yl-acetate. Fuscumol was first identified as the male-produced pheromone of species in the spondylidine genus *Tetropium* (Silk et al. 2007), and all three compounds are produced by males of South American lamiines *Hedypathes betulinus* (Klug) (Fonseca et al. 2010) and *Steirastoma breve* (Sulzer) (Liendo-Barandiaran et al. 2010). However, attraction to these compounds has yet to be confirmed for either species. Geranylacetone is achiral, but both fuscumol and fuscumol acetate are chiral, and their respective enantiomers each may serve different biological roles. In North America, the lamiine *Astyleiopus variegatus* (Haldeman) produces *S*-fuscumol and *S*-fuscumol acetate (Table 1.1; Hughes et al. 2013). Racemic fuscumol and fuscumol acetate are considered candidate pheromones for a number of North American lamiine species, because when deployed in the field they attract adult beetles of these species (Chapter 3). In the chapters that follow, I identify

pheromones for several more species of lamiines. These newly-identified pheromones are bolded in Table 1.1.

Mechanisms of reproductive isolation

The extensive pheromonal parsimony among cerambycid species described here raises the issue of how species with shared or similar pheromone components maintain reproductive isolation. There are three main mechanisms by which prezygotic reproductive isolation occurs in cerambycids: spatial, temporal, and chemical (Millar and Hanks 2017).

Sympatric species can avoid one another based on their larval host preferences, as pheromones are often strongly synergized by host plant odors. For example, adults that developed as larvae in oaks may be repelled by the odors associated with non-hosts, such as conifers, which may also antagonize attraction to pheromones (Collignon et al. 2016). In addition, some communities of cerambycids appear to be vertically stratified in forests, with adults of some species active primarily near ground level, other species flying within the upper canopy, while still others show no association with a particular canopy height (Graham et al. 2012; Dodds 2014; Webster et al. 2016).

Adult cerambycids in temperate zones are typically active only for a few weeks each year (Hanks and Wang 2017). Subsequently, species that do not overlap in seasonal phenology can use the same or similar pheromone components with little risk of interspecific attraction (Hanks and Millar 2013; Hanks et al. 2014; Handley et al. 2015; Mitchell et al. 2015). Some species that do overlap in seasonal phenology are segregated by differences in diel flight period. Cerambycid species may be strictly diurnal, nocturnal, or crepuscular in flight (Linsley 1959). Some cerambycine species even partition these periods further, into windows of activity lasting only a

few hours, providing another mechanism for segregating species that share pheromone components (Mitchell et al. 2015).

Pheromone chemistry also may serve to segregate cerambycids,even within groups of species that share the same dominant pheromone component. The minor pheromone component of one species may synergize attraction of conspecifics to the shared component while antagonizing attraction of the other species. For example, males of the cerambycine *Euderces pini* (Olivier) produce only (*R*)-3-hydroxyhexan-2-one, while males of *Phymatodes amoenus* (Say) produce the same compound along with smaller amounts of the synergist (*R*)-methylbutan-1-one, which also antagonizes attraction of *E. pini* to the ketol (Mitchell et al. 2015). Further, inhibition by different isomers of a shared component can act as a chemical mechanism of reproductive isolation. Examples of reproductive isolation via pheromone chemistry within the Lamiinae will be presented in the following chapters.

Pheromonal parsimony and invasive species

The world-wide conservation of pheromone components of cerambycids has important implications for invasion biology. Exotic cerambycid are introduced into new regions typically in quite small numbers as immatures in imported wood products, and adults typically do not emerge synchronously (Hanks and Millar 2016). Thus, Allee effects act against establishment of exotic species (Courchamp et al. 1999). If the pheromones of the exotic species comprise the common chemicals, pheromones released by much more abundant native species may impose a major barrier to reproduction of exotic species (Hanks and Millar 2016). On the other hand, exotic species with pheromones that are unique in the native cerambycid community will encounter “pheromone-free space”, allowing males and females to find one another, even at very low population densities, fostering establishment of the population (Hanks and Millar 2016). For

example, colonization and establishment of the Asian longhorned beetle, *Anoplophora glabripennis* Motschulsky, in multiple regions of North American and Europe (including France, Germany and Italy), may be explained by the unusual chemistry of the proposed pheromone produced by males: 4-(heptyloxy)butanol and the corresponding aldehyde, 4-(heptyloxy)butanal (Zhang et al. 2002). Traps baited with these compounds have attracted no native species in significant numbers during independent trapping surveys in various areas of the USA (DiGirolomo and Dodds 2014; Nehme et al. 2014), evidence that they represent a unique chemical signal for *A. glabripennis* in the areas of introduction.

Applications of cerambycid semiochemicals

Detection and monitoring

The primary benefits of using synthetic semiochemicals as attractants are their sensitivity and selectivity in quarantine monitoring for exotic species, as well as in assessing flight period and population densities of pest species (Witzgall et al. 2010). Multi-species blends of synthesized pheromone components can be used to attract many species at once, as already discussed, which increases the efficiency of sampling programs that aim to assess cerambycid community richness and diversity. Another advantage to semiochemical-baited traps is that they can be deployed with relative ease to different levels of the forest canopy, enabling the capture of species that fly within only a single vertical stratum within the forest (discussed above).

Because exotic species typically establish in low densities, it is essential that monitoring tools be both sensitive and selective. Pheromone-baited traps can not only detect new introductions before they expand beyond control, but they can also help to monitor the effectiveness of eradication efforts. It is just as necessary to monitor species in their native range,

such as *Monochamus* species that vector the pinewood nematode that is devastating coniferous forests in Europe (Alvarez et al. 2015; Rassati et al. 2012). Finally, pheromone-baited traps also may be used in conservation of cerambycid species that are threatened or endangered species, such as the North American elderberry longhorned borer *Desmocerus c. californicus* Horn (Ray et al. 2014).

Population regulation

Two predominant pheromone-based control strategies have been developed for controlling cerambycid pests: mating disruption and mass trapping. Mating disruption relies primarily on female-produced sex pheromones to permeate the area, which disrupts chemical communication and prevents mating (Witzgall et al. 2010). For example, populations of the prionine *P. californicus* can be managed by both mating disruption and mass trapping using traps baited with the female-produced sex pheromone prionic acid (Alston et al. 2015, Maki et al. 2011). However, Sweeney et al. (2010) have shown that populations of the exotic spondylidine species *Tetropium fuscum* (F.) can be reduced by mass trapping using the male-produced pheromone fuscumol, along with plant volatiles. These pheromone-based methods of reducing population densities of pest species probably are suitable only over small geographical areas (Hanks and Millar 2016). For example, colonization and establishment of the Asian longhorned beetle, *A. glabripennis* Motschulsky, in multiple regions of North American and Europe (including France, Germany and Italy), may be explained by the unusual chemistry of the proposed pheromone produced by males: 4-(heptyloxy)butanol and the corresponding aldehyde, 4-(heptyloxy)butanal (Zhang et al. 2002). Traps baited with these compounds have attracted no native species in significant numbers during independent trapping surveys in various areas of the

USA (DiGirolomo and Dodds 2014; Nehme et al. 2014), evidence that they represent a unique chemical signal for *A. glabripennis* in the areas of introduction.

The present work

In the chapters that follow, I focus on pheromones and chemical mechanisms of reproductive isolation in the Lamiinae. In Chapters 2, 3 and 4, I present research findings on the pheromone chemistry of eleven species native to the eastern USA, including two compounds that previously were not known to serve as pheromone components, sulcatone and sulcatol. These compounds represent a new class of pheromones for the Cerambycidae.

Tables

Table 1.1 Summary of research from volatile collection and field trials on pheromones of lamiines (Cerambycidae). References to chapters refer to sections of the present dissertation. Compounds in bold were identified in studies presented in the following chapters. "Generic pheromone blend" contained 25 mg of each isomer per 1 ml of solvent carrier (91% isopropanol) per lure: racemic 3-hydroxyhexan-2-one, monochamol, racemic (*E*)-fuscumol, and (*E*)-fuscumol acetate, *syn*-2,3-hexanediol, racemic 2-methylbutan-1-ol, citral, and racemic prionic acid (Handley et al. 2015)

Taxonomy	Compounds identified (Pher = confirmed attractant)	Attractant (Attr) Enhancer (Enh) Inhibitor (Inh)	Source
Acanthocinini			
Astyleiopus variegatus (Haldeman)	Pher: *S*-fuscumol, *S*-fuscumol acetate, **geranylacetone**	Attr: fuscumol + fuscumol acetate; fuscumol acetate Enh: geranylacetone	**Chapter 3**; Hanks and Millar 2013; Hanks et al. 2012; Hughes et al. 2013; Mitchell et al. 2011
Astylidius parvus (LeConte)	**Pher: *R*-, *S*-fuscumol, *R*-fuscumol acetate, Geranylacetone**	Attr: fuscumol; *R*-fuscumol	**Chapter 2**; Hanks et al. 2012; Hughes et al. 2016; Mitchell et al. 2011

Table 1.1 (cont.)

Taxonomy	Compounds identified (Pher = confirmed attractant)	Attractant (Attr) Enhancer (Enh) Inhibitor (Inh)	Source
Astylopsis macula (Say)	**Pher: sulcatone, S-sulcatol**	Attr: *S*-sulcatol Inh: fuscumol + fuscumol acetate	**Chapter 4**
Astylopsis sexguttata (Say)		**Attr: sulcatone + sulcatol + fuscumol acetate**	**Chapter 4**
Graphisurus despectus (LeConte)	**Pher: geranylacetone, fuscumol**	Attr: granylacetone Inh: fuscumol	**Chapter 3**
Graphisurus fasciatus (Degeer)	**Pher: geranylacetone, fuscumol acetate**	Attr: fuscumol + fuscumol acetate; fuscumol acetate	**Chapter 3**; Hanks and Millar 2013; Hanks et al. 2012; Mitchell et al. 2011;

Table 1.1 (cont.)

Taxonomy	Compounds identified (Pher = confirmed attractant)	Attractant (Attr) Enhancer (Enh) Inhibitor (Inh)	Source
Leptostylus transversus (Gyllenhal)	**Pher: sulcatone, *S*-sulcatol**	Attr: *S*-sulcatol Inh: fuscumol + fuscumol acetate	**Chapter 4**
Lepturges angulatus (LeConte)	**Pher: *R*-, *S*-fuscumol acetate, Geranylacetone**	Attr: fuscumol acetate; fuscumol + fuscumol acetate Enh: geranylacetone Inh: fuscumol	**Chapter 2**; Hanks and Millar 2013; Hanks et al. 2012; Mitchell et al. 2011
Lepturges confluens (Haldeman)		**Attr: fuscumol + fuscumol acetate;** ***S*-fuscumol + *S*-fuscumol acetate + geranylacetone**	**Chapter 3**; Hanks and Millar 2013

Table 1.1 (cont.)

Taxonomy	Compounds identified (Pher = confirmed attractant)	Attractant (Attr) Enhancer (Enh) Inhibitor (Inh)	Source
Sternidius alpha (Say)	**Pher: *S*-fuscumol, *R*-, *S*-fuscumol acetate**	Attr: fuscumol; fuscumol acetate; fuscumol + fuscumol acetate Inh: 3-C6-ketol	**Chapter 3**; Hanks et al. 2018; Hanks and Millar 2013; Mitchell et al. 2011;
Styloleptus b. biustus (LeConte)		Attr: fuscumol + fuscumol acetate; generic pheromone blend Inh: 3-C6-ketol	Handley et al. 2015; Hanks et al. 2018; Hanks and Millar 2013
Urgleptes querci (Fitch)		Attr: fuscumol + fuscumol acetate	Hanks and Millar 2013
Acanthoderini			

Table 1.1 (cont.)

Taxonomy	Compounds identified (Pher = confirmed attractant)	Attractant (Attr) Enhancer (Enh) Inhibitor (Inh)	Source
Acanthoderes quadrigibba (Say)		Attr: fuscumol + fuscumol acetate	Hanks and Millar 2013
Aegomorphus modestus (Gyllenhal)	**Pher: *R*-, *S*-fuscumol acetate, geranylacetone**	Attr: *S*-fuscumol, fuscumol acetate; fuscumol + fuscumol acetate; fuscumol acetate + geranylacetone Inh: fuscumol, 3-C6-ketol	**Chapter 3**; Handley et al. 2015; Hanks et al. 2018.; Hanks and Millar 2013; Hanks et al. 2012; Hughes et al. 2016; Mitchell et al. 2011;
Hedypathes betulinus (Klug)	*R*-fuscumol acetate, geranylacetone, *R*-, *S*-fuscumol		Fonseca et al. 2010; Vidal et al. 2010
Oplosia nubila (LeConte)		Attr: fuscumol Inh: 3-C6-ketol	Hanks et al. 2018

Table 1.1 (cont.)

Taxonomy	Compounds identified (Pher = confirmed attractant)	Attractant (Attr) Enhancer (Enh) Inhibitor (Inh)	Source
Steirastoma breve (Sulzer)	fuscumol		Liendo-Barandiaran et al. 2010
Agniini			
Acalolepta formosana (Breuning)		Attr: monochamol	Wickham et al. 2014
Desmiphorini			
Psenocerus supernotatus (Say)		Attr: generic pheromone blend	Handley et al. 2015
Dorcaschematini			

Table 1.1 (cont.)

Taxonomy	Compounds identified (Pher = confirmed attractant)	Attractant (Attr) Enhancer (Enh) Inhibitor (Inh)	Source
Dorcaschema alternatum (Say)		Attr: *anti*-C6-diol Inh: 3-C6-ketol + *syn*-C6-diol	Hanks and Millar 2013
Lamiini			
Pharsalia subgemmata (Thomson)		Attr: monochamol	Wickham et al. 2014
Pseudomacrochenus antennatus (Gahan)		Attr: monochamol	Wickham et al. 2014
Xenohammus bimaculatus Schwarzer		Attr: monochamol	Wickham et al. 2014
Monochamini			

Table 1.1 (cont.)

Taxonomy	Compounds identified (Pher = confirmed attractant)	Attractant (Attr) Enhancer (Enh) Inhibitor (Inh)	Source
Anoplophora chinensis (Forster)	Pher: 4-(*n*-heptyloxy)butan-1-ol		Hansen et al. 2015
Anoplophora glabripennis (Motschulsky)	Pher: 4-(*n*-heptyloxy)butanal, 4-(*n*-heptyloxy)butan-1-ol; (*E*,*E*)-α-farnesene		Crook et al. 2014; Nehme et al. 2010, 2014; Zhang et al. 2002
Monochamus alternatus Hope	Pher: monochamol	Attr: monochamol	Teale et al. 2011
Monochamus bimaculatus Gahan		Attr: monochamol	Wickham et al. 2014
Monochamus carolinensis (Olivier)	Pher: monochamol	Attr: monochamol; generic pheromone blend; syn-C6-diol Inh: 3-C6-ketol	Allison et al. 2012; Hanks and Millar 2013; Hanks et al. 2012; Ryall et al. 2015

Table 1.1 (cont.)

Taxonomy	Compounds identified (Pher = confirmed attractant)	Attractant (Attr) Enhancer (Enh) Inhibitor (Inh)	Source
Monochamus clamator (LeConte)		Attr: monochamol	Macias-Samano et al. 2012;
Monochamus galloprovincialis (Olivier)	Pher: monochamol		Pajares et al. 2010; Rassati et al. 2012
Monochamus leuconotus (Pascoe)	2-(4-heptyloxy-1-butyloxy)ethan-1-ol		Pajares et al. 2010
Monochamus marmorator Kirby		Attr: monochamol	Ryall et al. 2015
Monochamus mutator LeConte		Attr: monochamol	Ryall et al. 2015

Table 1.1 (cont.)

Taxonomy	Compounds identified (Pher = confirmed attractant)	Attractant (Attr) Enhancer (Enh) Inhibitor (Inh)	Source
Monochamus notatus (Drury)		Attr: monochamol	Fierke et al. 2012; Hanks and Millar 2013; Ryall et al. 2015
Monochamus o. obtusus Casey		Attr: monochamol	Macias-Samano et al. 2012
Monochamus s. scutellatus (Say)	Pher: monochamol	Attr: monochamol Inh: 3-C6-ketol	Fierke et al. 2012; Hanks and Millar 2013; Hanks et al. 2018; Macias-Samano et al. 2012; Ryall et al. 2015
Monochamus saltuarius (Gebler)		Attr: monochamol	Ryall et al. 2015
Monochamus sutor (L.)	Pher: monochamol	Attr: monochamol	Pajares et al. 2013

Table 1.1 (cont.)

Taxonomy	Compounds identified (Pher = confirmed attractant)	Attractant (Attr) Enhancer (Enh) Inhibitor (Inh)	Source
Monochamus urussovii (Fischer)		Attr: monochamol	Ryall et al. 2015;

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CHAPTER 2: SYNERGISM BETWEEN ENANTIOMERS CREATES SPECIES-SPECIFIC PHEROMONE BLENDS AND MINIMIZES CROSS-ATTRACTION FOR TWO SPECIES OF CERAMBYCID BEETLES[1]

Introduction

Recent research on the chemical ecology of cerambycid beetles has revealed that the pheromone chemistry of related species often is highly conserved, with many sympatric species sharing pheromone components, or even having identical attractant pheromones (reviewed by Millar and Hanks 2016). Within the subfamilies Cerambycinae, Lamiinae, and Spondylidinae, volatile aggregation pheromones are produced by males, and attract both sexes. Among species in the large subfamily Cerambycinae, even species on different continents often share pheromone components such as 3-hydroxyalkan-2-ones and the related 2,3-alkanediols, despite the species having diverged many thousands of years ago. Thus, traps baited with single pheromone components may attract multiple species of cerambycids in different parts of the world (e.g., Hayes et al. 2016; Sweeney et al. 2014; Wickham et al. 2014). The results from such screening trials have provided valuable leads for the subsequent full identification of pheromones for various target species (e.g., Hanks et al. 2007; Mitchell et al. 2013, 2015; Narai et al. 2015; Zou et al. 2016).

Interspecific attraction to shared pheromone components may be averted, or at least minimized among sympatric cerambycids by seasonal segregation: adults of each species typically are active for brief and discrete periods of the season (3 – 6 wk), and species emerge in orderly progression from early spring through late fall (e.g., see Handley et al. 2015; Hanks and Millar 2013; Hanks et al. 2014). Furthermore, species which overlap seasonally may be

[1] This chapter has already been published (Meier et al. 2016. J. Chem. Ecol. 42: 1181-1192)

segregated by diel phenology, because daily activity periods usually are limited to a few hours (Hanks and Wang 2016). Finally, those species which are fully synchronous, overlapping in both seasonal and diel phenology, may be segregated by the synergistic or antagonistic effects of the minor components of their pheromones (Mitchell et al. 2013, 2015).

Less is known about the pheromone chemistry of cerambycid species in the largest subfamily, the Lamiinae (reviewed by Millar and Hanks 2016; for taxonomy, see Švácha and Lawrence 2014). Several species in the lamiine genus *Monochamus* are known to use 2-(undecyloxy)ethanol (termed monochamol) as an aggregation pheromone, and a structural analog, 4-(heptyloxy)butan-1-ol, is reported to be a pheromone component of the Asian longhorned beetle, *Anoplophora glabripennis* (Motschulsky) (Zhang et al. 2002), and its congener, *A. chinensis* (Forster) (Hansen et al. 2015). In contrast, males of the South American lamiine *Hedypathes betulinus* (Klug) produce a blend of structurally related terpenoid derivatives, including (*R*)-(*E*)-6,10-dimethyl-5,9-undecadien-2-ol (fuscumol), (*R*)- and (*S*)-(*E*)-6,10-dimethyl-5,9-undecadien-2-yl acetate (fuscumol acetate), and (*E*)-6,10-dimethyl-5,9-undecadien-2-one (geranylacetone, Fig. 1; Fonseca et al. 2010; Vidal et al. 2010). Fuscumol also is produced by males of the South American species *Steirastoma breve* (Sulzer) (Liendo-Barandiaran et al. 2010).

Based on evidence that fuscumol and related compounds were pheromone components of lamiines, and the conserved nature of cerambycid pheromones in general, Mitchell et al. (2011) conducted a screening trial of racemic fuscumol and/or fuscumol acetate in three disparate areas of North America (east-central Illinois, northwestern Indiana, central Texas), attracting adults of several lamiine species. However, to date, a pheromone blend has been formally identified for

only one of these species, *Astyleiopus variegatus* (Haldeman), consisting of (*S*)-fuscumol + (*S*)-fuscumol acetate (Hughes et al. 2013).

It appears unlikely that interspecific attraction among the 12 lamiine species caught by Mitchell et al. (2011) would be averted by seasonal segregation because they all overlap broadly in seasonal phenology (during late June to early August; Handley et al. 2015; Hanks and Millar 2013; Hanks et al. 2014). Moreover, many of these species overlap in diel phenology as well, being crepuscular to nocturnal (unpub. data), a common trait among lamiines (Linsley 1959; Švácha and Lawrence 2014). Thus, it seemed likely that pheromone chemistry plays a critical role in averting interspecific attraction among these species, as suggested by differences among them as to whether the adults are attracted by racemic fuscumol or fuscumol acetate, or only by the blend of the two (Mitchell et al. 2011).

Here, we describe further screening trials of fuscumol and fuscumol acetate in east-central Illinois which were intended to target lamiine species for pheromone identification. Traps were baited with both racemic and chiral chemicals to provide more detailed insight into the nuances of their pheromone chemistry. The two species captured in greatest numbers were *Astylidius parvus* (LeConte) and *Lepturges angulatus* (LeConte). Mitchell et al. (2011) had already reported that adult *A. parvus* were attracted to racemic fuscumol, and adult *L. angulatus* to racemic fuscumol acetate, suggesting that the species differed in their pheromone chemistry. Larvae of both species are polyphagous, feeding within woody tissues of hardwood trees, shrubs, and vines of many families (Linsley and Chemsak 1995). Adults of both species are active from late June to early August (Hanks and Millar 2013; Hanks et al. 2014). Overlap in diel phenology was confirmed during the present study using capture data from traps that had been fitted with a

mechanism that changed collecting jars at programmable time intervals (see Methods and Materials).

We tested the hypothesis that cross attraction between *A. parvus* and *L. angulatus* is averted by differences in the chemistry of their respective pheromones. Thus, we identified the compounds produced by males, and confirmed that adults of each species were attracted only to their species-specific blends. Remarkably, both species were found to exhibit the rare phenomenon of synergism between the enantiomers of their pheromone components, with beetles being attracted only to the appropriate blends of the enantiomers, and not to the individual enantiomers.

Methods and Materials

Sources of Chemicals

(Fig. 1.1) Racemic (*E*)-fuscumol, (*E*)-fuscumol acetate, and (*E*)-geranylacetone were purchased from Bedoukian Research (Danbury, CN), and (*E/Z*)-geranylacetone from Sigma-Aldrich (St. Louis, MO, USA). (*R*)-Fuscumol (96.6% enantiomeric excess, ee), (*S*)-fuscumol (98% ee), (*R*)-fuscumol acetate (96.6% ee), and (*S*)-fuscumol acetate (98% ee) were synthesized by enzymatic kinetic resolution of the racemic compounds as described in Hughes et al. (2013).

Study Sites

Field work to identify pheromones of cerambycid species, and to characterize diel phenology and attraction to synthesized pheromones, was conducted at five study sites in east-central Illinois (Table 2.1), all of which were wooded with mature second-growth or successional

hardwoods and dominated by oaks (*Quercus* species), hickories (*Carya* species), maples (*Acer* species), and ash (*Fraxinus* species).

Diel Phenology of Adults

Diel flight phenology of adult *A. parvus* and *L. angulatus* was characterized by capturing beetles with cross-vane panel traps (black corrugated plastic, Alpha Scents, Portland, OR) coated with the fluoropolymer dispersion Fluon® (10% aqueous dilution; Northern Products, Woonsocket, RI, USA) to improve capture efficiency (for details on trapping methods, see Graham et al. 2010). Traps were hung from inverted L-shaped frames of polyvinylchloride pipe with trap bottoms ~0.5 m above the ground. Trap lures consisted of polyethylene sachets (5.1 × 7.6 cm, Bagettes® model 14770, Cousin Corp., Largo, FL, USA) which were loaded with 50 mg of synthesized pheromone dissolved in 1 ml isopropanol (a neutral solvent for cerambycids; Hanks et al. 2012), with separate lures for racemic fuscumol and fuscumol acetate. Traps also were baited with ethanol, because it synergizes attraction to pheromones for some lamiine species (Hanks et al. 2012). The ethanol lures consisted of polyethylene sachets (10 × 15 cm, 0.05 mm thick, Bagettes® model 14772, Cousin Corp.) loaded with 100 ml of ethanol. The supplied collection basins of traps were replaced with a mechanism which changed eight trap jars at programmable intervals (henceforth "timer traps"; model #2850, BioQuip Products, Rancho Dominguez, CA, USA). Individual traps were deployed at the Allerton Park and Brownfield Woods study sites (Table 1) during 16 June to 5 September 2013, except during periods of inclement weather. Timer traps were programmed to rotate jars at seven 1-h intervals, beginning at 19:00 h and ending at 2:00 h, so as to encompass the expected crepuscular to nocturnal activity periods of lamiines. The eighth jar was not rotated for 17 h (2:00 – 19:00), so as to confirm that

beetles were not active during the remainder of each 24 h cycle. The hour that beetles were caught was estimated as the median time that their trap jar was positioned under the trap. Overlap between the two species in diel flight period was assessed by calculating, for each species, the percentage of adults which were captured during the flight period of the other species.

Identification of Pheromones

Beetles for collection of headspace odors were captured alive with panel traps as described above, but with trap basins replaced with 2-l plastic jars with their bottoms replaced with aluminum screen to allow rainwater to drain. Trap lures consisted of polyethylene sachets as described above, but loaded with the blend of racemic fuscumol and fuscumol acetate (50 mg each) dissolved in 1 ml isopropanol. Single traps were deployed during May to August 2013 and 2014 at all four study sites (Table 2.1). Traps were serviced and beetles were collected every 1 or 2 d. Trap lures were replaced as needed, usually after 10-14 d.

Captured beetles were sexed by the morphology of the fifth abdominal sternite (Linsley and Chemsak 1995), or by pairing beetles in the laboratory and observing their behavior (i.e., males will attempt to mate by mounting females). Beetles were caged, separately by species and sex, under ambient laboratory conditions (~12:12 h L:D, ~20 °C). Adults of both study species feed on bark of oak branchlets (pers. obs.), and so caged beetles were provided sections of branches (2 – 5 cm in diameter, ~8 cm long) freshly cut from oak trees (*Quercus alba* L. and *Q. rubra* L.) at the same field sites where beetles were trapped. Beetles also were provided sugar water as a source of moisture (10% aqueous sucrose solution in a glass vial with cotton wick). Beetles were allowed to acclimate for at least 24 h before being aerated, and between aerations.

Volatiles produced by beetles were collected by aerating them in glass Mason-style canning jars placed adjacent to closed exterior windows (natural photoperiod, ~14:10 h L:D, ~20 °C). Clean air (1 l/min) was pulled through the jars by vacuum for 24 h. Headspace volatiles were collected with glass tube cartridges which contained a layer of the adsorbent polymer HayeSep® Q (150 mg; Sigma-Aldrich) between plugs of glass wool, attached to the chamber outlet. Beetles usually were aerated individually, but in some cases two or three beetles of the same sex were aerated together in case the presence of conspecifics enhanced pheromone release. Once it was confirmed that females did not produce volatile chemicals in detectable quantities (see Results), the sexes were sometimes aerated together, in case the presence of females stimulated males to call. Beetles usually were aerated with fresh twigs of oak (provided as food), and aerations of jars without beetles (including host material when appropriate) were run simultaneously as controls for system contaminants. Additional controls included jars containing twigs that had been damaged mechanically, by scraping or gouging the bark, to simulate feeding damage by beetles which may result in release of plant volatiles. Numbers of males and females that were aerated were 33 and 20 for *A. parvus*, and 16 and 8 for *L. angulatus*.

Insect-produced chemicals were recovered from adsorbent cartridges by extraction with 1.5 ml of dichloromethane. Extracts were analyzed with a gas chromatograph (GC) interfaced to a mass selective detector (Models 6890 and 5973, Hewlett-Packard, Palo Alto, CA, USA) fitted with a AT-5ms column (30 m × 0.25 mm i.d., 0.25 μm film; Alltech Associates Inc., Deerfield, IL, USA). The GC oven was programmed from 35 °C/1 min, 10 °C/min to 210 °C, hold 3 min. Injections were made in splitless mode, with an injector temperature of 250 °C, and helium carrier gas. Sex-specific compounds were identified by comparing spectra and retention times to those of authentic standards.

The enantiomeric ratio and absolute configuration of insect-produced fuscumol acetate and the double bond configuration of geranylacetone were determined by analyzing aliquots of extracts with an HP 5890 GC fitted with a chiral stationary phase Cyclodex B column (30 m × 0.25 mm i.d., 0.25 μm film; Agilent Technologies, Inc., Santa Clara, CA, USA). The oven temperature was programmed from 50 °C/1 min, 2.5 °C/min to 200 °C, hold 5 min, with an injector temperature of 210 °C. Structures were confirmed by coinjection of an aliquot of aeration extract with the mixture of synthetic stereoisomers (Millar et al. 2009); (*R*)- and (*S*)-fuscumol acetate (retention times 51.33 and 51.72 min, respectively) and (*Z*)- and (*E*)-geranylacetone were resolved to baseline (retention times 14.53 and 14.74 min, respectively).

Because the enantiomers of fuscumol did not resolve on the chiral column, fuscumol in extracts was esterified with (*S*)-*O*-acetyl lactic acid chloride (Slessor et al. 1985), with the method modified as described by Hughes et al. (2013). The resulting diastereomeric derivatives were separated to baseline on an achiral DB-5 GC column (30 m × 0.25 mm i.d., 0.25 μ film, J&W Scientific, Folsom, CA, USA). The oven temperature was programmed from 40 °C/1 min, 10 °C/min to 250 °C, hold 5 min, with an injector temperature of 250 °C, and helium carrier gas. The retention times of the diastereomeric esters of (*R*)- and (*S*)-fuscumol were 18.94 minutes and 19.03 minutes, respectively. Samples of racemic and (*R*)-fuscumol were esterified under the same conditions to distinguish between the diastereomers, and to verify that the derivatizing reagent was enantiomerically pure. Overall, chirality determinations were made with four samples from male *A. variegatus* and five samples from *A. parvus*.

Field Bioassays of Pheromones

Attraction of beetles to synthesized pheromones was tested with four independent field bioassays because the large number of possible combinations of compounds would have resulted in excessively long trap lines if they had all been tested at once. Beetles were caught with panel traps as described above, but with the trap basins filled with ~300 ml of saturated aqueous NaCl solution to kill and preserve captured beetles. Traps were positioned 10 m apart in linear transects, with one treatment per transect randomly assigned to one trap on the first day. Traps were serviced every 1-3 d, at which time treatments were rotated one position along transects to control for positional effects.

All four experiments shared two treatments for comparative purposes (Table 2.2): racemic fuscumol and, separately, racemic fuscumol acetate (which simulated the pheromone of *L. angulatus*, see Results). Experiment 1 was designed as a follow-up to the screening trial of Mitchell et al. (2011), but with treatments added to test the influence of geranylacetone (see Results), and to test for attraction to geranylacetone alone (Table 2.2). In this experiment, synthesized pheromones were dispensed from sachets made from heat-sealed polyethylene tubing (Associated Bag, Milwaukee, WI, USA), which were loaded with 1 ml of neat chemical. Lure release rates were standardized to ~20 mg/d by using sachets with different wall thicknesses and adding a cotton roll (1 × 4 cm dental wick, Patterson Dental Supply, Inc., St. Paul, MN, USA) to some lures, as follows: fuscumol (1.5 mil [38 µm] wall thickness, with cotton roll); fuscumol acetate (3 mil [76 µm] wall thickness, with cotton roll), geranylacetone (3 mil wall thickness, without cotton roll). An empty 3 mil wall thickness sachet served as a blank control. The experiment was conducted during three years: 5 August to 5 September 2013 at Vermilion River Observatory (Table 1), 22 June to 6 August 2014 at Allerton Park and Brownfield Woods

(one transect at each site), and 15 July to 16 September 2015 at Forest Glen Preserve. Lures were replaced every 14 d.

For the remaining three experiments, trap lures consisted of the Bagettes® model 14772 polyethylene sachets loaded with dilute synthesized pheromones. The experiments were conducted at Brownfield Woods and Nettie Hart Woods (Table 2.1). Experiment 2 (25 July to 3 September 2014 and 15 June to 17 September 2015) tested attraction to various blends of racemic and chiral fuscumol and fuscumol acetate, including simulations of the pheromone blends of both species (Table 2.2; see Results). Experiment 3 (15 June to 17 September 2015) was similar to the second experiment, but included different combinations of chiral compounds (Table 2.2). Experiment 4 (15 June to 17 September 2015) tested a third combination of treatments so as to assess attraction to individual chiral compounds versus individual racemic blends (Table 2.2).

For each experiment, differences between treatment means, blocked by site and date, were tested separately for each species using the nonparametric Friedman's Test (PROC FREQ, option CMH; SAS Institute 2011) because data violated homoscedasticity assumptions of ANOVA (Sokal and Rohlf 1995). Thus, replicates were defined by study site and collection date. Assuming a significant overall Friedman's test, pairs of treatment means were compared with the nonparametric Dunn-Nemenyi multiple comparison test (Elliot and Hynan 2011; Zar 2010). Replicates that contained no specimens of the species in question were dropped from analyses.

Taxonomy of captured beetles follows Monné and Hovore (2005). Specimens of species that were represented in the data set are available from the laboratory collection of LMH, and voucher specimens have been deposited with the collection of the Illinois Natural History Survey, Champaign, IL.

Results

Diel Phenology of Adults

Timer traps captured 26 adult *A. parvus* and 45 adult *L. angulatus*, with most adults of both species caught between 29 June and 28 August 2013. Both species showed a strongly skewed frequency distribution (Fig. 2.2), with most beetles caught within the first hour, which was between 1950 and 2050 h for *A. parvus*, and 2050 and 2150 h for *L. angulatus*. Thus, adults became active with the onset of complete darkness (solar radiation fell to zero between 1800 and 2000 h during the trapping period; Water and Atmospheric Resources Monitoring Program, Illinois Climate Network 2014, Illinois State Water Survey, Champaign, IL: http://dx.doi.org/10.13012/J8MW2F2Q). The two species overlapped broadly in diel phenology, with ~61% of *A. parvus* caught during the activity period of *L. angulatus*, and 100% of *L. angulatus* caught during the activity period of *A. parvus*.

Identification of Pheromones

Extracts of volatiles emitted by males of both species contained fuscumol and/or fuscumol acetate, and (*E*)-geranylacetone, with detectable quantities in 13 of 33 extracts from male *A. parvus* and 11 of 16 extracts from male *L. angulatus*. These compounds were not detected in any aeration extracts from females, nor any system controls. Male *A. parvus* produced (*R*)- + (*S*)-fuscumol, (*R*)-fuscumol acetate, and (*E*)-geranylacetone in a ratio of ~3:1:1 (*R*:*S*-fuscumol ranging from 1:10 to 10:1; N = 4), and male *L. angulatus* produced (*R*)- + (*S*)-fuscumol acetate and (*E*)-geranylacetone in a ratio of ~3:1 (*R*:*S*-fuscumol acetate ranging from 2:1 to 8:3; N = 5).

Field Bioassays of Pheromones

Exactly 1,800 cerambycid beetles of 46 species were trapped during the four field experiments (Table 2.3), including representatives of the subfamilies Cerambycinae, Lamiinae, Lepturinae, Parandrinae, Prioninae, and one species in the closely related family Disteniidae. Most of the trapped beetles (~91%) were lamiines, with the species caught in largest numbers being the two targeted species, *A. parvus* and *L. angulatus*, followed by three species in the same tribe (Acanthocinini), *Graphisurus fasciatus* (Degeer), its congener *G. despectus* (LeConte), and *Astylopsis macula* (Say), as well as *A. variegatus* in the tribe Acanthoderini (Table 2.3). Both sexes of *A. parvus* and *L. angulatus* were caught, for example with sex ratios of 43 and 50% female in Experiment 1.

In all four experiments, adults of both *A. parvus* and *L. angulatus* were significantly influenced by the experimental treatments, and generally in accordance with their pheromone chemistry. In Experiment 1, the greatest number of adult *A. parvus* were captured by traps baited with racemic fuscumol, with or without fuscumol acetate and/or geranylacetone (Fig. 2.3; treatments F, F+Ga, F+Fa, and F+Ga+Fa), consistent with males producing both enantiomers of fuscumol. These findings suggest that both enantiomers of fuscumol are necessary and sufficient for attraction, that the (*R*)-fuscumol acetate component is not essential, and that geranylacetone does not influence attraction and is not attractive alone. This experiment also indicated that the non-natural (*S*)-fuscumol acetate was not antagonistic. The remaining experiments supported these conclusions (Figs. 2.4A, 2.5A, 2.6A), confirming that both enantiomers of fuscumol are necessary, and that neither enantiomer of fuscumol acetate influences attraction.

In contrast, Experiment 1 revealed that adult *L. angulatus* were significantly attracted to racemic fuscumol acetate alone (Fig. 2.3B), consistent with males producing both enantiomers,

but attraction was significantly enhanced by geranylacetone. Fuscumol antagonized attraction to both fuscumol acetate (in treatment F+Fa, Fig. 2.3B), and the blend of fuscumol acetate and geranylacetone (in treatment F+Fa+Ga). There was some support for this antagonism in Experiment 2 (Fig. 2.4B), with the mean for the blend of racemic fuscumol and fuscumol acetate being not significantly different from the mean for the control, although the individual enantiomers of fuscumol apparently did not influence attraction. Nevertheless, there was no evidence that racemic fuscumol antagonized attraction of *L. angulatus* to fuscumol acetate in Experiment 3 (Fig. 2.5B). Finally, Experiment 4 reconfirmed that both enantiomers of fuscumol acetate were necessary for attraction of *L. angulatus* (Fig. 2.6B).

Discussion

Species in subfamilies other than Lamiinae generally were represented by few specimens which probably were captured by random encounters with traps. The fact that 41 adults of the cerambycine *Xylotrechus colonus* (F.) were caught might suggest significant attraction, but that species is among the most common and abundant cerambycids in eastern North America, and even low levels of attraction would have resulted in capture of much greater numbers of beetles (e.g., see Hanks et al. 2014). Other non-lamiine species that were fairly numerous are among those that typically are captured by panel traps regardless of how they are baited, such as the cerambycine *Elaphidion mucronatum* (Say), the parandrine *Neandra brunnea* (F.), and the prionine *Orthosoma brunneum* (Forster) (e.g., see Hanks and Millar 2013).

Astylidius parvus and *L. angulatus* overlap broadly in the phenology of their adults, as revealed in earlier studies of their seasonal phenology (Hanks et al. 2014), and the study of diel phenology reported here. Identification of the possible pheromone components produced by the

males and field bioassays of racemic and chiral pheromone components supported the hypothesis that interspecies attraction is minimized by differences in the chemistry of their pheromones. Thus, adult *A. parvus* required both enantiomers of fuscumol for attraction, neither of which are produced by *L. angulatus*, and adult *L. angulatus* required both enantiomers of fuscumol acetate for attraction, whereas male *A. parvus* produce only the (*R*)-enantiomer. Therefore, the mechanisms that prevent interspecific attraction are complementary, effectively preventing attraction in either direction, as has been reported among some sympatric cerambycine species (Mitchell et al. 2015).

Although the two enantiomers of fuscumol were essential for attraction of adult *A. parvus*, neither the (*R*)-fuscumol acetate nor the geranylacetone found in the male-produced volatiles appeared to play a role in attraction of conspecifics. However, geranylacetone did enhance attraction of *L. angulatus* to fuscumol acetate, indicating that for this species, it is indeed a pheromone component. Geranylacetone is also a component of the volatile blend produced by males of the South American *H. betulinus*, possibly being sequestered directly from host plants of the adults (Fonseca et al. 2010) as a biosynthetic precursor of fuscumol and fuscumol acetate (Zarbin et al. 2013).

Synergism between enantiomers of pheromone components, such as that shown by *A. parvus* and *L. angulatus*, is uncommon in insects (Mori 2007), but most of the known examples are from the Coleoptera. In the first example reported, females of the ambrosia beetle *Gnathotrichus sulcatus* LeConte (Curculionidae: Scolytinae) produced a 35:65 blend of (*R*)- and (*S*)-sulcatol, and field bioassays showed that beetles were attracted only to blends of the enantiomers, and not to either pure enantiomer (Borden et al. 1976; Bordon et al. 1980; Byrne et al. 1974). Bordon et al. (1980) also found that the sympatric congener *G. retusus* (LeConte)

produced and responded only to (*S*)-sulcatol, demonstrating how differences in the enantiomeric composition created species-specific pheromone channels. Even more intriguing, Miller et al. (1996) found that in the pine engraver, *Ips pini* (Say) (Scolytinae), the enantiomeric ratio of ipsdienol varied among geographic populations, possibly resulting from character displacement where *I. pini* was sympatric with other species that also used ipsdienol as a pheromone component. In another beetle family (Laemophloeidae), the stored products pest *Cryptolestes turcicus* (Grouvelle) uses nonracemic mixtures of the enantiomers of macrocyclic lactones in its pheromone blend (Millar et al. 1985, Oehlschlager et al. 1987).

These precedents for enantiomeric synergism raise the possibility that the manipulation of enantiomeric ratios to create unique aggregation pheromone channels may be common among sympatric cerambycid species that produce fuscumol and fuscumol acetate as pheromone components. For example, in addition to the examples described in this paper, males of *H. betulinus* produce an 82:18 blend of (*R*)- and (*S*)-fuscumol, but pure (*R*)-fuscumol acetate (Vidal et al. 2010). However, field bioassays of the enantiomers, alone or in blends, have not yet been reported for *H. betulinus*. In contrast, males of the North American *A. variegatus* produce pure (*S*)-fuscumol and (*S*)-fuscumol acetate (Hughes et al. 2013, 2016), although bioassays comparing the racemic and enantiomeric forms of the two compounds have not yet been reported. Moreover, racemic fuscumol and fuscumol acetate, individually or in combination, have been shown to attract a number of other sympatric lamiine species, plus a species in the Cerambycinae, *Obrium maculatum* (Olivier) (e.g., Hanks and Millar 2013; Hanks et al. 2012, Mitchell et al. 201l). As the pheromones of these lamiine species are identified, other examples of enantiomeric synergism may be found, as well as examples of inhibition by enantiomers.

Similarly, among European species in the subfamily Spondylidinae, males of *Tetropium fuscum* (F.) produce (*S*)-fuscumol (Silk et al. 2007), and adults of the congener *T. castaneum* (L.) are attracted by the same compound when released along with host plant volatiles (Sweeney et al. 2010). These two species overlap in their geographical distribution in Europe (Bense 1995), suggesting that mechanisms other than pheromone chemistry serve to limit interspecific attraction.

Previous research already had shown that adult *A. parvus* were attracted by fuscumol and not fuscumol acetate, and vice versa for *L. angulatus* (Mitchell et al. 2011), suggesting that the composition of their pheromones could have been predicted beforehand. However, trap catches in field bioassays of blends of synthetic pheromones may sometimes be misleading, because even weak attraction to incorrect ratios, or blends that lack synergistic components, may nevertheless result in statistical significance relative to unbaited controls. For example, Mitchell et al. (2011) found that adult *A. variegatus* were significantly attracted to racemic fuscumol acetate in comparison to controls, despite the absence of the (*S*)-fuscumol component of its pheromone. However, those authors subsequently discovered that attraction to racemic fuscumol fell to insignificant levels in the presence of lures containing a blend of racemic fuscumol + fuscumol acetate (Mitchell et al. 2011). It still remains to be seen what effect the "non-natural" (*R*)-enantiomers of fuscumol and fuscumol acetate have on attraction of *A. variegatus*, and to what degree attraction is influenced by the ratios of the various components.

Identification of the pheromones of *A. parvus*, *L. angulatus*, and previously of *A. variegatus* (Hughes et al. 2013), and earlier reports that adults of other species in the tribes Acanthocinini and Acanthoderini are attracted by fuscumol and/or fuscumol acetate (Mitchell et al. 2011), indicate that these compounds comprise another conserved pheromone motif within

the Cerambycidae. However, there are nuances at play, because the fuscumol/fuscumol acetate pheromone channel appears to be quite finely partitioned among these species. If indeed species specificity in pheromone composition could be afforded only by the chirality of fuscumol and fuscumol acetate, and assuming that pheromones could be composed of one to four components (i.e., a single enantiomer of either fuscumol and fuscumol acetate, versus both enantiomers of each), there are 14 unique combinations available as species-specific pheromone blends, even without considering differences in blend ratios. Moreover, the role of geranylacetone in enhancing attraction of *L. angulatus* to the other pheromone components suggests that it may further extend the number of unique pheromone blends that are available. Thus, different subsets and ratios of the five compounds can accommodate a substantial number of blends, more than enough to minimize cross attraction within individual communities of sympatric and synchronic lamiine species that use fuscumol, fuscumol acetate, and/or geranylacetone as pheromone components.

Acknowledgements

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Tables

Table 2.1. Study sites for field bioassays conducted in east-central Illinois during 2013-2015

County	Name	GPS coordinates (lat., lon.)	Area (ha)
Champaign	Brownfield Woods[a]	40.145, -88.165	26
Champaign	Nettie Hart Memorial Woods[a]	40.229, -88.358	16
Champaign	Trelease Woods[a]	40.132, -88.141	29
Piatt	Robert Allerton Park[b]	39.996, -88.651	600
Vermilion	Forest Glen Preserve[c]	40.015, -7.568	3

[a] University of Illinois natural area (http://research.illinois.edu/cna/)
[b] Property of the University of Illinois
[c] Vermilion County Conservation District (http://www.vccd.org/)

Table 2.2. Experimental treatments for four field bioassays that tested attraction of adult *Astylidius parvus* and *Lepturges angulatus* to various blends of racemic and chiral pheromone components (for structures, see Fig. 1)

	Treatment
Expt. 1	Racemic fuscumol
	Racemic fuscumol + (*E/Z*)-geranylacetone
	Racemic fuscumol acetate (= pheromone of *L. angulatus*)
	Racemic fuscumol acetate + (*E/Z*)-geranylacetone
	Racemic fuscumol + fuscumol acetate
	Racemic fuscumol + fuscumol acetate + (*E/Z*)-geranylacetone
	(*E/Z*)-geranylacetone
	Solvent control
Expt. 2	Racemic fuscumol
	Racemic fuscumol acetate (= pheromone of *L. angulatus*)
	Racemic fuscumol + racemic fuscumol acetate
	Racemic fuscumol + (*R*)-fuscumol acetate (= pheromone of *A. parvus*)
	Racemic fuscumol + (*S*)-fuscumol acetate
	(*R*)-fuscumol + racemic fuscumol acetate
	(*S*)-fuscumol + racemic fuscumol acetate
	Solvent control
Expt. 3	Racemic fuscumol
	Racemic fuscumol acetate (= pheromone of *L. angulatus*)
	Racemic fuscumol + fuscumol acetate
	(*R*)-fuscumol + (*R*)-fuscumol acetate
	(*R*)-fuscumol + (*S*)-fuscumol acetate
	(*S*)-fuscumol + (*S*)-fuscumol acetate
	(*S*)-fuscumol + (*R*)-fuscumol acetate
	Solvent control
Expt. 4	Racemic fuscumol
	(*R*)-fuscumol
	(*S*)-fuscumol
	Racemic fuscumol acetate (= pheromone of *L. angulatus*)
	(*R*)-fuscumol acetate
	(*S*)-fuscumol acetate
	Solvent control

Table 2.3. Total numbers of adult cerambycid beetles that were captured during field experiments conducted in east-central Illinois

Taxonomy	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Total
Cerambycinae					
Clytini					
Clytoleptus albofasciatus (Laport and Gory)	1				1
Neoclytus a. acuminatus (F.)	2			1	3
Neoclytus scutellaris (Olivier)				2	2
Xylotrechus colonus (F.)	11	12	13	5	41
Elaphidiini					
Anelaphus villosus (F.)		3	2		5
Elaphidion mucronatum (Say)	15	9	5	3	32
Parelaphidion aspersum (Haldeman)		1			1
Neoibidionini					
Heteracthes quadrimaculatus Haldeman	1				1
Parelaphidion incertum (Newman)	1		1	1	3
Obriini					
Obrium rufulum Gahan		1			1
Smodicini					
Smodicum cucujiforme (Say)		1			1
Lamiinae					
Acanthocinini					
Astyleiopus variegatus (Haldeman)	67	49	16	1	133
Astylidius parvus (LeConte)	338	119	28	17	502
Astylopsis macula (Say)	22	51	24	13	110
Astylopsis sexguttata (Say)	1				1
Graphisurus despectus (LeConte)	65	8	3	4	80
Graphisurus fasciatus (Degeer)	125	50	14	5	194
Hyperplatys aspersa (Say)	1				1

Table 2.3 (cont.)

Hyperplatys maculata Haldeman	2	1	1	2	6
Leptostylus transversus (Gyllenhal)	4	2	2		8
Lepturges angulatus (LeConte)	229	77	19	7	332
Lepturges confluens (Haldeman)	37	1	4	1	43
Lepturges pictus (LeConte)	6	4	1	1	12
Lepturges regularis (LeConte)	8				8
Lepturges symmetricus (Haldeman)	7	8	7	1	23
Sternidius alpha (Say)		43	5	7	55
Urgleptes querci (Fitch)	6	3	8	2	19
Urgleptes signatus (LeConte)	1	2	1	1	5
Acanthoderini					
Aegomorphus modestus (Gyllenhal)	70	7	6	2	85
Oplosia nubila (LeConte)	1				1
Desmiphorini					
Psenocerus supernotatus (Say)	2				2
Dorcaschematini					
Dorcaschema cinereum (Olivier)		1	1	1	3
Monochamini					
Microgoes oculatus (LeConte)	2	2			4
Pogonocherini					
Ecyrus d. dasycerus (Say)	1	1	1	3	6
Saperdini					
Saperda imitans Felt and Joutel	1		1		2
Saperda lateralis F.		2			2
Saperda tridentata Olivier	3		1		4
Lepturinae					
Lepturini					
Analeptura lineola (Say)		1			1
Strangalia luteicornis (F.)	1			1	2
Strophiona nitens (Forster)	1				1

Table 2.3 (cont.)

Typocerus lugubris (Say)		3	1		4
Typocerus v. velutinus (Olivier)	1	2			3
Parandrinae					
Parandrini					
Neandra brunnea (F.)		28	3	4	35
Prioninae					
Prionini					
Orthosoma brunneum (Forster)	4	4	6	6	20
Disteniidae					
Disteniini					
Elytrimitatrix undata (F.)		2			2
Total	1,037	497	175	91	1,800

Figures

Fig. 2.1. Structures of geranylacetone and stereoisomers of fuscumol and fuscumol acetate.

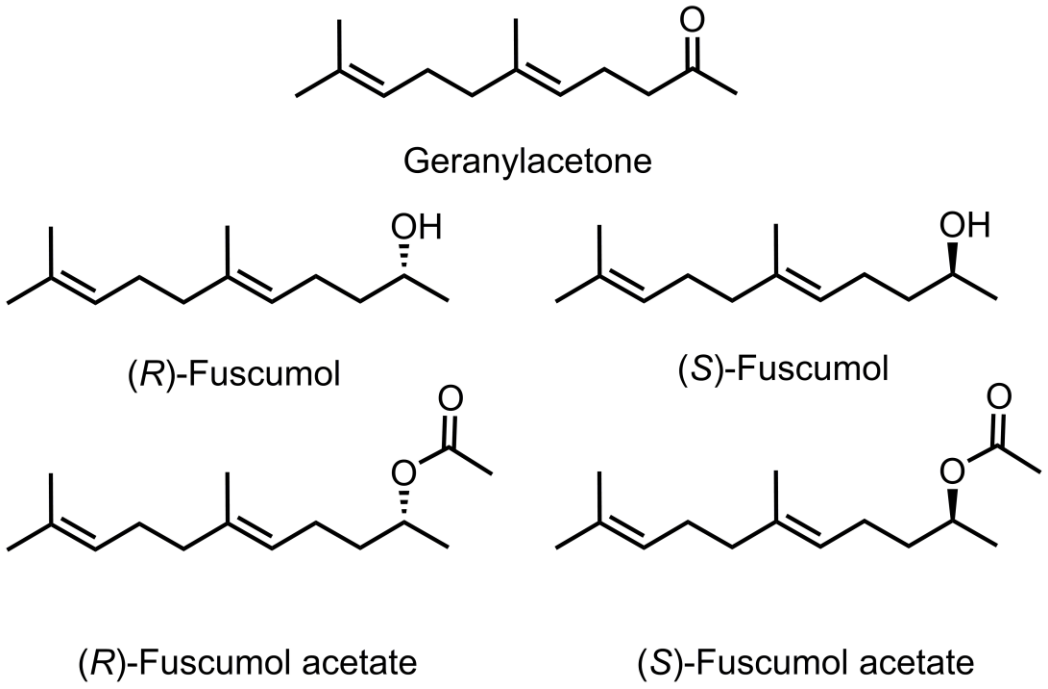


Fig. 2.2. Diel phenology of adult *Astylidius parvus* and *Lepturges angulatus* as determined by the time of day that beetles were captured by traps which were fitted with a mechanism which changed collection jars at hourly intervals. Time of day (h) represents time class midpoint.

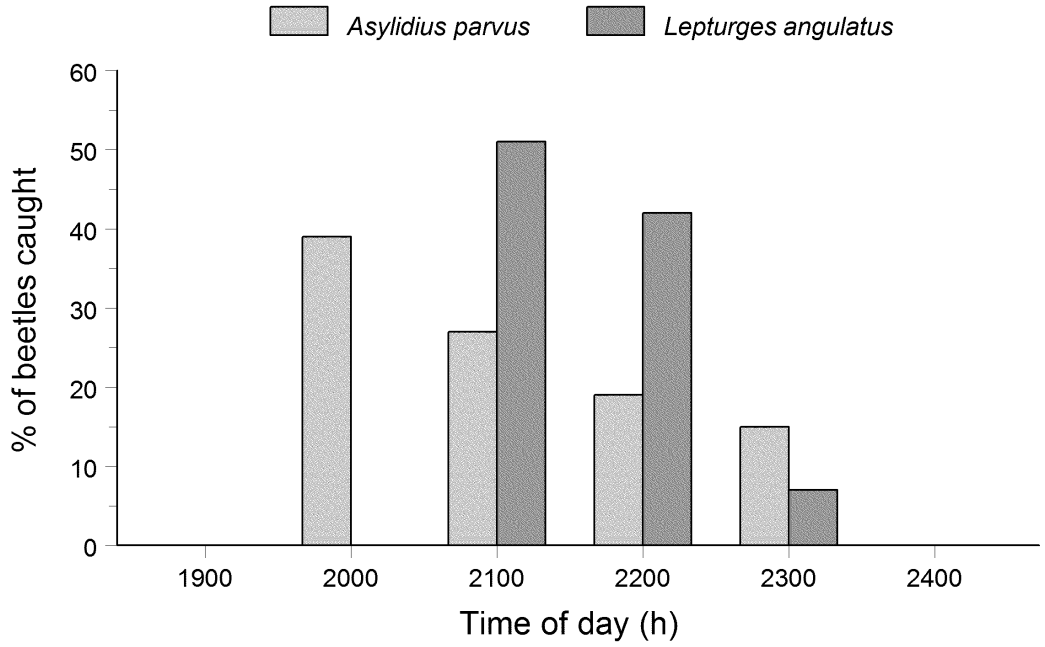


Fig. 2.3. Mean (± SE) number of adult *Astylidius parvus* (A) and *Lepturges angulatus* (B) that were caught per replicate during Experiment 1. Chemical abbreviations: F = racemic fuscumol, Fa = racemic fuscumol acetate, Ga = geranylacetone. Means significantly different: Friedman's $Q_{7,297} = 118.2$, $P < 0.001$, and $Q_{7,249} = 106.9$, $P < 0.001$, respectively. Means with different letters are significantly different (Dunn-Nemenyi multiple comparison test, $P < 0.05$).

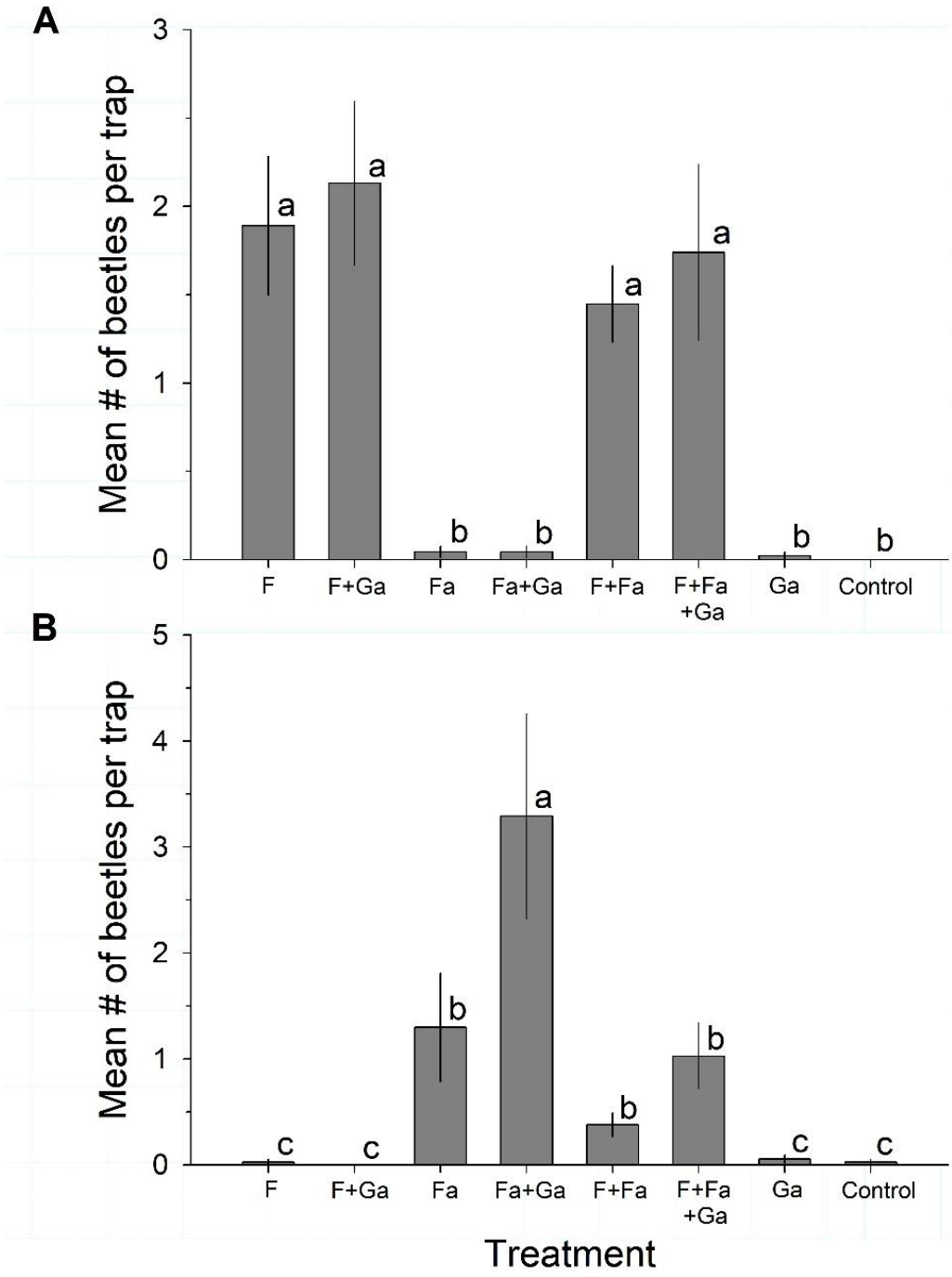


Fig. 2.4. Mean (± SE) number of adult *Astylidius parvus* (A) and *Lepturges angulatus* (B) that were caught per replicate during Experiment 2. Chemical abbreviations: F = racemic fuscumol, Fa = racemic fuscumol acetate, with enantiomers indicated by *R* and *S*. Means significantly different: Friedman's $Q_{7,96}$= 29.7, $P < 0.001$, and $Q_{7,120} = 49.6$, $P < 0.001$, respectively. Means with different letters are significantly different (Dunn-Nemenyi multiple comparison test, $P < 0.05$).

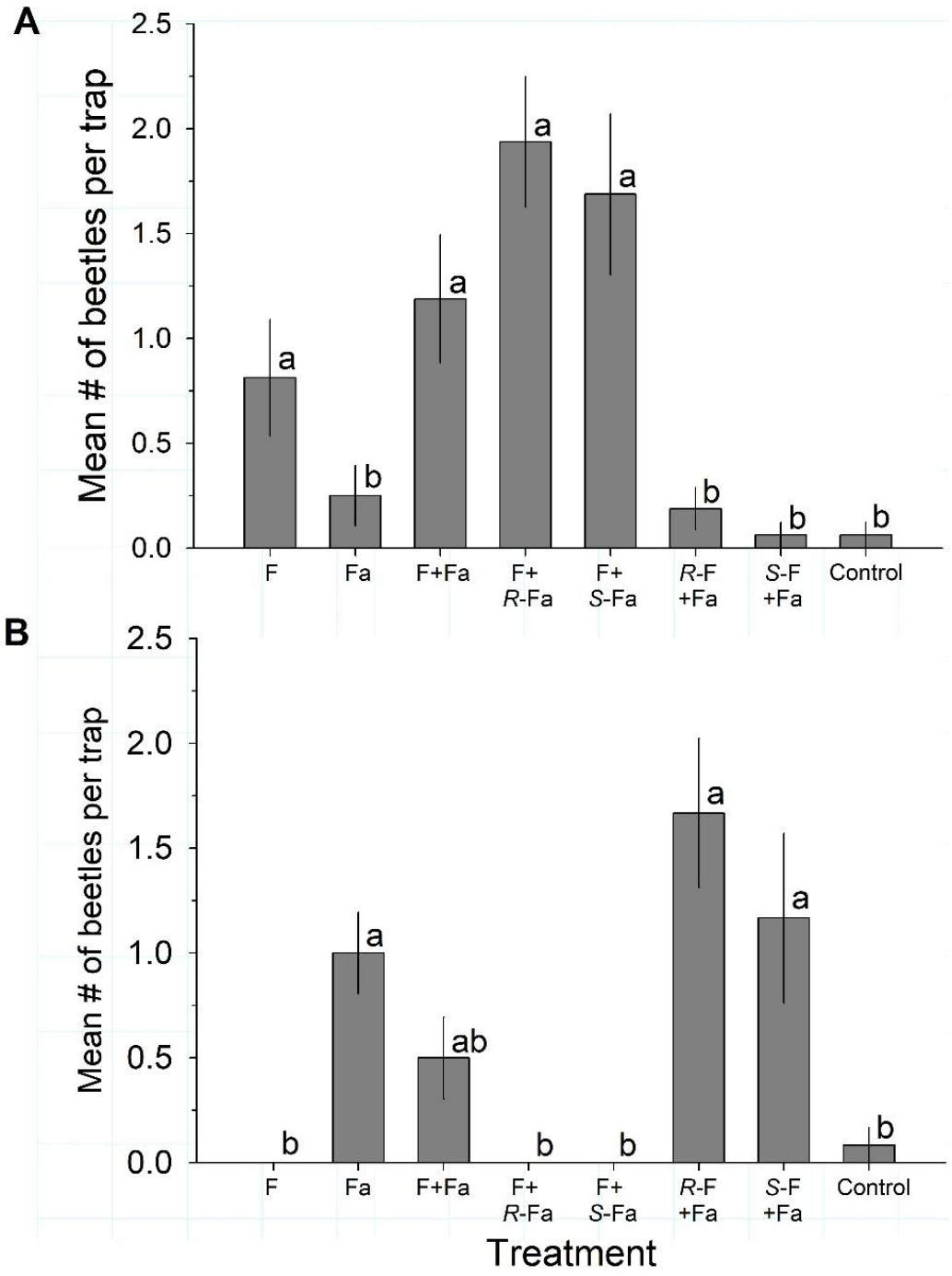


Fig. 2.5. Mean (± SE) number of adult *Astylidius parvus* (A) and *Lepturges angulatus* (B) that were caught per replicate during Experiment 3. Chemical abbreviations: F = racemic fuscumol, Fa = racemic fuscumol acetate, with enantiomers indicated by *R* and *S*. Means significantly different: Friedman's $Q_{7,240} = 56.0$, $P < 0.001$, and $Q_{7,264} = 68.6$, $P < 0.001$, respectively. Means with different letters are significantly different (Dunn-Nemenyi multiple comparison test, $P < 0.05$).

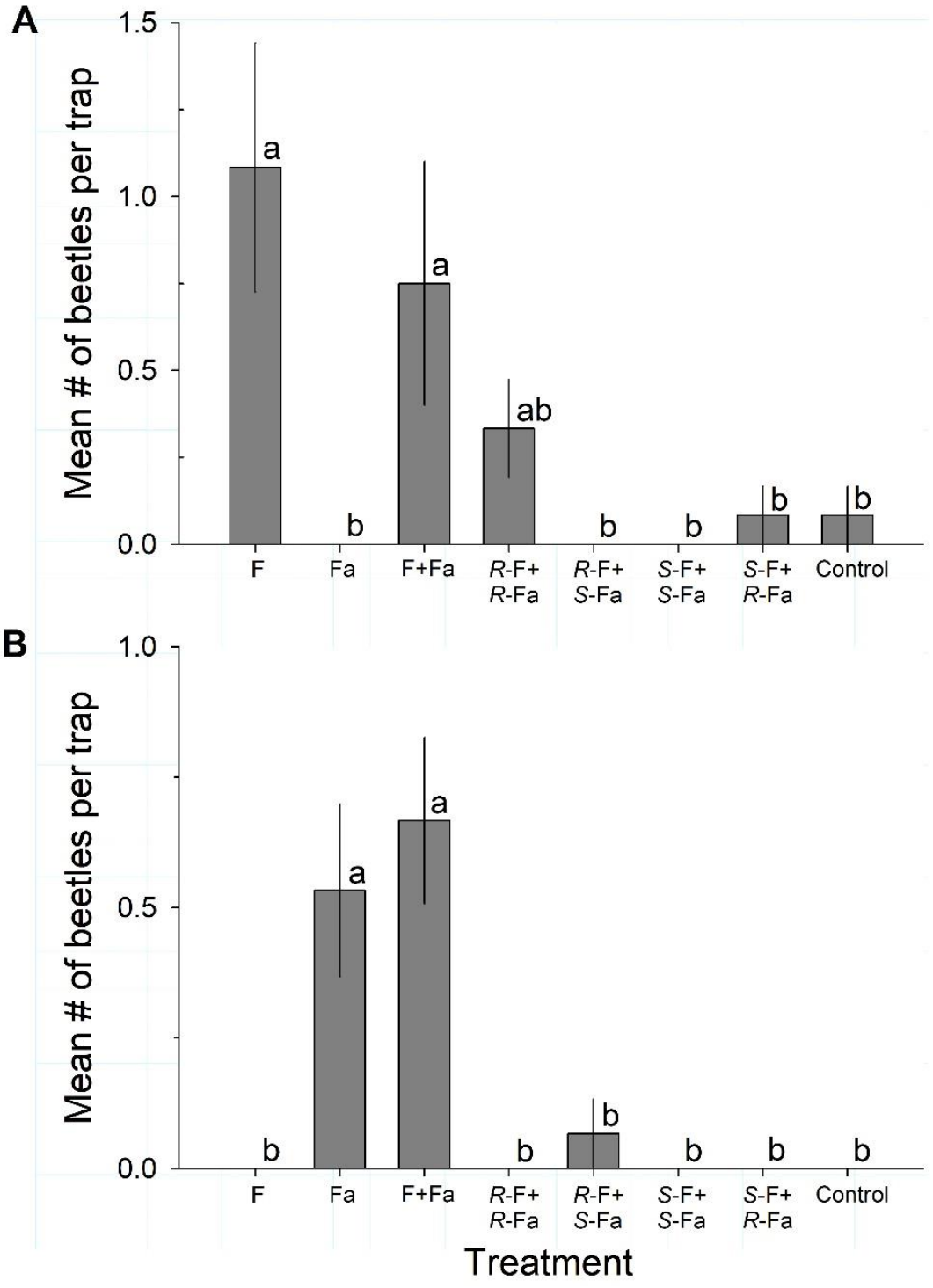
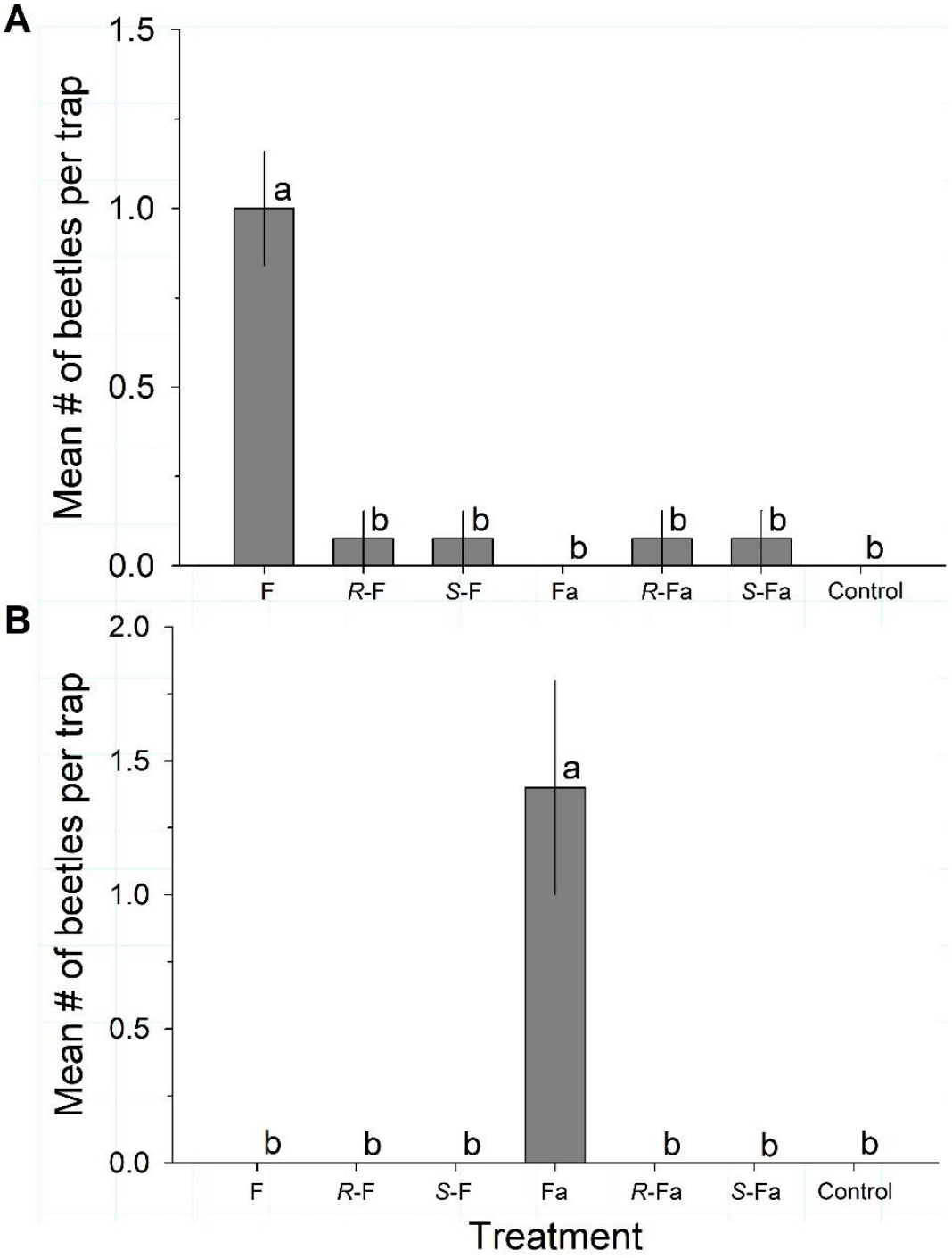


Fig. 2.6. Mean (± SE) number of adult *Astylidius parvus* (A) and *Lepturges angulatus* (B) that were caught per replicate during Experiment 4. Chemical abbreviations: F = racemic fuscumol, Fa = racemic fuscumol acetate, with enantiomers indicated by *R* and *S*. Means significantly different: Friedman's $Q_{6,91} = 52.1$, $P < 0.001$, and $Q_{6,35} = 33.9$, $P < 0.001$, respectively. Means with different letters are significantly different (Dunn-Nemenyi multiple comparison test, $P < 0.05$).



Erratum to: Synergism between enantiomers creates species-specific pheromone blends and minimizes cross-attraction for two species of cerambycid beetles

Erratum to: J Chem Ecol DOI 10.1007/s10886-016-0782-z

In the original version of this article, a line on page 36, first paragraph, second sentence, has the retention times for (*R*)- and (*S*)- fuscumol acetate reversed. The correct sentence should read, “Structures were confirmed by coinjection of an aliquot of aeration extract with the mixture of synthetic stereoisomers (Millar et al. 2009); (*S*) - and (*R*)-fuscumol acetate (retention times 51.33 and 51.72 min, respectively) and (*Z*) - and (*E*)- geranylacetone were resolved to baseline (retention times 14.53 and 14.74 min, respectively).” The authors regret this error.

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CHAPTER 3: PHEROMONE COMPOSITION OF FIVE SPECIES PROVIDES INSIGHT INTO CHEMICAL COMMUNICATION AND AVOIDANCE OF INTERSPECIFIC ATTRACTION IN LAMIINE CERAMBYCID BEETLES

Introduction

The chemical ecology of beetles in the family Cerambycidae has been extensively studied during the last 15 years, with pheromones at least tentatively identified for nearly 300 species (Millar and Hanks 2017). Most research has focused on the Cerambycinae, the second largest subfamily, which includes many important pests of woody plants throughout the world (Švácha and Lawrence 2014). To date, all known pheromones of cerambycines are produced by males and attract both sexes (Millar and Hanks 2017) and thus are considered aggregation-sex pheromones (*sensu* Cardé 2014). One important paradigm that has emerged from this work is that the pheromone chemistry of cerambycines is often highly conserved, even among sympatric species that would seem to be subject to interspecific attraction due to their similar, and in some cases identical, pheromone chemistry (reviewed by Millar and Hanks 2016). For example, many cerambycine species native to different continents use pheromones composed of hydroxyalkanones and the related alkanediols (e.g., Narai et al. 2015; Ray et al. 2015; Silva et al. 2018). The ubiquity of these compounds as pheromones of cerambycids is further indicated by attraction of multiple species to traps baited with single pheromone components during field screening bioassays in Asia, Europe, Australia, South America, and North America (Fan et al. 2018; Hayes et al. 2016; Imrei et al. 2013; Millar et al. 2018; Silva et al. 2017; Sweeney et al. 2014; Wickham et al. 2014). Nevertheless, there is growing evidence that some species of cerambycines use pheromones of different structural classes that may be shared with few other

species, or possibly are species-specific (e.g., Millar et al. 2017; Silva et al. 2016; Zou et al. 2015).

Among sympatric cerambycine species of the eastern United States that share pheromone components, differences in seasonal and/or daily flight period may serve to prevent interspecific attraction (Mitchell et al. 2015). Each species flies during discrete periods of the year, for 3 to 6 weeks, with an orderly progression of species emerging as adults from early spring through fall (Handley et al. 2015; Hanks and Millar 2013; Hanks et al. 2014). Studies in east-central Illinois reveal that most of these species fly for fewer than four hours per day, and species vary as to whether they are active in late morning, afternoon, evening, or into the night (Mitchell et al. 2015; L. M. Hanks, unpub. data). Among those species that are truly synchronous, flying at the same time of year and same time of day, interspecific attraction to shared pheromone components may be prevented by minor pheromone components that are strong synergists for conspecifics, or strong antagonists for heterospecifics (Mitchell et al. 2013, 2015).

Despite the relatively thorough knowledge of chemical ecology in cerambycines, less is known about the pheromone chemistry of cerambycids in the largest subfamily, the Lamiinae (reviewed by Millar and Hanks 2016; for taxonomy, see Monné and Hovore 2005). As with the cerambycines, all known pheromones of lamiines are male-produced aggregation-sex pheromones, and pheromone chemistry has been conserved across continents. Lamiine pheromones identified to date are either based on hydroxyethers or terpenoids. An example of the hydroxyether pheromone class is 2-(undecyloxy)ethanol, with the common name monochamol, the pheromone for several species in the genus *Monochamus* (tribe Monochamini) that are native to North America and Eurasia (e.g., Ryall et al. 2015). The terpenoid-based pheromones are degradation products of geranylacetone (hereafter referred to as “the

geranylacetone class"), the first identified being (*E*)-6,10-dimethyl-5,9-undecadien-2-ol, known as fuscumol, the *S*-enantiomer of which was identified as the pheromone of *Tetropium* species in the subfamily Spondylidinae (Silk et al. 2007; Sweeney et al. 2010). The acetate ester of fuscumol, (*E*)-6,10-dimethyl-5,9-undecadien-2-yl acetate ("fuscumol acetate"), and lesser amounts of fuscumol and geranylacetone were subsequently identified from the South American lamiine *Hedypathes betulinus* (Klug) (Fonseca et al. 2010).

Field screening trials of synthesized racemic fuscumol and fuscumol acetate in the eastern USA have revealed that several native lamiine species are attracted to traps baited with the individual compounds or only to the blend (Hanks and Millar 2013; Hanks et al. 2012, 2018; Millar et al. 2018; Mitchell et al. 2011). Nine of those species are in the tribe Acanthocinini and two in the Acanthoderini. There remains no evidence, however, that either fuscumol or fuscumol acetate serves as pheromones of lamiine species native to the Old World, with no species attracted to the synthesized compounds during a screening trial in Australia (Hayes et al. 2016), and multiple trials in China (Sweeney et al. 2014; Teale al. 2016; Wickham et al. 2014).

Attraction of multiple lamiine species to traps baited with fuscumol and/or fuscumol acetate suggests that these compounds are shared among species as pheromone components, as with the Cerambycinae, and that there is a potential for interspecific attraction. However, among lamiine species of the eastern USA, there would seem to be less opportunity for seasonal segregation of species, because most of the species fly during summer and overlap broadly in flight period (Handley et al. 2015; Hanks and Millar 2013; Hanks et al. 2014). In addition, these lamiine species fly at night, as is common among lamiines in general (Švácha and Lawrence 2014), and thus also appear to be less likely to be segregated by differences in daily activity period. The focal species of the present study feed within trees, shrubs and vines within a wide

range of families as immatures and show considerable overlap in host plant use (Linsley and Chemsak 1995), with Fabaceae (legumes, including Black Locust), Juglandaceae (walnut/hickory) and Sapindaceae (includes *Acer* species) being the most common host plant families (Table 3.1; Wong 2016). It therefore seems probable that these species are segregated by differences in the subtleties of their pheromone chemistry.

Pheromones have been identified for only three of the sympatric species that were attracted by fuscumol and/or fuscumol acetate during earlier field bioassays in the eastern USA, but these preliminary findings nevertheless lend support to the notion that sympatric and synchronous species are segregated by pheromone chemistry. That is, males of *Astyleiopus variegatus* (Haldeman) produce *S*-fuscumol + *S*-fuscumol acetate (Hughes et al. 2013), and both sexes are attracted only to the binary blend (Hughes et al. 2016). Males of *Astylidius parvus* (LeConte) produce *R/S*-fuscumol + *R*-fuscumol acetate + geranylacetone, but adults of both sexes were attracted only by the blend of the fuscumol enantiomers, not to either enantiomer separately, and were not influenced by either enantiomer of fuscumol acetate or by geranylacetone (Meier et al. 2016). Similarly, males of *Lepturges angulatus* (LeConte) produce *R/S*-fuscumol acetate + geranylacetone, and the adults were attracted by the blend of both enantiomers, and attraction was enhanced by geranylacetone but not influenced by either enantiomer of fuscumol (Meier et al. 2016). Thus, all three of these species were attracted only to combinations of enantiomers that simulated their pheromones and not to the pheromone blends of the other species.

Identification of the pheromones of *A. variegatus*, *A. parvus*, and *L. angulatus* accounts for the findings of earlier screening trials of the racemic materials in the eastern USA (see Hanks and Millar 2013; Hanks et al. 2012; Millar et al. 2018; Mitchell et al. 2011, Schmeelk et al.

2016). In fact, attraction of beetles to traps baited with the racemic materials has facilitated identification of their pheromones. For example, adults of *A. parvus* have been attracted by racemic fuscumol, whether presented alone or combined with fuscumol acetate in binary, or more complicated blends of cerambycid pheromones. These findings are consistent with attraction of this species to *R*/*S*-fuscumol and the lack of any effect by either enantiomer of fuscumol acetate. Similarly, adults of *L. angulatus* have been attracted by racemic fuscumol acetate alone, because both enantiomers are essential, even when it was combined with fuscumol and other cerambycid pheromones. On the other hand, adults of *A. variegatus* have responded to neither fuscumol nor fuscumol acetate when tested separately in the same field bioassays, but were attracted only when the two were blended and the essential *S*-enantiomers brought together.

That the pheromones of the three lamiine species represents a unique combination of the enantiomers of fuscumol and fuscumol acetate, as well as geranylacetone, suggests that combinations within the geranylacetone class offer a limited number of unique pheromones. Assuming that pheromones may be composed of one to all five of these chemicals, there would be 31 unique permutations. This limit would seem to account for the species diversity of lamiines in the area of these studies (midwestern USA) that are most likely to use these pheromones, the Acanthocinini and Acanthoderini, which number at least 23 species (Hanks et al. 2014). Of course, these arguments take a rather narrow view of how pheromone chemistry evolves. For example, the unique pheromone composition of a particular species likely is the product of disruptive selection, due to competition for pheromone channels with multiple species in different parts of its range. In any particular part of its range, the lamiine community may be missing certain of these influential species, and thus there likely would be incomplete representation of all possible permutations of pheromone composition.

Here, I examine the pheromone chemistry of lamiine species native to east-central Illinois, with the goal of further testing the hypothesis that species that communicate via the geranylacetone class of compounds are segregated by the subtleties of their pheromone chemistry. The goal of this project is to elucidate coexistence mechanism for seven species of cerambycids that are broadly polyphagous as larvae and overlap in their host species ranges (Linsley and Chemsak 1995). I conducted four experiments to test for attraction of beetles to 19 unique combinations of the enantiomers of fuscumol and fuscumol acetate, along with geranylacetone. I tested all 31 possible combinations of these compounds, assuming that some species may respond only to the exact simulations of their pheromones. This research resulted in the identification of pheromones of an additional four species: *Graphisurus despectus* (LeConte), *Graphisurus fasciatus* (Degeer), and *Sternidius alpha* (Say) of the Acanthocinini, and *Aegomorphus modestus* (Gyllenhal) of the Acanthoderini. I also show that the pheromone of *A. variegatus* includes geranylacetone and provide preliminary evidence of the pheromone chemistry of *Lepturges confluens* (Haldeman).

Methods and Materials

Sources of Chemicals

Racemic (*E*)-fuscumol and (*E*)-fuscumol acetate were purchased from Bedoukian Research (Danbury, CT, USA), and (*E/Z*)-geranylacetone from Sigma-Aldrich (St. Louis, MO, USA). (*R*)-fuscumol (96.6%), (*S*)-fuscumol (98%), (*R*)-fuscumol acetate (96.6%), and (*S*)-fuscumol acetate (98%) were synthesized as described in Hughes et al. (2013).

Study Sites

Field work to identify pheromones of cerambycid species and attraction to synthesized pheromones was conducted at four study sites in east-central Illinois (Table 3.2), all of which were wooded with mature second-growth or successional hardwoods and dominated by oaks (*Quercus* species), hickories (*Carya* species), maples (*Acer* species), and ash (*Fraxinus* species).

Identification of Pheromones

Live beetles for collection of headspace volatiles were caught with cross-vane panel traps (black corrugated plastic, Alpha Scents, Portland, OR, USA) coated with the fluoropolymer dispersion Fluon® (10% aqueous dilution; Northern Products, Woonsocket, RI, USA) to improve capture efficiency (for details on trapping methods, see Graham et al. 2010). The collection buckets that are supplied with the traps were replaced with plastic jars having aluminum screen bottoms to allow rainwater to drain. Trap lures consisted of polyethylene sachets (5.1 × 7.6 cm, Bagettes® model 14770, Cousin Corp., Largo, FL),, USA) loaded with 50 mg of the racemic fuscumol or fuscumol acetate (i.e., 25 mg per enantiomer) dissolved in 1 ml isopropanol, and separate lures for the two compounds. Single traps were deployed during May to August 2013 – 2017 at all four study sites (Table 3.2). Traps were serviced and beetles were collected every 1 – 2 d. Trap lures were replaced after 10 – 14 d.

Beetles were sexed based on the morphology of the fifth abdominal sternite (Linsley and Chemsak 1995). In the laboratory, they were caged individually under ambient conditions (~12:12 h L:D, ~20 °C). Adults of the study species feed on bark of oak branchlets or maple (pers. obs.), and so caged beetles were provided sections of branches (2 – 5 cm in diameter, ~8 cm long) freshly cut from oak or maple trees (*Quercus alba* L., *Q. rubra* L., and *Acer saccharum*

Marshall) at the same field sites where beetles were trapped. As a source of moisture, beetles also were provided sugar water (10% aqueous sucrose solution in a glass vial with a cotton wick). Beetles were allowed an acclimation period of 24 h under laboratory conditions before their first aeration and between subsequent aerations.

Volatiles produced by beetles were collected by aerating them in glass Mason-style canning jars near closed exterior windows (natural photoperiod, ~14:10 h L:D, ~20 °C). Jars were swept with clean air (1 l/min) for 24 h. Headspace volatiles were collected with glass vial cartridges attached to the chamber outlet, which contained a layer of the adsorbent polymer HayeSep® Q (150 mg; Sigma-Aldrich, St. Louis, MO, USA) between plugs of glass wool. Beetles usually were aerated individually, but in some cases two or three beetles of the same sex were aerated together in case the proximity of conspecifics encouraged them to release pheromone. Beetles usually were aerated with pieces of freshly cut oak or maple branches (provided as food and as a perch). Aerations of jars without beetles (including host material and/or sugar water) were run simultaneously to control for system contaminants. Additional controls included jars containing branch pieces that had been mechanically injured, by scraping or gouging the bark, to simulate feeding damage by beetles that could result in the release of plant volatiles. Numbers of males and females that were aerated were 17 and 12 for *A. modestus*, 16 and 4 for *A. variegatus*, 19 and 3 for *G. despectus*, 54 and 8 for *G. fasciatus*; and also 37 beetles of *S. alpha* that were of unknown sex due to difficulties in differentiating males from females.

Chemicals were extracted from aeration cartridges with 1.5 ml of dichloromethane, and extracts were analyzed with a gas chromatograph (GC) interfaced to a mass selective detector (Models 6809 and 5973, Hewlett-Packard, Palo Alto, CA, USA) fitted with a AT-5ms column

(30 m × 0.25 mm i.d., 0.25 μm film; Alltech Associates, Inc., Deerfield, IL, USA). The GC oven was programmed from 35 °C/1 min, 10 °C/min to 210 °C, hold 3 min. Injections were made in splitless mode, with an injector temperature of 250 °C and helium carrier gas. Sex-specific peaks were identified by comparing spectra and retention times to those of authentic standards.

The absolute configurations of insect-produced fuscumol acetate were determined by analyzing aliquots of extracts with an HP 5890 GC fitted with a chiral stationary phase Cyclodex B column (30 m × 0.25 mm i.d., 0.25 μm film; Agilent Technologies, Inc., Santa Clara, CA, USA). The oven temperature was programmed from 50 °C/1 min, 2.5 °C/min to 200 °C, hold 5 min, with an injector temperature of 210 °C. Aliquots of aeration extracts were co-injected with synthetized stereoisomers of fuscumol acetate to determine which enantiomers were being produced by beetles (Millar et al. 2009). On the chiral column, (*R*)- and (*S*)-fuscumol acetate showed retention times of 51.33 and 51.72 min, respectively.

Because the two enantiomers of fuscumol did not separate on the chiral column, fuscumol in extracts was esterified with (*S*)-*O*-acetyl lactic acid chloride following the method of Slessor et al. (1985) and modified as described in Hughes et al. (2013). The resulting esterified enantiomers separated on an achiral DB-5 column (30 m × 0.25 mm i.d., 0.25 μ film, J&W Scientific, Folsom, CA, USA), and enantiomers were identified by comparing retention times with esterified synthesized standards. The retention times of the diastereomeric esters of (*R*)- and (*S*)-fuscumol were 18.94 minutes and 19.03 minutes, respectively.

Few of the aeration extracts contained sufficient quantities of pheromone for determining chirality of either fuscumol or fuscumol acetate, resulting in smaller sample sizes (four samples from male *A. variegatus* and a single sample from a male *S. alpha*, respectively; see Results).

Field Experiments

Four independent field bioassays were conducted to test attraction of beetles to synthesized pheromones (study sites and dates in Table 3.2). Beetles were caught with the same panel traps as previously described, but with the original trap basins containing ~300 ml of saturated aqueous NaCl solution to kill and preserve captured beetles. Traps were set 10 m apart in linear transects, with treatments randomly assigned to traps on the first day. Traps were serviced every 1-3 d, at which time treatments were shifted one position along transects to control for small-scale positional effects. Trap lures usually were replaced every 14 d, as needed.

The four experiments tested 19 unique combinations of racemic and chiral materials (Table 3.3), including many that had not been tested in earlier experiments (Hughes et al. 2016; Meier et al. 2016; Mitchell et al. 2011). Some treatments were added to experiments specifically to provide a means of assessing the contribution of individual components to blends, or as reconstructions of the pheromones of target species *A. variegatus*, *A. modestus*, and *S. alpha* (Table 3.3). However, *G. despectus* and *G. fasciatus* could not be targeted specifically without information on the chirality of fuscumol and fuscumol acetate in their respective pheromones (Table 3.4). Nevertheless, blending of the racemic materials with geranylacetone in Experiment 1 provided a preliminary test of attraction to their candidate pheromones.

Trap lures for Experiment 1 were composed of sachets made from heat-sealed polyethylene tubing (Associated Bag, Milwaukee, WI), which were loaded with 1 ml of neat chemicals. Lure release rates were standardized to ~20 mg/d by using sachets with different wall thicknesses and adding a cotton roll (1 × 4 cm dental wick, Patterson Dental Supply, Inc., St. Paul, MN) to some lures, as follows: fuscumol (1.5 mil, 38 µm wall thickness, with cotton roll); fuscumol acetate (3 mil, 76 µm wall thickness, with cotton roll), geranylacetone (3 mil, 76 µm

wall thickness, without cotton roll). Three empty sachets that matched the types used for the three chemicals were placed together on a trap as a blank control. Lures were replaced every 14 d. For the remaining three experiments, trap lures were made from heat-sealed polyethylene tubing, but loaded with diluted chemicals, as described in Identification of Pheromones, above.

Data violated homoscedasticity assumptions of ANOVA (Sokal and Rohlf 1995), so differences between treatment means, blocked by site and date, were tested separately for species represented by at least eight specimens using the nonparametric Friedman's Test (PROC FREQ, option CMH; SAS Institute 2011). If the overall Friedman's test was significant, pairs of treatment means were compared using the non-parametric Ryan-Einot-Gabriel-Welsch Q (REGWQ) multiple comparison test (SAS Institute 2011). Replicates with zero specimens of the species of interest, such as due to inclement weather, were dropped from analyses.

Taxonomy of captured beetles follows Monné and Hovore (2005). Representative specimens were deposited in the laboratory collection of LMH, and voucher specimens were placed in the collection of the Illinois Natural History Survey, Champaign, IL. Supporting data from additional independent field experiments are presented in the Supplement.

Results

Identification of Pheromones

Compounds of the geranylacetone class were present in aerations from males of the five targeted species, with unique combinations for each species, as well as for the two species whose pheromones were identified previously, *A. parvus* and *L. angulatus* (Table 3.4). These compounds were absent in all aeration extracts from females and from system controls. Males of *A. variegatus* produced geranylacetone, along with the *S*-fuscumol and *S*-fuscumol acetate

reported previously (Hughes et al. 2013). *Sternidius alpha* was the only species that lacked geranialacetone in aeration extracts.

Field Experiments

Traps caught 2,135 cerambycid beetles of 53 species during the four field experiments (Table 3.5), including species in the subfamilies Cerambycinae, Lamiinae, Lepturinae, Parandrinae, Prioninae, and four specimens of a species in the closely related family Disteniidae (see Švácha and Lawrence 2014). Most of the trapped beetles (~92%) were lamiines. Species in other subfamilies that were caught in numbers sufficient for statistical analysis showed no significant treatment effects in any experiment (Friedman's test $P > 0.05$). These species included the cerambycines *Elaphidion mucronatum* (Say) and *Xylotrechus colonus* (F.), which are among the most widespread and common cerambycid beetles of eastern North America (Hanks et al. 2014; Lingafelter 2007), and the prionine *Orthosoma brunneum* (Forster), which apparently is captured passively by traps, regardless of how they are baited (Hanks et al. 2014).

Eight species of lamiines were caught in numbers sufficient for statistical analysis of treatment effects in at least one experiment. These species included the three that were the subjects of earlier studies on pheromone identification (*A. parvus*, *L. angulatus*, *A. variegatus*; see Introduction), as well as the four of species for which pheromones are identified here for the first time (*A. modestus*, *G. despectus*, *G. fasciatus*, *S. alpha*). Analysis of treatment effects for an additional species, *L. confluens*, provides tentative insight into its pheromone chemistry.

With respect to the species for which pheromones had been previously identified, adults of *A. variegatus* (pheromone: *S*-fuscumol, *S*-fuscumol acetate, geranylacetone) were significantly attracted only to the complete blend of racemic fuscumol, fuscumol acetate, and geranylacetone

in Experiment 1 (Fig. 3.1A), attesting to the synergistic influence of the latter component, which had been missed in earlier work (Hughes et al. 2013). In fact, this synergism apparently overshadowed attraction to fuscumol + fuscumol acetate that was reported previously.

Adults of *A. parvus* (pheromone: *R/S*-fuscumol, *R*-fuscumol acetate, geranylacetone) were attracted by racemic fuscumol during Experiment 3 and not influenced by either *R*-fuscumol acetate or geranylacetone (Fig. 3.1B). These findings are consistent with earlier research on this species (Meier et al. 2016).

Adults of *L. angulatus* (pheromone: *R*/*S* fuscumol acetate, geranylacetone) were significantly attracted during Experiment 3 only by the blend of racemic fuscumol and fuscumol acetate (Fig. 3.1C; this experiment lacked a treatment for racemic fuscumol acetate alone). Geranylacetone had the unanticipated effect of antagonizing the attraction of *L. angulatus* to fuscumol + fuscumol acetate, despite its being a component of the pheromone. This finding is inconsistent with an earlier report that geranylacetone is a powerful synergist for this species when paired with fuscumol acetate (Chapter 2), but the antagonism could be due to interference by the non-natural fuscumol.

Adults of *A. modestus* (chemicals produced: *R/S*-fuscumol acetate, geranylacetone) were attracted during Experiment 1 only to the synthetized blend that included all three components (Fig. 3.2A). Neither racemic fuscumol acetate nor geranylacetone were attractive when tested alone, and fuscumol antagonized attraction to the pheromone reconstruction. However, Experiment 2 revealed that adults of this species were attracted to fuscumol acetate, perhaps due to the absence of traps baited with the complete blend, with both racemic fuscumol and its individual enantiomers acting as antagonists (Fig. 3.2B).

During Experiment 1, adults of *G. despectus* (pheromone: geranylacetone, trace fuscumol) were attracted by geranylacetone alone (Fig. 3.2C), and, in a surprising finding, attraction to geranylacetone was strongly antagonized by fuscumol. The non-natural fuscumol acetate did not significantly influence attraction.

Adults of *G. fasciatus* (pheromone: geranylacetone, trace fuscumol acetate) were attracted only to blends containing fuscumol acetate, and geranylacetone neither attracted beetles when tested alone, nor did it synergize attraction to fuscumol acetate. The non-natural fuscumol had no apparent effect on attraction (Fig. 3.3A). During Experiment 3, adults of this species were attracted to all treatments that contained racemic fuscumol acetate but were not attracted to those treatments that included one of the enantiomers (Fig. 3.3B). This finding is further evidence of enantiomeric synergism, as was reported earlier for *A. parvus* and *L. angulatus* (Meier et al. 2016).

Adults of *L. confluens* (pheromone chemistry unknown) were attracted to the blend of fuscumol, fuscumol acetate, and geranylacetone in Experiment during 1 (Fig. 3.4A), and only to the blend of *S*-fuscumol, *S*-fuscumol acetate, and geranylacetone in Experiment 3 (Fig. 3.4B). This response to treatments is similar to that of *A. variegatus*, suggesting the two species have similar pheromones.

Adults of *S. alpha* (pheromone: *S*-fuscumol, *R*/*S*-fuscumol acetate) were attracted to racemic fuscumol in Experiment 1, and an unexpected finding was that attraction was antagonized by fuscumol acetate (Fig. 3.5). Geranylacetone did no influence the response of *S. alpha* to fuscumol.

Discussion

Attraction of adult beetles to traps baited with reconstructed blends of compounds produced by their conspecific males confirmed the pheromone chemistries of *A. modestus*, *G. despectus*, *G. fasciatus*, and *S. alpha*. In addition, this research has demonstrated that geranylacetone is an important synergistic pheromone component for *A. variegatus*. All five of these species differed in the composition of their pheromones, supporting the hypothesis that the community of acanthocinine and acanthoderine species are segregated by differences in their pheromone chemistries. However, results of this study suggest that males of *A. modestus* produce the same pheromone as do males of *L. angulatus*, *R/S*-fuscumol + geranylacetone (Meier et al. 2016).

For many of the lamiine species that have been studied, only certain components of the volatiles produced by males appear to be essential for attraction (see Table 3.6 for a summary of the findings from the present and earlier studies). For instance, males of *A. parvus* produce a blend of *R*/*S*-fuscumol, *R*-fuscumol acetate, and geranylacetone, but attraction of beetles in the field was not influenced by the latter two components (Meier et al. 2016). Similarly, males of *S. alpha* produced *S*-fuscumol and *R*/*S*-fuscumol acetate, but the latter components antagonized attraction to fuscumol in the present study.

The species-specific pheromone chemistry of lamiine species and the response of beetles to particular compounds accounts for chemical mechanisms that prevent interspecific attraction among most of the lamiine species. Moreover, the mechanisms that prevent attraction between species are complementary, operating in both directions, and for some species there appear to be multiple factors involved (Table 3.6). For example, the pheromones of *A. modestus* and *A. variegatus* have in common *S*-fuscumol acetate and geranylacetone; however, adults of *A.*

modestus should not be attracted to the pheromone of the other species because it lacks the essential *R*-fuscumol acetate and also includes the inhibitory *S*-fuscumol. On the other hand, adults of *A. variegatus* should not be attracted to the pheromone of the other species because it lacks the essential *S*-fuscumol.

The uncertain chirality of the fuscumol component of the pheromone produced by *G. despectus* and the chirality of fuscumol acetate component of *G. fasciatus* are responsible for most of the cases in which the mechanisms that would prevent interspecific attraction could not be determined (question marks in Table 3.6). These mechanisms could be surmised for *L. confluens*, even though its pheromone has yet to be identified, based on its attraction only to the tertiary blend of *S*-fuscumol, *S*-fuscumol acetate, and geranylacetone. The same three compounds are produced by males of *A. variegatus*, but adults of that species do not require geranylacetone for attraction. Nevertheless, it is not yet clear why adults of *L. confluens* would not be attracted by the pheromone of the other species.

The same is true for *A. modestus* and *L. angulatus*, males of which both produce and are strongly attracted to the blend of *R*/*S*-fuscumol acetate and geranylacetone. However, sharing the same pheromone may be possible if species are segregated by some other factor. For example, volatile chemicals associated with host plants of the larvae may synergize attraction of cerambycid beetles to their pheromones (e.g., Ryall et al. 2015; Sweeney et al. 2010), while volatiles from non-hosts may antagonize attraction (Collignon et al. 2016, Wong et al. 2017). Larvae of both *A. modestus* and *L. angulatus* are reported to be polyphagous on hardwoods of several families (Table 3.1; Linsley and Chemsak 1984, 1995), which suggests that they are unlikely to be segregated on the basis of host plant volatiles. However, the two species may differ in the condition of the larval host that their larvae require (living, moribund, dead), in

which case different types of plant volatiles could influence the response of the adult beetles to pheromones, providing a mechanism of species segregation.

Cerambycids partition their forest habitat vertically, such that some species are active primarily within the canopy while others rarely stray from the understory (Schmeelk et al. 2016, Wong and Hanks 2016). This form of spatial segregation may allow sympatric species to share the same pheromone chemistry.

The seven species featured in this study represent only about one-third of the acanthocinine and acanthoderine species that are native to the area of my studies, the species that presumably are most likely to have pheromones of the geranylacetone class of compounds. Given that pheromones of these lamiines are now known to comprise unique combinations of the available components, there remain many combinations out of the 31 possible blends to be tested. For example, *A. variegatus* was targeted to confirm it pheromone by baiting traps with *S*-fuscumol + *S*-fuscumol acetate + geranylacetone, but the blend of *R*-fuscumol + *R*-fuscumol acetate + geranylacetone has yet to be tested. Likewise, the blend of compounds that is produced by males of *S. alpha*, *S*-fuscumol and *R/S*-fuscumol acetate, has yet to be tested in combination with geranylacetone. It also should be noted that some of the unique combinations have not be thoroughly tested, at multiple field sites and throughout the entire flight seasons of lamiines. Lastly, it is likely that some of the lamiine species present in central Illinois are simply not abundant enough to yield statistically significant treatment effects. For example, the species *Lepturges pictus* (LeConte), *L. regularis* (LeConte), and *L. symmetricus*, and *Oplosia nubila* (LeConte) were caught in small numbers, if at all, during the field experiments, and are generally regarded as uncommon (e.g., Lingafelter 2007).

There also is evidence that the pheromone produced by males of *G. fasciatus*, reported here to be composed of fuscumol acetate and geranylacetone, may include a component of a different class, an 11-carbon dien ketol that have yet to be tested in the field. The addition of even one additional compound to the list of potential pheromone ingredients would more than double the number of unique blends (N = 63) available to the community of lamiine species native to the eastern USA.

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Tables

Table 3.1. Plant families that have been recorded as larval hosts for the cerambycid study species

Species of Beetle

Host Plant Family	*Aegomorphus modestus*	*Astyleiopus variegatus*	*Astylidius parvus*	*Graphisurus despectus*	*Graphisurus fasciatus*	*Lepturges angulatus*	*Lepturges confluens*	*Sternidius alpha*
Altingiaceae	■				■	■		
Anacardiaceae			■		■			
Asparagaceae							■	
Betulaceae	■				■			■
Cannabaceae		■				■		■
Fabaceae	■	■	■			■		■
Fagaceae		■			■			
Juglandaceae				■	■	■	■	■
Magnoliaceae	■							
Malvaceae	■				■			
Moraceae			■					
Oleaceae	■				■			■
Pinaceae	■	■			■			
Platanaceae						■		
Rosaceae	■					■		■
Sapindaceae	■	■	■			■		
Ulmaceae		■						
Vitaceae		■						

Table 3.2. Study sites for field bioassays conducted in east-central Illinois during 2013-2015, and 2017

County	Name	GPS coordinates (lat., long.)	Area (ha)
Champaign	Brownfield Woods[a]	40.145, -88.165	26
Champaign	Nettie Hart Memorial Woods[a]	40.229, -88.358	16
Champaign	Trelease Woods[a]	40.132, -88.141	29
Piatt	Allerton Park[b]	39.996, -88.651	600
Vermillion	Vermillion River Observatory[a]	40.066, -87.561	192

[a] University of Illinois natural area (http://research.illinois.edu/cna/)

[b] Property of the University of Illinois

Table 3.3. Experimental treatments for three field experiments that tested attraction of adults of lamiine species to various blends of chiral fuscumol and fuscumol acetate (enantiomers indicated by *R* and *S*), the racemic materials, and geranylacetone. Species whose pheromones are simulated by a particular synthetic blend are indicated in parentheses.

Compound (species represented)	**Expt 1**	**Expt 2**	**Expt 3**
Fuscumol	X	X	
R-fuscumol			
S-fuscumol			
Fuscumol acetate (*L. angulatus*)	X	X	
R-fuscumol acetate			
S-fuscumol acetate			
Geranylacetone	X		X
Fuscumol + fuscumol acetate	X	X	X
Fuscumol + *R*-fuscumol acetate	X	X	X
Fuscumol + *S*-fuscumol acetate		X	
R-Fuscumol + fuscumol acetate		X	
S-Fuscumol + fuscumol acetate		X	
R-fuscumol + *R*-fuscumol acetate			
S-fuscumol + *S*-fuscumol acetate			X
Fuscumol + geranylacetone	X		
Fuscumol acetate + geranylacetone (*A. modestus*)	X		
Fuscumol + fuscumol acetate + geranylacetone	X		X
Fuscumol + *R*-fuscumol acetate + geranylacetone (*A. parvus*)			X
S-fuscumol + *S*-fuscumol acetate + geranylacetone (*A. variegatus*)			X
Solvent control	X	X	X

Table 3.4. Approximate relative proportions of compounds detected in the headspace samples from males of seven species of cerambycid beetles in the Lamiinae native to eastern North America. *S* and *R* indicate chirality of fuscumol and fuscumol acetate. (Chirality could not be determined for compounds present in trace amounts.) Numbers of successful aerations (total number of aerations): 9 (18) for *Aegomorphus modestus*, 9 (14) for *Astyleiopus variegatus*, 4 (17) for *Graphisurus despectus*, 6 (36) for *Graphisurus fasciatus*, and 4 (33) for *Sternidius alpha*. Data for *Astylidius parvus* and *Lepturges angulatus* are from Meier et al. (2016).

	Fuscumol		Fuscumol acetate		
	R	*S*	*R*	*S*	Geranylacetone
Aegomorphus modestus			2.25	0.75	1
Astyleiopus variegatus		3		3	1
Astylidius parvus	1.5	1.5	1		1
Graphisurus despectus	Trace				1
Graphisurus fasciatus			Trace		1
Lepturges angulatus			2	1	1
Sternidius alpha		1	1	1	

Table 3.5. Total numbers of adult cerambycid beetles that were captured during four field experiments conducted in east-central Illinois that assessed attraction of beetles to synthesized pheromones

Taxonomy	Expt. 1	Expt. 2	Expt. 3	Total
Cerambycinae				
Anaglyptini				
Cyrtophorus verrucosus (Olivier)			1	1
Clytini				
Clytoleptus albofasciatus (Laporte & Gory)	1			1
Neoclytus a. acuminatus (F.)	2			2
Neoclytus scutellaris (Olivier)				
Xylotrechus colonus (F.)	14	12		26
Elaphidiini				
Anelaphus parallelus (Newman)				
Anelaphus villosus (F.)		3		3
Elaphidion mucronatum (Say)	18	9	15	42
Parelaphidion aspersum (Haldeman)		1	4	5
Parelaphidion incertum (Newman)	1		1	2
Hesperophanini				
Tylonotus bimaculatus			1	1
Neoibidionini				
Heterachthes quadrimaculatus Haldeman	1			1
Parelaphidion incertum (Newman)	1			1
Obriini				
Obrium rufulum Gahan		1	1	2
Smodicini				
Smodicum cucujiforme (Say)		1		1
Lamiinae				
Acanthocinini				
Astyleiopus variegatus (Haldeman)	72	49	60	181
Astylidius parvus (LeConte)	377	119	64	560
Astylopsis macula (Say)	23	51	5	79

Table 3.5 (cont.)

Astylopsis sexguttata (Say)	1			1
Graphisurus despectus (LeConte)	65	8	20	93
Graphisurus fasciatus (Degeer)	133	50	6	189
Hyperplatys aspersa (Say)	1			1
Hyperplatys maculata Haldeman	2	1	1	4
Leptostylus transversus (Gyllenhal)	4	2	1	7
Lepturges angulatus (LeConte)	249	77	35	361
Lepturges confluens (Haldeman)	45	1	43	89
Lepturges pictus (LeConte)	6	4	6	16
Lepturges regularis (LeConte)	8		1	9
Lepturges symmetricus (Haldeman)	7	8	3	18
Sternidius alpha (Say)	139	43	7	189
Urgleptes querci (Fitch)	6	3	3	12
Urgleptes signatus (LeConte)	1	2	1	4
Acanthoderini				
Aegomorphus modestus (Gyllenhal)	79	7		86
Oplosia nubila (LeConte)	1		5	6
Desmiphorini				
Psenocerus supernotatus (Say)	2			2
Dorcaschematini				
Dorcaschema cinereum (Olivier)		1		1
Dorcaschema nigrum (Say)				
Monochamini				
Microgoes oculatus (LeConte)	2	2		4
Pogonocherini				
Ecyrus d. dasycerus (Say)	1	1	12	14
Saperdini				
Saperda discoidea Fabricius				
Saperda imitans Felt and Joutel	1			1
Saperda lateralis F.		2		2

Table 3.5 (cont.)

Saperda tridentata Olivier	3		1	4
Saperda vestita (Say)			3	3
Lepturinae				
Lepturini				
Analeptura lineola (Say)		1		1
Strangalia luteicornis (F.)	1			1
Strophiona nitens (Forster)	1			1
Typocerus lugubris (Say)		3	1	4
Typocerus v. velutinus (Olivier)	1	2	2	5
Parandrinae				
Parandrini				
Neandra brunnea (F.)	2	28	13	43
Prioninae				
Prionini				
Orthosoma brunneum (Forster)	4	4	3	11
Disteniidae				
Disteniini				
Elytrimitatrix undata (F.)	11	2		13
Total	***1286***	***498***	***319***	***2,103***

Table 3.6. Chemical mechanisms that may prevent interspecific attraction among eight sympatric species of cerambycid beetles that share pheromone components. Entries within the table body indicate the factors that would prevent responding species (columns) from being attracted by the pheromones of calling species (rows), including the lack of essential attractants or synergists, and inhibition of attraction by non-natural compounds ("inhib."). Question marks indicate instances in which pheromone chemistry appears not to prevent interspecific attraction, or if effects cannot be confirmed due to the lack of data on chirality. Columns for responding species indicate the pheromone components that are essential and sufficient for attraction, synergistic components, and non-natural compounds of other species that inhibit attraction based on the findings of field bioassays. Some information for *A. modestus*, *A. parvus*, and *L. angulatus* is from Hughes et al. (2016) and Meier et al. (2016), and the pheromone chemistry of *L. confluens* is not known. Chemical abbreviations: F = racemic fuscumol, Fa = racemic fuscumol acetate (*R* and *S* indicating chirality), Ga = geranylacetone.

			Responding species							
			A. modestus	*A. variegatus*	*A. parvus*	*G. despectus*	*G. fasciatus*	*L. angulatus*	*S. alpha*	*L. confluens*
		Components essential for attraction	*R*- + *S*-Fa	*S*-F + *S*-Fa	*R*- + *S*-F	Ga	*R*- + *S*-Fa	*R*- + *S*-Fa	*S*-F	*S*-F + *S*-Fa + Ga
		Synergists	Ga	Ga						
		Inhibitors	*R*- or *S*-F			*R*- and/or *S*-F	*R*- or *S*-F	*R*-, *S*-F, Ga	*R*- and/or *S*-Fa	
Calling species and pheromone composition	*A. modestus*	*R*,*S*-Fa, Ga		Lacks *S*-F	Lacks *R*- and *S*-F	?	?	Inhib. Ga	Lacks *S*-F	Lacks *S*-F
	A. variegatus	*S*-F, *S*-Fa, Ga	Lacks *R*-Fa, Inhib. *S*-F		Lacks *R*-F	Inhib. *S*-F	?	Lacks *R*-Fa, inhib. *S*-F, Ga	Inhib. *S*-Fa?	?
	A. parvus	*R*,*S*-F, *R*-Fa, Ga	Lacks *S*-Fa, Inhib. *R*,*S*-F	Lacks *S*-Fa		Inhib. *R*- or S-F?	Inhib. *R*-, or *S*-F	Lacks *S*-Fa, inhib. *R*- or *S*-F, Ga	Inhib. *R*-Fa	Lacks *S*-Fa
	G. despectus	*R*- and/or *S*-F, Ga	Lacks *R*,*S*-Fa, Inhib *S*-F?	Lacks *S*-Fa	Lacks *R*- or *S*-F?		Lacks Fa, inhib. *R*- or *S*-F	Lacks *R*- and *S*-Fa, inhib. *R*- or *S*-F, Ga	?	Lacks *S*-Fa
	G. fasciatus	*R*- and/or *S*-Fa, Ga	?	Lacks *S*-F	Lacks *R*- and *S*-F	?		Inhib. Ga	Lacks *S*-F, inhib. *R*- or *S*-Fa	Lacks *S*-F
	L. angulatus	*R*,*S*-Fa, Ga	?	Lacks *S*-F	Lacks *R*- and *S*-F	?	?		Lacks *S*-F, inhib. *R*- or *S*-Fa	Lacks *S*-F
	S. alpha	*S*-F, *R*,*S*-Fa	Lacks Ga, Inhib. *S*-F?	Lacks Ga	Lacks *R*-F	Lacks Ga, inhib. *S*-F	?	Inhib. *S*-F		Lack Ga

Figures

Fig. 3.1. Mean (± SE) numbers of adult beetles caught per replicate during field bioassays of synthesized pheromones for the three species whose pheromones were identified previously (Hughes et al. 2013; Meier et al. 2016): A) *Astyleiopus variegatus* (Experiment 1, Friedman's $Q_{7,104} = 49.2$, $P < 0.0001$); B) *Astylidius parvus* (Experiment 3, $Q_{7,80} = 32.2$, $P < 0.0001$); C) *Lepturges angulatus* (Experiment 3, $Q_{7,56} = 29.2$, $P < 0.0001$). Means within figures with different letters are significantly different (REGWQ test, $P < 0.05$). Chemical abbreviations: F = racemic fuscumol, Fa = racemic fuscumol acetate, Ga = geranylacetone. *R* and *S* indicate enantiomers of fuscumol and fuscumol acetate.

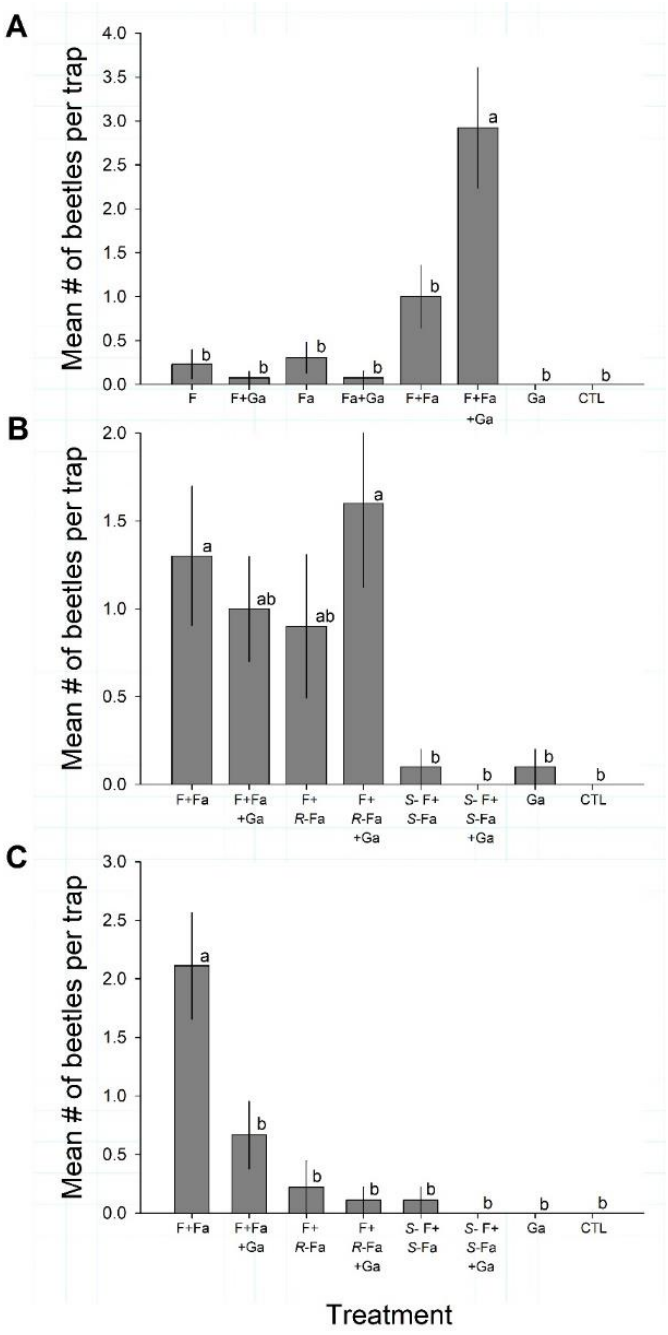


Fig. 3.2. Mean (± SE) numbers of adult beetles caught per replicate during field bioassays of synthesized pheromones for: A) *Aegomorphus modestus* (Experiment 1, Friedman's $Q_{7,88}$= 43.3, P < 0. 0001); B) *A. modestus* (Experiment 2, $Q_{7,48}$= 27.3, P < 0.001); and C) *Graphisurus despectus* (Experiment 1, $Q_{7,64}$= 33.3, P < 0. 0001). Means within figures with different letters are significantly different (REGWQ test, P < 0.05). Chemical abbreviations: F = racemic fuscumol, Fa = racemic fuscumol acetate, Ga = geranylacetone. *R* and *S* indicate enantiomers of fuscumol and fuscumol acetate.

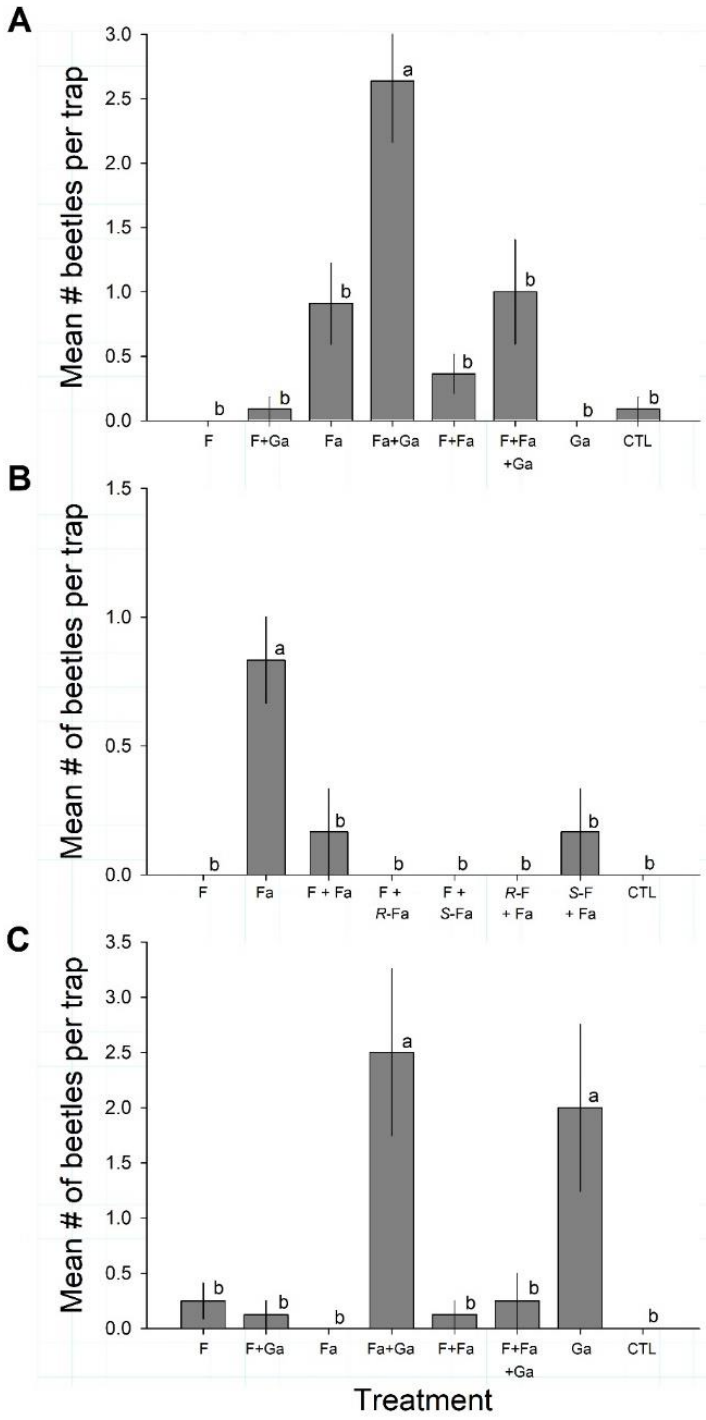


Fig. 3.3. Mean (± SE) numbers of adult beetles caught per replicate during field bioassays of synthesized pheromones for *Graphisurus fasciatus*: A) Experiment 1 (Friedman's $Q_{7,144} = 38.2$, $P < 0.0001$), B) Experiment 2 ($Q_{7,192} = 26.7$, $P < 0.001$). Means within figures with different letters are significantly different (REGWQ test, $P < 0.05$). Chemical abbreviations: F = racemic fuscumol, Fa = racemic fuscumol acetate, Ga = geranylacetone. *R* and *S* indicate enantiomers of fuscumol and fuscumol acetate.

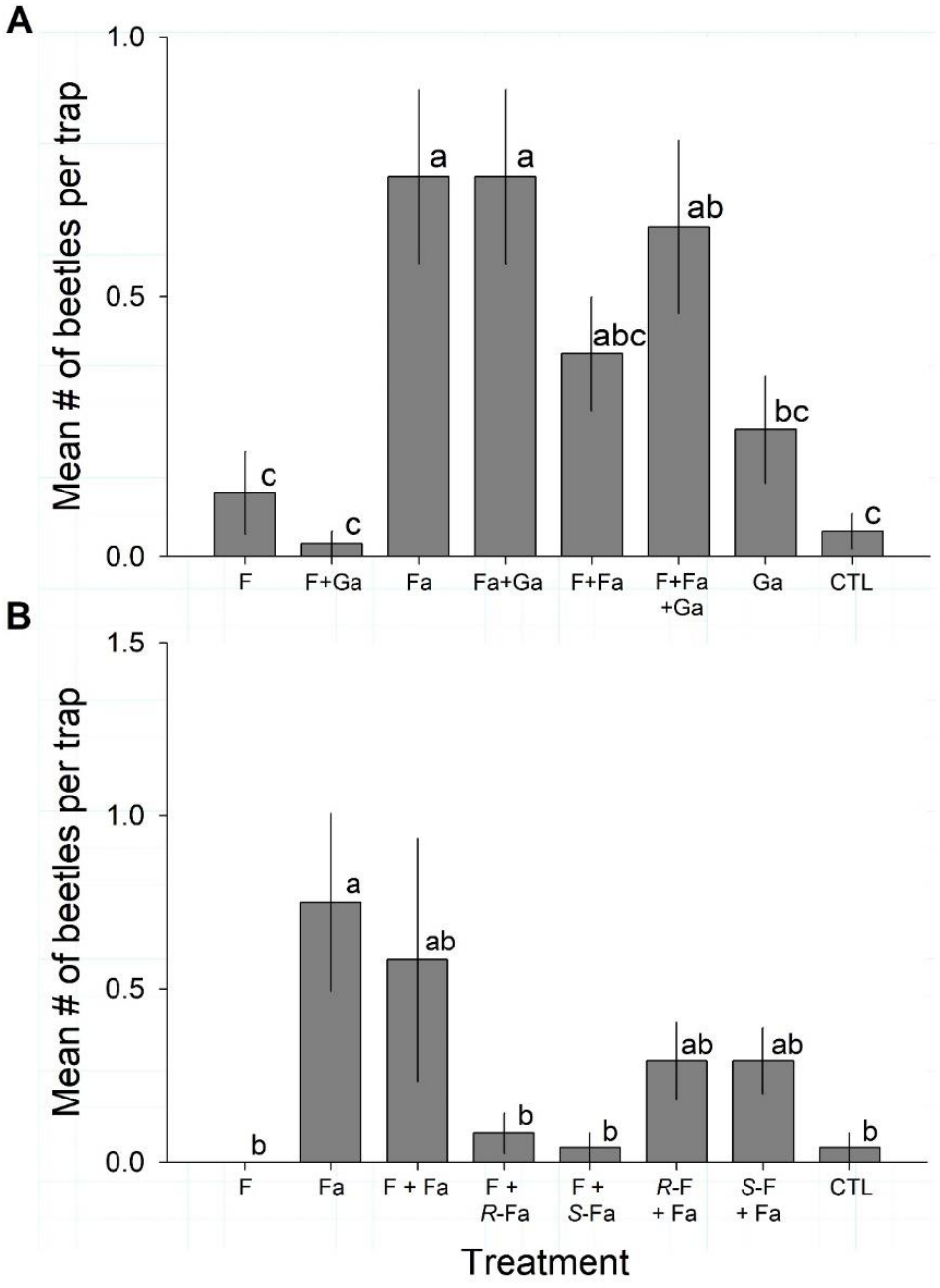


Fig. 3.4. Mean (± SE) numbers of adult beetles caught per replicate during field bioassays of synthesized pheromones for *Lepturges confluens* A) Experiment 1 (Friedman's $Q_{7,72} = 24.9$, $P < 0.001$); B) Experiment 3 ($Q_{7,56} = 29.2$, $P < 0.0001$). Means within figures with different letters are significantly different (REGWQ test, $P < 0.05$). Chemical abbreviations: F = racemic fuscumol, Fa = racemic fuscumol acetate, Ga = geranylacetone. *R* and *S* indicate enantiomers of fuscumol and fuscumol acetate.

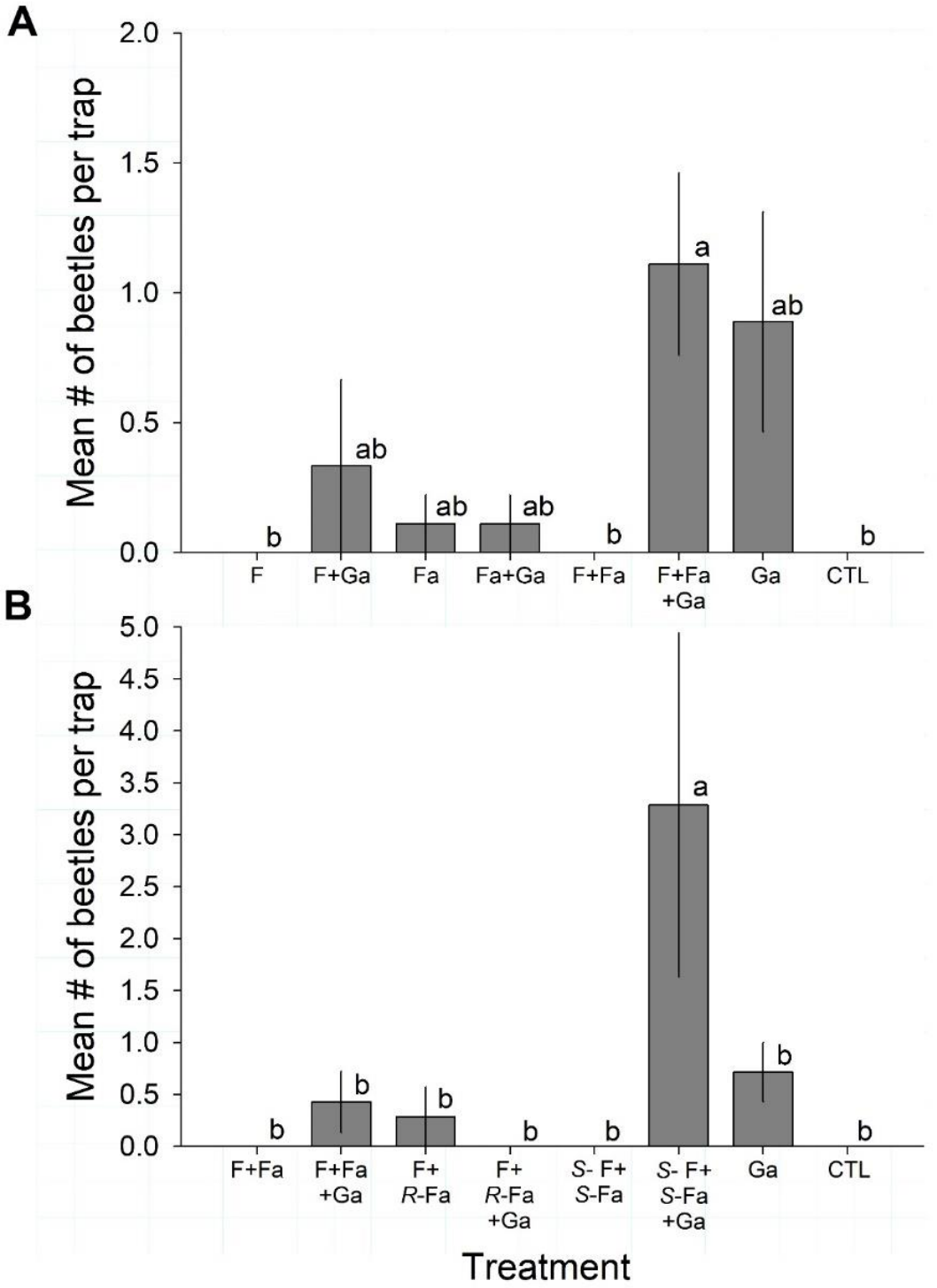
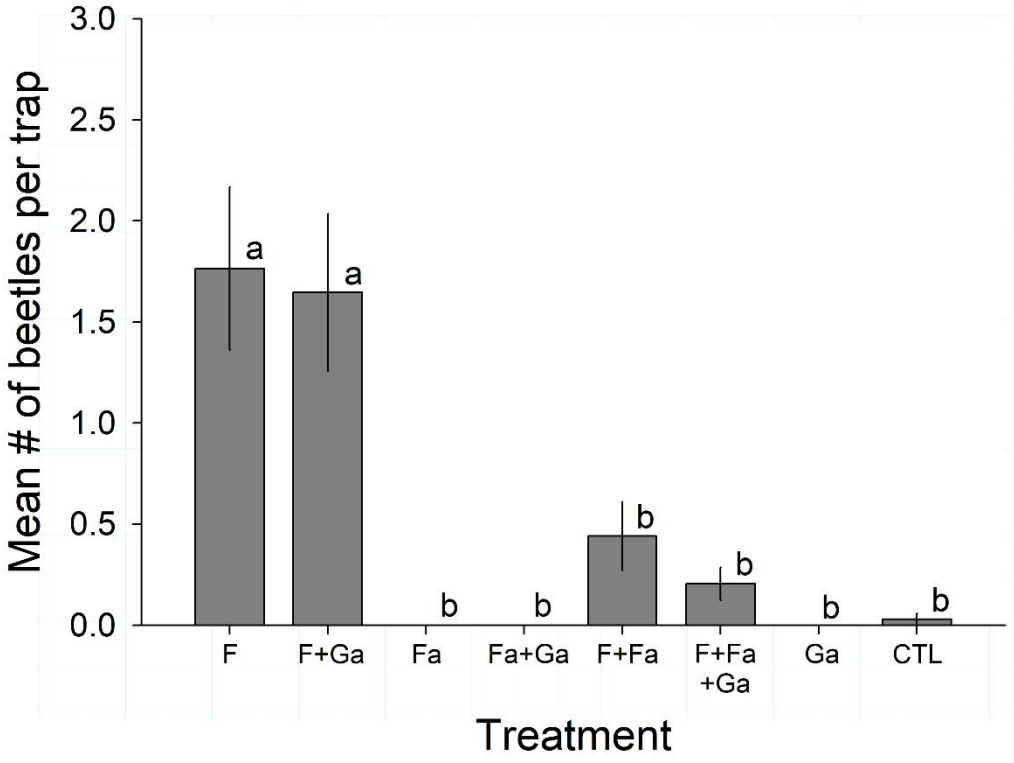


Fig. 3.5. Mean (± SE) numbers of adult beetles caught per replicate during field bioassays of synthesized pheromones for *Sternidius alpha* during Experiment 1 (Friedman's $Q_{7,80} = 58.8$, $P < 0.0001$). Means with different letters are significantly different (REGWQ test, $P < 0.05$). Chemical abbreviations: F = racemic fuscumol, Fa = racemic fuscumol acetate, Ga = geranylacetone. *R* and *S* indicate enantiomers of fuscumol and fuscumol acetate.



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CHAPTER 4: SULCATONE AND SULCATOL ARE PHEROMONE COMPONENTS FOR TWO SPECIES OF CERAMBYCID BEETLES IN THE SUBFAMILY LAMIINAE

Introduction

Research on pheromone chemistry of cerambycid beetles suggests that specific chemicals are evolutionarily conserved globally with sympatric species sharing dominant pheromone components, or at least compounds of the same structural class (reviewed by Millar and Hanks 2017). For example, males of many species in the large subfamily Lamiine, native to South and North America, produce aggregation-sex pheromones (*sensu* Cardé 2014) composed of three related structures (Fig. 1): the sesquiterpene degradation product geranylacetone, the corresponding alcohol (known as fuscumol), and its acetate ester (fuscumol acetate; Chapter 3; Mitchell et al. 2011; Hanks and Millar 2016; Hughes et al. 2013, 2016; Meier et al. 2016; Millar et al. 2018). These species belong to the tribes Acanthocinini and Acanthoderini.

Cross attraction among sympatric cerambycid species that share dominant pheromone components may be averted by differences in the seasonal or daily activity periods of the adults, or by minor pheromone components that synergize attraction of conspecifics and/or antagonize attraction of heterospecifics (Mitchell et al. 2015). A study in east-central Illinois demonstrated that synergism among enantiomers of fuscumol and fuscumol acetate imparts species specificity to the pheromones of the sympatric and synchronic acanthocinines *Astylidius parvus* (LeConte) and *Lepturges angulatus* (LeConte) (Meier et al. 2016). That is, males of *A. parvus* produce both *R*- and *S*-fuscumol + *R*-fuscumol acetate, while males of *L. angulatus* produce *R*- and *S*-fuscumol acetate, and adults of both species respond only to the combinations of enantiomers that simulate their pheromones. The results presented in Chapter 3 further demonstrate that a number of acanthocinine and acanthoderine species avoid cross-attraction through differences in their

pheromone chemistry. However, there are a limited number of unique and potentially species-specific blends available to the community.

Here, I show that pheromone components of another structural class, also based on a sesquiterpene degradation product, 6-methyl-5-hepten-2-one (sulcatone; Fig. 4.1), play a role in segregating communities of lamiine species. The study focused on the species *Astylopsis macula* (Say) and *Leptostylus transversus* (Gyllenhal) (both Acanthocinini), which are sympatric throughout much of the eastern United States (Linsley and Chemsak 1995). The two species also overlap broadly in seasonal activity period, *L. transversus* being the earliest lamiine to fly in east central Illinois and is active from May through July, while adults of *A. macula* fly from mid-June through early July (Hanks et al. 2014). Larvae of both species are polyphagous on hardwoods trees of several families (Linsley and Chemsak 1995). The pheromones of these species were not known, although adults of *A. macula* are attracted to traps baited with racemic fuscumol and fuscumol acetate, separately and blended together (Hanks and Millar 2013, Millar et al. 2018), and specifically to *S*-fuscumol (Hughes et al. 2016). Adults of *L. transversus* were attracted by racemic fuscumol in an earlier study (Mitchell et al. 2011).

I provide evidence that males of *A. macula* and *L. transversus* produce pheromones of similar composition, a blend of sulcatone and only the *S*-enantiomer of the corresponding alcohol, the chiral compound sulcatol. Attraction to the synthesized candidate pheromone was confirmed with a field experiment. The hypothesis that the sulcatone pheromone class is partitioned into species-specific pheromones was tested using two lines of evidence: 1) that the species are segregated by differences in their pheromone chemistry; and 2) that attraction of a species to a synthesized reconstruction of its pheromone would be inhibited by non-natural compounds that are pheromone components of other species. In addition, I hypothesized that the

geranylacetone and sulcatone classes interact by serving as antagonists of one another, thereby reinforcing reproductive isolation.

Methods and Materials

Sources of Chemicals

Sulcatone, racemic sulcatol, (*R*)-sulcatol, and (*S*)-sulcatol were purchased from Alfa Aesar (Haverhill, MA, USA), and racemic (*E*)-fuscumol and (*E*)-fuscumol acetate from Bedoukian Research (Danbury, CN, USA).

Study Sites

Field work to identify pheromones of *A. macula* and *L. transversus* and to confirm attraction to synthesized pheromones was conducted at two study sites in east-central Illinois: Brownfield Woods Co., 40.145 lat., -88.165 long.; ~26 h; property of the University of Illinois) and Forest Glen Preserve (Vermilion Co., 40.015 lat., -7.568 long., ~3 hectares; Vermilion Co. Conservation District). Both sites were wooded with mature second-growth or successional hardwoods and dominated by oaks (*Quercus* species) and hickories (*Carya* species), common larval hosts of *A. macula* and *L. transversus* (Linsley and Chemsak 1995), as well as maples (*Acer* species; common hosts of *A. macula*) and ash (*Fraxinus* species).

Identification of Pheromones

Adults of *A. macula* and *L. transversus* were captured alive for collection of headspace odors using flight intercept traps (black corrugated plastic, cross-vane, Alpha Scents, Portland, OR) coated with Fluon® (10% aqueous dilution; for details, see Allison et al. 2016). Trap basins

were replaced with 2-l plastic jars with the bottoms replaced with aluminum screen to permit drainage of rainwater. Trap lures were polyethylene sachets (5.1 × 7.6 cm, Bagettes® model 14770, Cousin Corp., Largo, FL) loaded with 25 mg of sulcatone blended with 50 mg of sulcatol (i.e., 25 mg per enantiomer) in 1 ml isopropanol. Single traps were deployed during May to August 2015 at the two study sites, and serviced every 1-2 d. Trap lures were replaced as needed, usually every 10 – 14 d.

Captured adults of *A. macula* and *L. transversus* were sexed by the morphology of the fifth abdominal sternite (Linsley and Chemsak 1995), or by caging pairs of beetles and checking for mating behavior (i.e., males mount females). Beetles were caged, separately by species and sex, under ambient laboratory conditions (~12:12 h L:D, ~20 °C). Preliminary observations indicated that adults of both species will feed on the tender bark of their larval hosts, *A. macula* on branches of maple (*Acer saccharum* Marshall) and *L. transversus* on branches of hickory (*Carya ovata* [Mill.] K. Koch). Therefore, the caged beetles were provided branches (2 – 5 cm in diameter, ~8 cm long) of maple and hickory, respectively, which had been freshly cut from trees at our study sites. Beetles also were provided 10% aqueous sucrose solution (in a glass vial with cotton wick) for moisture and nutrition. They were allowed at least 24 h to acclimate prior to their first aeration, and between subsequent aerations.

Volatiles produced by beetles were collected by aerating them in glass Mason-style canning jars that were positioned near closed exterior windows (natural photoperiod, ~14:10 h L:D, ~20 °C). Beetles usually were aerated individually, but in some cases two or three beetles of the same sex were aerated together to improve the chances of collecting pheromones. Once it was confirmed that only the males produced volatile chemicals (see Results), the sexes were sometimes aerated together in case the presence of females encouraged males to call. Beetles

were often aerated with small sections of their host plants, so we ran control aerations using the same methods but without beetles to determine if we were picking up chemicals from the plants. Additional controls were used to detect plant volatiles released by feeding beetles, using jars that contained twigs that had been damaged mechanically, by scraping or gouging the bark.

Aeration jars were swept with clean air (1 l/min) for 24 h, and headspace volatiles were collected on a trap of the adsorbent polymer HayeSep® Q (150 mg; Sigma-Aldrich, St. Louis, MO, USA). The polymer traps were extracted with 1.5 ml of dichloromethane into silanized glass vials that were stored at -20 °C. Total numbers of aerations of males and females were 14 and 2 for *A. macula*, and 32 and 5 for *L. transversus*.

Aeration extracts from adult beetles were analyzed with a gas chromatograph interfaced to a mass selective detector (Models 6809 and 5973, Hewlett-Packard, Palo Alto, CA, USA; AT-5ms column, 30 m × 0.25 mm i.d., 0.25 μm film, Alltech Associates, Inc., Deerfield, IL, USA). The GC oven was programmed from 35 °C/1 min, 10 °C/min to 210 °C, hold 3 min. Injections were made in splitless mode, with an injector temperature of 250 °C, and helium the carrier gas. Sex-specific compounds were tentatively identified by interpreting mass spectra, and structures were confirmed by comparing retention times and spectra with those of authentic standards.

The absolute configurations of insect-produced chemicals were determined by analyzing aliquots of extracts with an HP 5890 gas chromatograph equipped with a chiral stationary phase Cyclodex B column (30 m × 0.25 mm i.d., 0.25 μm film; Agilent Technologies, Inc., Santa Clara, CA, USA) with the oven temperature programmed from 50 °C/1 min, 2.5 °C/min to 125 °C, hold 3 min, and an injector temperature of 210 °C. Absolute configurations of sulcatol were confirmed by co-injection of an aliquot of aeration extract with the mixture of both enantiomers (Millar et al. 2009), and *S*- and *R*-sulcatol were resolved to baseline (retention times 26.1 and

26.38 min, respectively). Five extracts from each of the two species were analyzed to determine the chirality of sulcatol.

Daily Activity Period of Adult Beetles

Diel phenology of the two species was compared by estimating the time of day that beetles were captured using the same type of trap described above, but fitted with a mechanism (model #2850, BioQuip Products, Rancho Dominguez, CA, USA) that rotated the collecting jars at programmable intervals. The timer mechanism was set to rotate the eight trap jars at seven 1-h intervals, beginning at 19:00 h and ending at 2:00 h, in order to encompass the expected crepuscular to nocturnal activity periods of the beetle species. The eighth jar was not rotated for 17 h (2:00 – 19:00), to confirm that beetles were not active during the remainder of each 24-h cycle. The hour that beetles were caught was estimated as the median time that their trap jar was positioned under the trap. Timer traps were run between 15 May and 5 July 2017 at the Brownfield Woods study site. Activity periods of the species were compared by calculating 95% confidence intervals around mean capture times, with overlap in confidence intervals indicating that means were not significantly different (Di Stefano 2004).

Field Bioassays of Pheromones

Attraction of adults of *A. macula* and *L. transversus*, and other lamiine species native to the study area, to synthesized pheromones was tested with four independent field bioassays. Synthesized pheromones were dispensed from sachets made from heat-sealed polyethylene tubing (Associated Bag, Milwaukee, WI, USA), which were loaded with 50 mg of sulcatone, 25mg of particular enantiomers of sulcatol, or 50 mg of racemic sulcatol (25mg per enantiomer),

diluted in 1 ml isopropanol. All lures also contained a cotton roll (1 × 4 cm dental wick, Patterson Dental Supply, Inc., St. Paul, MN, USA), which prevented leakage and stabilized release rates. Separate lures were used for each compound and combined on the same trap to create blends. Release rates of each compound was standardized to ~20 mg/d by using sachets of different wall thicknesses, as follows: fuscumol (1.5 mil, 38 µm wall thickness); fuscumol acetate (3 mil, 76 µm wall thickness), sulcatone (1.5 mil, 38 µm wall thickness), sulcatol (1.5 mil, 38 µm wall thickness), and sulcatone + sulcatol (1.5 mil, 38 µm wall thickness). Control traps were baited with three sachets of the same design as the pheromone lures, but loaded with 1 ml neat isopropanol. Lures were replaced every ~14 d.

Experiment 1 tested for attraction of *A. macula* and *L. transversus* to sulcatone, racemic sulcatol, *R*-, and *S*-sulcatol individually and in binary blends, including the candidate pheromone of the two study species, sulcatone + *S*-sulcatol (Table 4.1). The experiment was conducted at Forest Glen Preserve site from 22 April to 25 July 2016 and at the Brownfield Woods site from 22 April to 29 July 2016.

The remaining three experiments (2A, B, and C) had traps baited with sulcatone and racemic sulcatol, fuscumol, and fuscumol acetate, individually, in all possible binary and trinary combinations, and as the complete quaternary blend (Table 4.1). The objective was to test fuscumol and fuscumol acetate with sulcatone (Experiment 2A), with sulcatol (Experiment 2B), and with both sulcatone and sulcatol (Experiment 2C). Experiments 2A and 2B were conducted concurrently at the Brownfield Woods site from 22 April to 12 August 2016, while 2C was conducted at the Forest Glen Preserve site from 19 July to 31 August 2015, and at the Brownfield Woods site from 22 April to 12 August 2016.

Supporting data from additional independent field experiments are presented in the Supplement.

Statistical Analyses

For each experiment, differences between treatment means were tested separately for all species that were represented by at least eight specimens, blocked by site and date, using the nonparametric Friedman's Test (PROC FREQ, option CMH; SAS Institute 2011) because data violated homoscedasticity assumptions of ANOVA (Sokal and Rohlf 1995). Thus, the analyses were blocked by site and date, excluding replicates that contained no specimens of the species in question due, for example, to inclement weather. Comparisons of treatment means were protected (i.e., assuming a significant overall Friedman's test), using the Ryan-Einot-Gabriel-Welsch Q multiple comparison test (REGWQ, SAS Institute 2011).

Captured beetles were identified to species using keys in Lingafelter (2007), with taxonomy following Monné and Hovore (2005). Specimens of species that were represented in the data set are available from the laboratory collection of LMH, and voucher specimens have been deposited with the collection of the Illinois Natural History Survey, Champaign, IL, USA.

Results

Identification of Pheromones

Extracts of volatiles emitted by males of both species contained sulcatone and sulcatol, with detectable quantities in 14 of 22 extracts from males of *A. macula* and 23 of 39 extracts from males of *L. transversus*. These compounds were not detected in any aeration extracts from females or in system controls. Males of both species produced only the *S*-enantiomer of sulcatol,

and the average percentages of sulcatone to *S*-sulcatol (± SD) were 61.5 ± 26.3 and 23.8 ± 10.7 for *A. macula* and *L. transversus*, respectively. Surprisingly, sulcatone was also identified in three extracts from males of *Astylidius parvus* (LeConte) (Acanthocinini) and one extract from males of *Aegomorphus modestus* (Gyllenhal) (Acanthoderini), for which pheromones had already been identified, but perhaps only partially so (Chapters 2 and 3, respectively).

Daily Activity Period of Adult Beetles

Timer traps captured only eight adults of *A. macula* and 16 adults of *L. transversus*, primarily due to inclement weather during the sample period. Nevertheless, the timer trap data suggested overlap in activity periods (Fig. 4.2); that is, adults of *A. macula* were captured during 1800 – 2100 h (mean ± 1 SD: 1850 ± 141) while adults of *L. transversus* were captured during 1800 – 2300 h (2056 ± 165 h). The overlapping 95% confidence intervals suggest that the means were not significantly different.

Field Bioassays of Pheromones

A total of 1,242 beetles of 47 cerambycid species were trapped during the four field experiments (Table 4.2), including representatives of the subfamilies Cerambycinae, Lamiinae, Lepturinae, Parandrinae, and Prioninae, as well six individuals of a species in the closely related Disteniidae. Most of the trapped beetles (~87%) were lamiines, and among the most numerous species were the two targeted for pheromone identification, *A. macula* and *L. transversus*. Other common species included three species for which pheromones were already characterized, *Astyleiopus variegatus* (Haldeman), *A. parvus*, and *L. angulatus* (Hughes et al. 2016; Meier et al. 2016), as well as four species whose pheromones were identified in Chapter 3: *A. modestus*,

Graphisurus despectus (LeConte), *Graphisurus fasciatus* (Degeer), *Sternidius alpha* (Say). Both sexes of *A. macula* and *L. transversus* were caught by traps, with respective sex ratios of 63 and 59% female in Experiment 2C, for example, confirming that the males produce aggregation-sex pheromones.

The four experiments varied as to which lamiine species were captured in sufficient numbers for statistical analysis, including the species targeted for pheromone identification, *A. macula* and *L. transversus*, in Experiments 1, 2B, and 2C, and various others of the dominant species in Experiments 2A, 2B, and 2C (see below). Adults of *A. macula* were significantly attracted to traps baited with lures that emitted *S*-sulcatol during Experiment 1 (Fig. 4.3A), with no evidence of synergism by sulcatone or inhibition by the non-natural *R*-sulcatol. In Experiment 2B, adults of this species were again attracted only by sulcatol, and attraction apparently was strongly antagonized by fuscumol acetate (Fig. 4.3B). In Experiment 3, the adults were attracted only by the blend of sulcatone and sulcatol (Fig. 4.3C), indicating that fuscumol and fuscumol acetate were strong antagonists.

Across experiments, *L. transversus* responded in a fashion similar to that of *A. macula*. In Experiment 1, the beetles were significantly attracted by lures that emitted *S*-sulcatol but were not significantly antagonized by the unnatural *R*-enantiomer present in racemic sulcatol (Fig. 4.4A). Sulcatone was not attractive alone but appeared to antagonize attraction to *S*-sulcatol. Attraction of *L. transversus* to sulcatol or the blend of sulcatone + sulcatol was antagonized by fuscumol acetate in Experiment 2B (Fig. 4.4B) and by both fuscumol and fuscumol acetate in Experiment 2C (Fig. 4.4C).

Adults of *A. parvus* were caught in numbers sufficient for analysis only in Experiments 2A and 2C. The pheromone of this species (*R/S*-fuscumol, *R*-fuscumol acetate, geranylacetone)

was identified by Meier et al. (2016). In Experiment 2A, adults of this species were significantly attracted only by lures that emitted the trinary blend of sulcatone, fuscumol, and fuscumol acetate (Figs. 4.5A,B). In Experiment 2C, adults of *A. parvus* were attracted to the blend of sulcatone + sulcatol + fuscumol, and attraction was not influenced by fuscumol acetate (Fig. 4.5B). Taken together, these findings suggest that attraction to fuscumol is synergized by sulcatone only in the presence of fuscumol acetate and not by the combination of sulcatone + sulcatol. This is consistent with the pheromone of this species (see Results).

Adults of *A. modestus* also were caught in numbers sufficient for analysis only in Experiments 2A and 2C. The pheromone of this species (*R/S*-fuscumol acetate, geranylacetone) was identified in Chapter 3. Beetles were significantly attracted only to the fuscumol acetate treatment in Experiment 2A, with strong antagonism by both fuscumol and sulcatol (Fig. 4.6A). In Experiment 2C, adults of this species were significantly attracted only to the trinary blend of fuscumol acetate, sulcatone, and sulcatol (Fig. 4.6B), and attraction to this blend again was antagonized by fuscumol. Taken together, these findings suggest that sulcatol is an antagonist, but its effect can be masked by strong synergism by sulcatone. As with *A. parvus*, the pheromone composition of this species also contains sulcatone, and previous reports (Chapter 3) of the pheromone chemistry of *A. modestus* must be revised.

Adults of *Astylopsis sexguttata* (Say), for which the pheromone is unknown, showed a similar response to treatments in Experiment 2C (Fig. 4.7A), responding only to the tertiary blend of sulcatone, sulcatol, and fuscumol acetate, not to either of the compounds alone, with significant antagonism by fuscumol.

The remaining two species responded to treatments as would be expected from their pheromone chemistry and earlier research (Meier et al. 2016; Chapter 3). That is, adults of *L.*

angulatus (pheromone: *R/S*-fuscumol acetate, geranylacetone) were attracted only to the fuscumol acetate treatment in Experiment 2C (Fig. 4.7B), and attraction was antagonized by both the blend of sulcatone and sulcatol, and by fuscumol. Adults of *S. alpha* (*S*-fuscumol, *R/S*-fuscumol acetate) were attracted by fuscumol (Fig. 4.7C) and attraction was antagonized by the blend of sulcatone and sulcatol, and by fuscumol acetate. The antagonistic effect of fuscumol acetate, despite both enantiomers being components of the presumed pheromone, was also observed in an independent experiment (Chapter 3).

Discussion

Attraction of adult males and females of *A. macula* and *L. transversus* to traps baited with the synthesized reconstruction of their pheromones, sulcatone plus *S*-sulcatol, confirmed that these chemicals serve as an aggregation-sex pheromone, as has been shown for many cerambycid species in the subfamilies Lamiinae, Cerambycinae, and Spondylidinae (Hanks and Millar 2016).

The field experiments further revealed that both species were attracted to any blend of chemicals that contained *S*-sulcatol, that attraction was not antagonized by the non-natural *R*-enantiomer, and that sulcatone neither influenced attraction nor was an attractant when tested alone.

Both sulcatone and sulcatol are common semiochemicals in insects (El-Sayed 2018). For example, sulcatone serves as a pheromone for more than 80 species of insects in the orders Hemiptera, Coleoptera, Hymenoptera, Lepidoptera, and Trichoptera, in addition to other classes of arthropods, and even some vertebrates (see El-Sayed 2018). Sulcatol is a pheromone component for a similar broad taxonomic diversity of insects. Both compounds serve as pheromones for wood-boring beetles of the Platypodidae and the Scolytinae of the

Curculionidae, but this is the first report of their presence as pheromone components of cerambycid beetles.

The similar pheromone composition of *A. macula* and *L. transversus* and the considerable amount of overlap in their seasonal and daily flight periods suggest that mechanisms other than differences in pheromone chemistry and phenology must be responsible for segregating the species in nature and averting interspecific attraction. One potentially important factor is the relative ratio of pheromone components. In other insects, particular ratios of components may confer species-specificity to blends of components (Den Otter 1977, Leal et al. 1994), but these ratios have been largely overlooked in earlier research on pheromones of cerambycid beetles (Millar and Hanks 2017).

Attraction of both *A. macula* and *L. transversus* to their pheromones was antagonized by both fuscumol and fuscumol acetate, which has important implications for the pheromone chemistry of the lamiine community native to east-central Illinois. These antagonistic effects are evidence that the two species are capable of detecting compounds of both the sulcatone and geranylacetone classes, and that this ability plays an important role in fitness. The same can be said for the antagonistic effects of sulcatone and sulcatol on attraction of *L. angulatus* and *S. alpha* to their geranylacetone class pheromones. These findings lend support to the hypothesis that the geranylacetone and sulcatone classes act in tandem in comprising lamiine pheromones, and that pheromones of some species may include components from both classes. Further support for the discovery (in this study) of sulcatone in headspace volatiles of both *A. modestus* and *A. parvus*. Attraction to their geranylacetone class pheromones was strongly synergized by sulcatone, suggesting that this compound is a pheromone component that has been overlooked in earlier research. Lastly, attraction of *A. sexguttata*, a congener of *A. macula*, only to the blend of

sulcatone and geranylacetone class compounds, and not to any of the compounds when tested separately, is reliable evidence that the pheromone of this species is composed of both classes of compounds.

Addition of the sulcatone class of compounds as pheromone components available to lamiine species greatly increases the number of unique combinations that can impart species specificity. All possible combinations of the enantiomers of fuscumol and fuscumol acetate, along with geranylacetone offers a total of 31 unique combinations (Chapter 3). However, with sulcatone and the enantiomers there would be 127 unique combinations, apparently offering an excess of species-specific pheromones for the community of acanthocinine and acanthoderine species native to the area, which number fewer than 30 species (Hanks et al. 2014).

Acknowledgments

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Tables

Table 4.1 Experimental treatments for four field experiments that test attraction of adults of *Astylopsis macula* and *Leptostylus transversus*, to synthesized pheromones, and various blends thereof, conducted at two study sites in east-central Illinois. Fusc. = fuscumol and F. acetate = fuscumol acetate. The chiral compounds fuscumol, fuscumol acetate, and sulcatol are racemic mixtures, except for the individual enantiomers of sulcatol in Expt. 1.

Compound	Expt 1	Expt 2A	Expt 2B	Expt 2C
Solvent control	X	X	X	X
Sulcatone	X	X		
Sulcatol	X		X	
Sulcatone + sulcatol	X			X
R-sulcatol	X			
S-sulcatol	X			
R-sulcatol + sulcatone	X			
S-sulcatol + sulcatone	X			
Fusc.		X	X	X
Fusc. + sulcatone		X		
Fusc. + sulcatol			X	
Fusc. + sulcatone + sulcatol				X
F. acetate		X	X	X
F. acetate + sulcatone		X		
F. acetate + sulcatol			X	
F. acetate + sulcatone + sulcatol				X
Fusc. + F. acetate		X	X	X
Fusc. + F. acetate + sulcatone		X		
Fusc. + F. acetate + sulcatol			X	
Fusc. + F. acetate + sulcatone + sulcatol				X

Table 4.2 Taxonomy of beetles trapped during four field experiments in east-central Illinois that tested attraction to synthesized aggregation-sex pheromones

Taxonomy	Expt 1 2016	Expt 2A 2016	Expt 2B 2016	Expt 2C 2015	Expt 2C 2016	Total
Cerambycinae						
Anaglyptini						
Cyrtophorus verrucosus (Olivier)	21	9	8		3	41
Callidiini						
Phymatodes amoenus (Say)			1			1
Clytini						
Clytus ruricola (Olivier)		5	1			6
Megacyllene caryae (Gahan)			1			1
Neoclytus a. acuminatus (F.)	1	1	2			4
Xylotrechus colonus (F.)	12	12	11	2	15	52
Elaphidiini						
Anelaphus pumilus (Newman)	1					1
Anelaphus villosus (F.)				1		1
Elaphidion mucronatum (Say)	3			3	2	8
Parelaphidion aspersum (Haldeman)					1	1
Parelaphidion incertum (Newman)					1	1

Table 4.2 (cont.)

Neoibidionini						
Heterachthes quadrimaculatus (Haldeman)		1				1
Lamiinae						
Acanthocinini						
Astyleiopus variegatus (Haldeman)	2		3	1	23	29
Astylidius parvus (LeConte)	1	12	7	43	22	85
Astylopsis collaris (Haldeman)			3			3
Astylopsis macula (Say)	156	4	30	22	69	281
Astylopsis sexguttata (Say)				8		8
Graphisurus despectus (LeConte)	2	22	8	11		43
Graphisurus fasciatus (Degeer)	18	115	79	20	12	244
Graphisurus triangulifer (Haldeman)		2				2
Leptostylus transversus (Gyllenhal)	110	7	36	1	32	186
Lepturges angulatus (LeConte)		14	3	9	20	46

Table 4.2 (cont.)

Lepturges confluens (Haldeman)		3	1	3		7
Lepturges regularis (LeConte)					1	1
Lepturges symmetricus (Haldeman)					3	3
Sternidius alpha (Say)		2	3	8	10	23
Sternidius misellus (LeConte)		1				1
Urgleptes querci (Fitch)	1	1	1	2	1	6
Urgleptes signatus (LeConte)		1	1		1	3
Acanthoderini						
Aegomorphus modestus (Gyllenhal)	8	23	21	12	17	81
Oplosia nubila (LeConte)		4	5		5	14
Dorcaschematini						
Dorcaschema nigrum (Say)		1				1
Monochamini						
Microgoes oculatus (LeConte)	1	4	3			8
Pogonocherini						
Ecyrus d. dasycerus (Say)		1			3	4

Table 4.2 (cont.)

Saperdini						
Saperda discoidea F.		1				1
Saperda imitans Felt & Joutel			2			2
Saperda lateralis F.		1				1
Lepturinae						
Lepturini						
Bellamira scalaris LeConte		1				1
Brachyleptura rubrica (Say)	3		1			4
Strangalia luteicornis (F.)					1	1
Strophiona nitens (Forster)		1				1
Typocerus lugubris (Say)	1	1				2
Typocerus v. velutinus (Olivier)	1	2	1			4
Rhagiini						
Gaurotes cyanipennis (Say)		2	2			4
Stenocorus cinnamopterus (Randall)			1			1
Parandrinae						
Parandrini						
Neandra brunnea (F.)	2	2		4	9	17

Table 4.2 (cont.)

Prioninae						
Prionini						
Orthosoma brunneum (Forster)	4		1		1	6
Disteniidae						
Disteniini						
Elytrimitatrix undata (F.)		2	1	3		6
Total	**348**	**258**	**237**	**153**		**1248**

Figures

Fig. 4.1. Structures of geranylacetone, fuscumol, and fuscumol acetate (left) and structures of sulcatone and sulcatol (right).

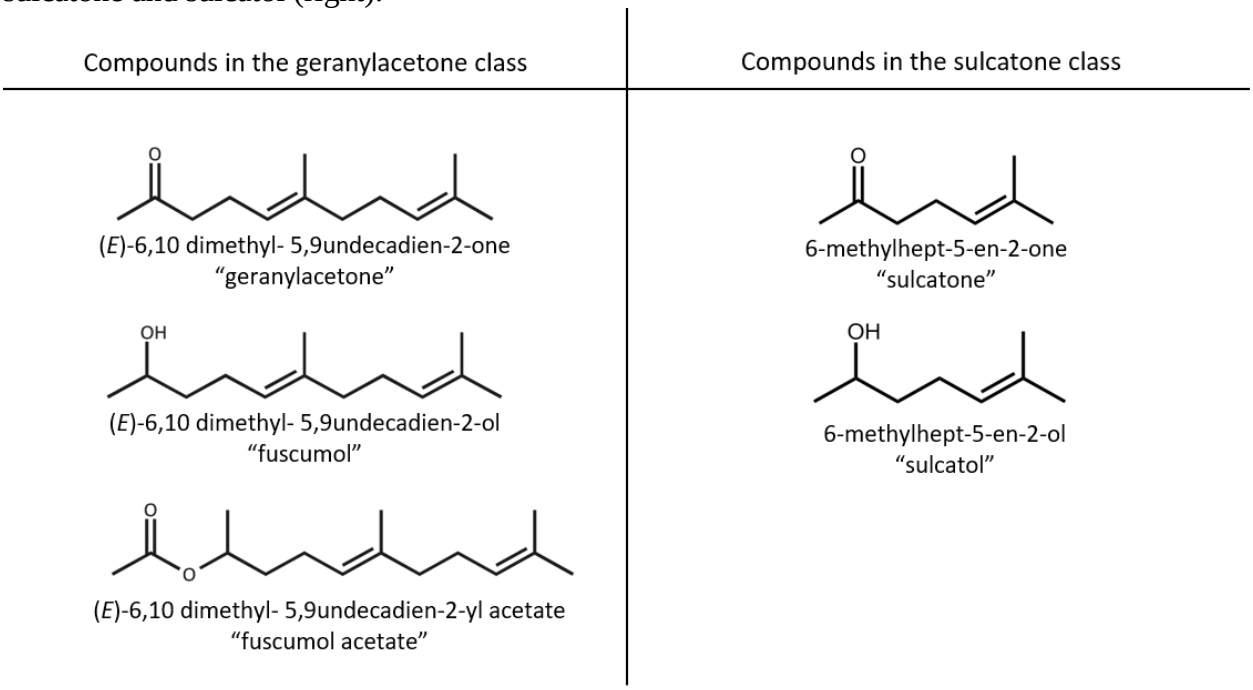


Fig. 4.2. Diel phenology of *Astylopsis macula* and *Leptostylus transversus* as determined by the time of day that beetles were captured by timer traps.

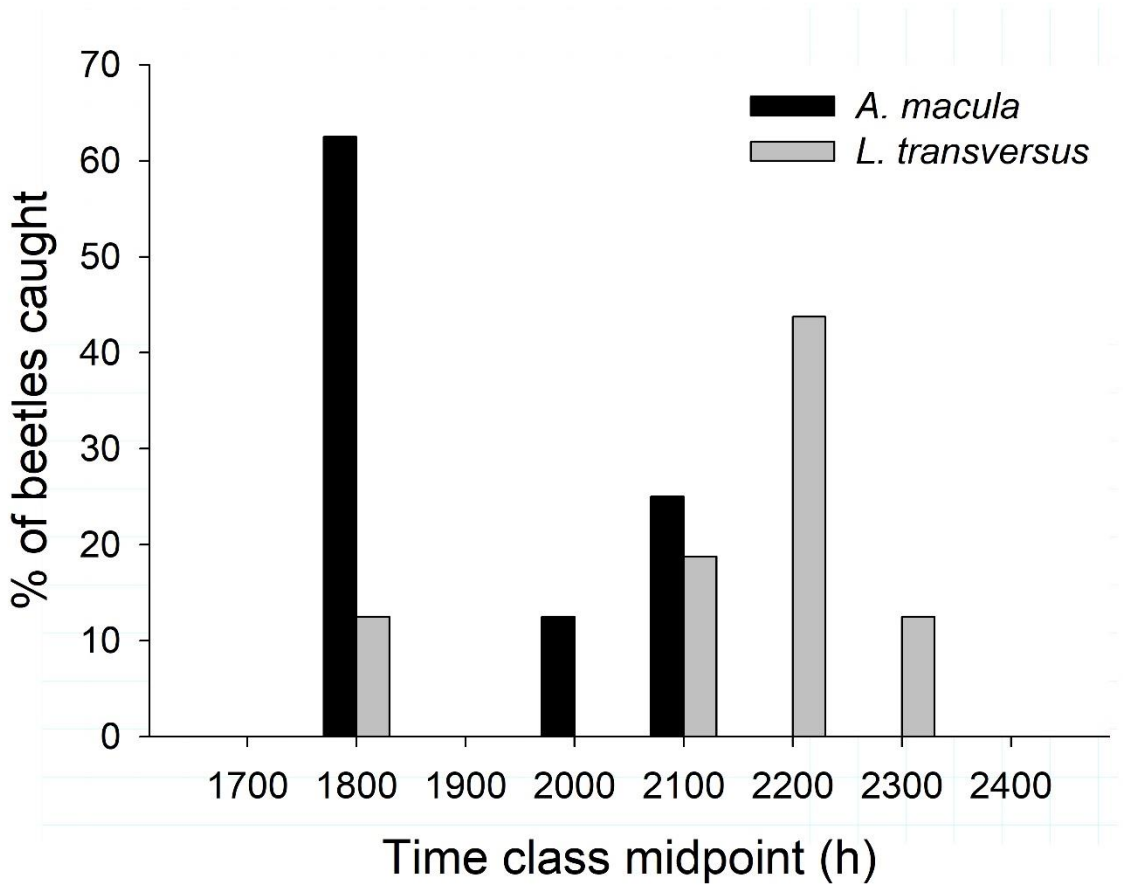


Fig. 4.3. Mean (± SE) number of adults of *Astylopsis macula* that were caught per replicate during A) Experiment 1 (Friedman's $Q_{7,88} = 43.4$, $P < 0.0001$), B) Experiment 2B ($Q_{7,72} = 33.5$, $P < 0.0001$), and C) Experiment 2C ($Q_{7,72} = 31.4$, $P < 0.0001$). Chemical abbreviations: One = sulcatone, *R*-Ol = *R*-sulcatol, *S*-Ol = *S*-sulcatol, *R/S*-Ol and Ol = racemic sulcatol. Means within experiments with different letters are significantly different (REGWQ test, $P < 0.05$).

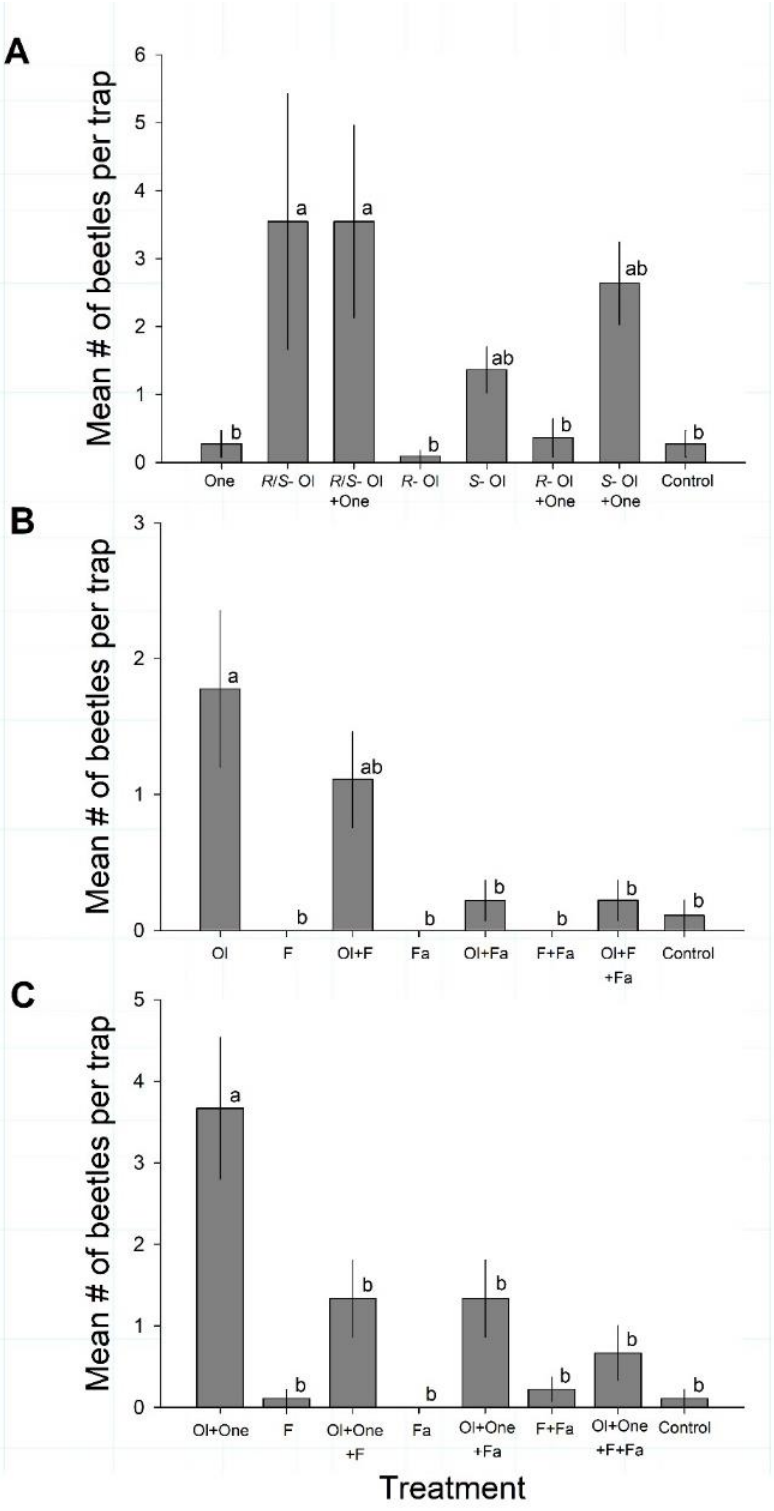


Fig. 4.4. Mean (± SE) number of adults of *Leptostylus transversus* that were caught per replicate in Experiment 1 (A), Experiment 2B (B), and Experiment 2C (C). Chemical abbreviations: One = sulcatone, *R*-Ol = *R*-sulcatol, *S*-Ol = *S*-sulcatol, *R/S*-Ol and Ol = racemic sulcatol. Friedman's $Q_{7,64} = 37.8$, $P < 0.0001$, $Q_{7,72} = 25.7$, $P < 0.001$, and $Q_{7,88} = 25.1$, $P < 0.001$, respectively. Means with different letters are significantly different (REGWQ test, $P < 0.05$).

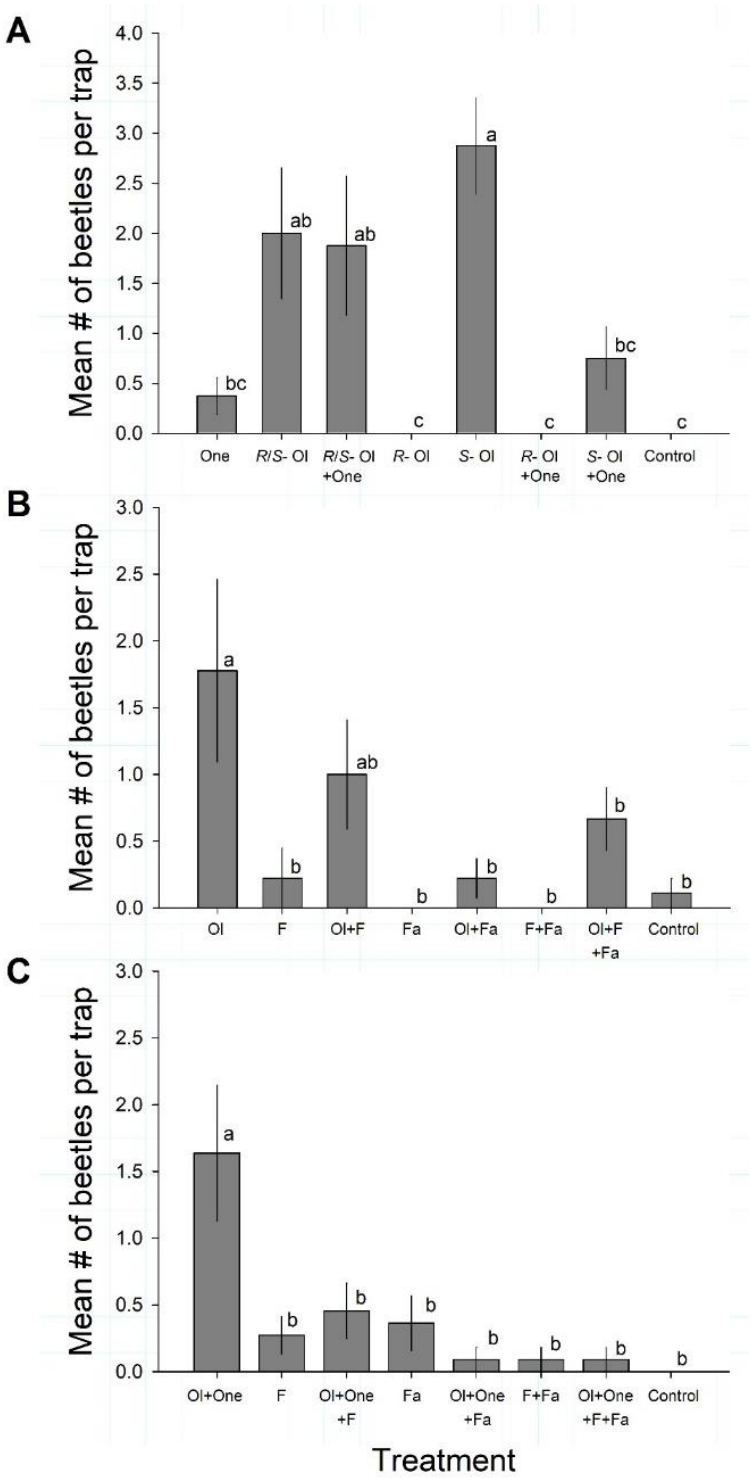


Fig. 4.5. Mean (± SE) number of adults of *Astylidius parvus* that were caught per replicate in Experiment 2A (A) and Experiment 2C (B). Chemical abbreviations: One = sulcatone, *R*-Ol = *R*-sulcatol, *S*-Ol = *S*-sulcatol, *R/S*-Ol and Ol = racemic sulcatol. $Q_{7,64} = 30.5$, $P < 0.0001$ and $Q_{7,88} = 37.8$, $P < 0.0001$, respectively. Means with different letters are significantly different (REGWQ test, $P < 0.05$).

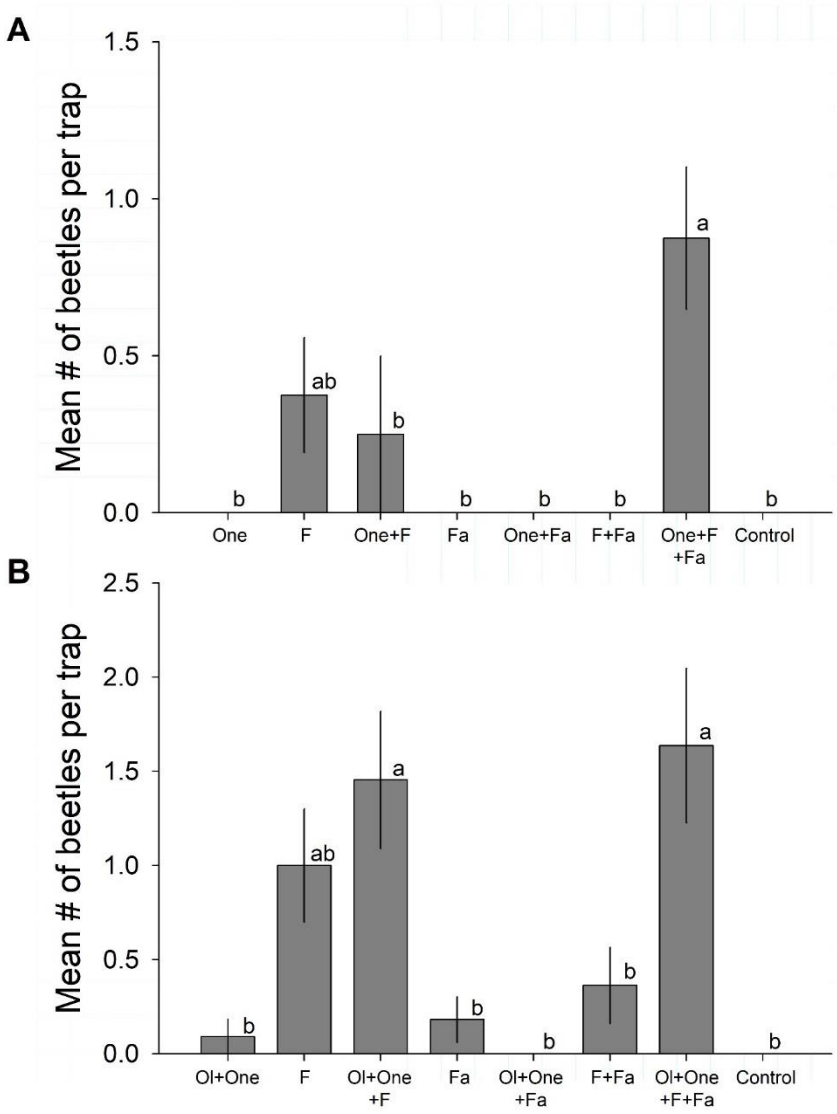


Fig. 4.6. Mean (± SE) number of adults of *Aegomorphus modestus* that were caught per replicate in Experiment 2B (A) and Experiment 2C (B). Chemical abbreviations: One = sulcatone, *R*-Ol = *R*-sulcatol, *S*-Ol = *S*-sulcatol, *R/S*-Ol and Ol = racemic sulcatol. Friedman's $Q_{7,64} = 27.6$, $P < 0.001$ and $Q_{7,64} = 30.4$, $P < 0.0001$, respectively. Means with different letters are significantly different (REGWQ test, $P < 0.05$).

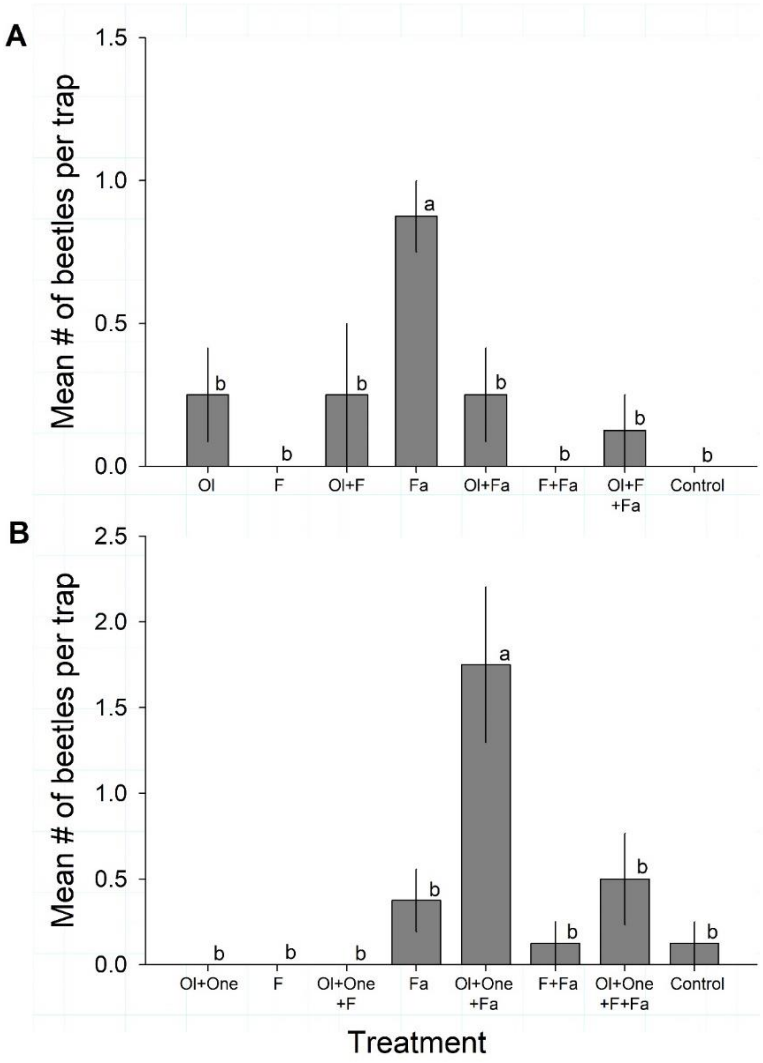
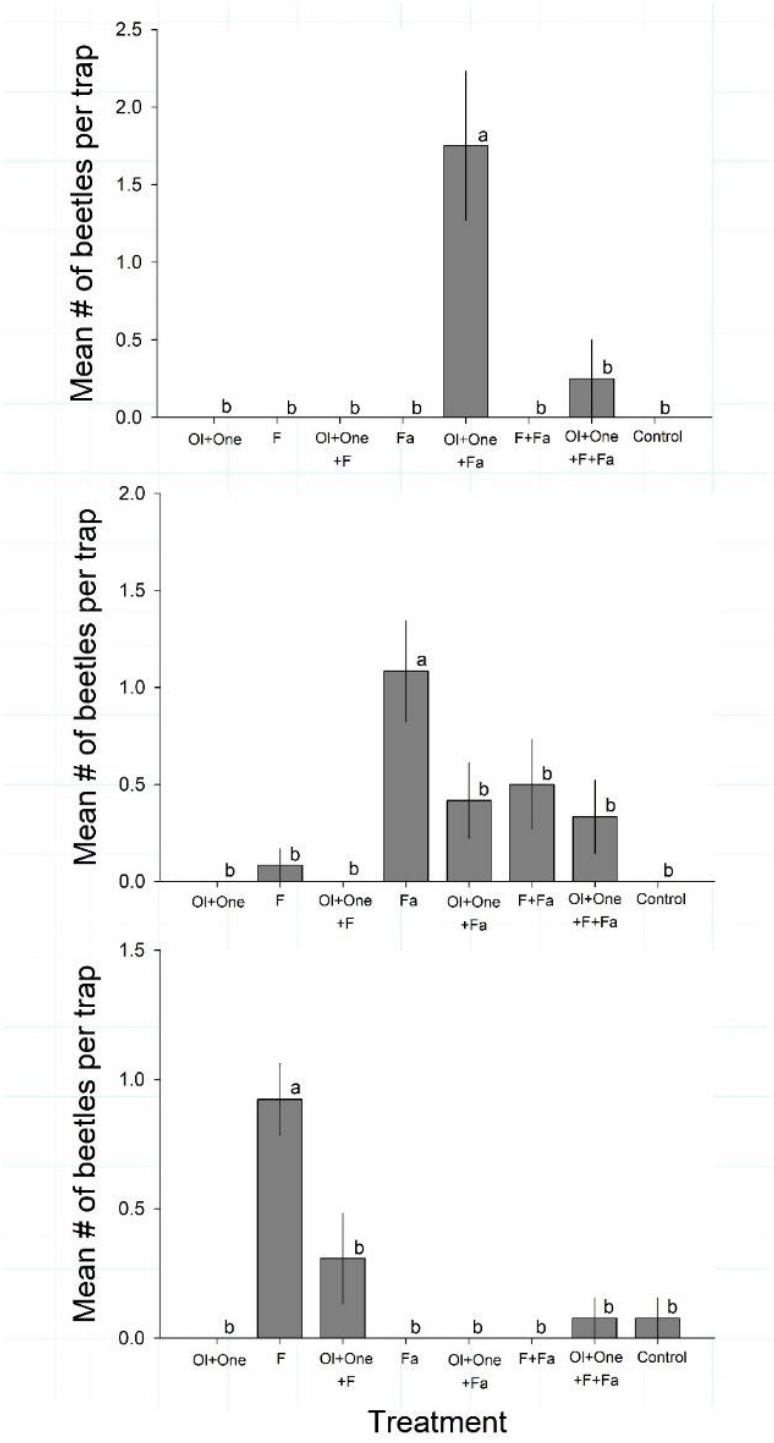


Fig. 4.7. Mean (± SE) number of adult beetles that were caught per replicate in Experiment 2C for the species: A) *Astylopsis sexguttata* (Friedman's $Q_{7,32} = 25.9$, $P < 0.001$), B) *Lepturges angulatus* ($Q_{7,96} = 32.7$, $P < 0.0001$), and C) *Sternidius alpha* ($Q_{7,104} = 57.9$, $P < 0.0001$). Chemical abbreviations: One = sulcatone, *R*-Ol = *R*-sulcatol, *S*-Ol = *S*-sulcatol, and Ol = racemic sulcatol. Means within species with different letters are significantly different (REGWQ test, $P < 0.05$).



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APPENDIX: SUPPLEMENTARY INFORMATION

Here are summarized the results of four field bioassays that yielded results that were redundant with the bioassays whose data are featured in Chapters 3 and 4.

Methods and Materials

Sources of Chemicals

(*E*)-fuscumol and (*E*)-fuscumol acetate were purchased from Bedoukian Research (Danbury, CT, USA), (*E/Z*)-geranylacetone from Sigma-Aldrich (St. Louis, MO, USA), and sulcatone, racemic sulcatol, *R*-sulcatol, and *S*-sulcatol were purchased from Alfa Aesar (Haverhill, MA, USA). *R*-fuscumol (96.6%), *S*-fuscumol (98%), *R*-fuscumol acetate (96.6%), and *S*-fuscumol acetate (98%) were synthesized as described in Hughes et al. (2013).

Study Sites

Experiments to identify pheromones of cerambycid species were conducted at five study sites in east-central Illinois (Table S-1), including four natural areas owned by the University of Illinois, all of which were wooded with mature second-growth or successional hardwoods and dominated by oaks (*Quercus* species), hickories (*Carya* species), maples (*Acer* species), and ash (*Fraxinus* species). The remaining study site was the backyard of a private residence in the city of Urbana, IL.

General Methods of Trapping

Attraction of beetles to candidate pheromones was tested using cross-vane panel traps (black corrugated plastic, Alpha Scents, Portland, OR) with their surfaces coated with a lubricant (Fluon® fluoropolymer dispersion; 10% aqueous dilution; Northern Products, Woonsocket, RI,

USA). Traps were suspended ~0.5 m above the ground from inverted L-shaped frames of polyvinylchloride pipe. Trap basins were partly filled with ~300 ml of saturated aqueous NaCl solution to preserve captured beetles. Test chemicals were dispensed from polyethylene sachets (5.1 × 7.6 cm, Bagettes® model 14770, Cousin Corp., Largo, FL, USA) with loadings of 50 mg of achiral compounds, 25 mg of individual enantiomers of chiral compounds, and 50 mg of racemic compounds (i.e., 25 mg per enantiomer) in 1 ml isopropanol. Binary and tertiary combinations of compounds were created either by baiting traps with sachets containing the blended components (Experiment 3), or by using separate sachets for each component (Experiments 1 and 4). (Experiment 2 did not include any combinations of compounds). Lures used in Experiments 2, 3, and 4 also contained a cotton wick (1 × 4 cm dental wick, Patterson Dental Supply, Inc., St. Paul, MN, USA), which served to prevent leakage and stabilize release rate.

The four experiments tested 19 unique combinations of racemic and chiral materials (Table S-2) as part of an on-going program to test all possible combinations of pheromone components known to be produced by males of acanthocinine and acanthoderinie lamiine species native to the study area. Experiment 1 tested various combinations of the individual enantiomers of fuscumol and fuscumol acetate and was conducted at the Nettie Hart, Trelease Woods, and Forest Glen sites from 15 August to 1 September 2014. Experiment 2 tested racemic fuscumol and fuscumol acetate, as well as their individual enantiomers, and was conducted at the Brownfield Woods and Nettie Hart sites during 15 June to 17 September 2015. Experiment 3 tested combinations of sulcatone with racemic and chiral sulcatol and was conducted at the Trelease Woods site and as well as the private residence during 9 June to 24 July 2015. Lastly, Experiment 4 tested various combinations of racemic fuscumol, fuscumol acetate, and sulcatol,

with geranylacetone and sulcatone, and was conducted at the Brownfield Woods and Trelease Woods sites during 12 June to 23 August 2017.

On the first day of each experiments, treatments were assigned randomly to traps that were set up 10 m apart in linear transects. For each experiment, traps were serviced at intervals of 1-3 d, and on each date treatments were shifted one position along transects to control for small-scale positional effects. Lures were replaced as needed, usually every 14 d.

Because the data violated homoscedasticity assumptions of ANOVA (Sokal and Rohlf 1995), differences between treatment means were tested separately, blocked by site and date, for each species using the nonparametric Friedman's Test (PROC FREQ, option CMH; SAS Institute 2011). Given a significant overall Friedman's test, pairs of treatment means were compared using the non-parametric Ryan-Einot-Gabriel-Welsch Q (REGWQ) multiple comparison test (SAS Institute 2011). Replicates that had no specimens of the species of interest, such as due to inclement weather, were not included in analyses.

Results

A total of 586 cerambycid beetles were captured during the four field experiments (Table S3), most of which were in the subfamily Lamiinae. Seven species showed statistically significant treatment effects in one of the experiments (Table S4), in most cases consistent with the findings of experiments summarized in Chapters 3 and 4.

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Table A.1. Study sites for four field bioassays conducted in east-central Illinois during 2014-2017

County	Name	GPS coordinates (lat., long.)	Area (ha)
Champaign	Brownfield Woods[a]	40.145, -88.165	26
Champaign	Nettie Hart Memorial Woods[a]	40.229, -88.358	16
Champaign	Trelease Woods[a]	40.132, -88.141	29
Champaign	Private residence	40.135, -88.176	1
Vermillion	Forest Glen Preserve[b]	40.0152, -87.568	728

[a] University of Illinois natural area (http://research.illinois.edu/cna/)

[b] Vermilion County Conservation District

Table A.2. Experimental treatments for four field experiments that tested attraction of adults of lamiine species to various blends of chiral fuscumol, fuscumol acetate, and sulcatol (enantiomers indicated by *R* and *S*), the racemic materials, geranylacetone, and sulcatone.

Compound used as bait	**Expt 1**	**Expt 2**	**Expt 3**	**Expt 4**
Fuscumol		X		
R-fuscumol		X		
S-fuscumol		X		
Fuscumol acetate		X		X
R-fuscumol acetate		X		
S-fuscumol acetate		X		
Geranylacetone				X
R-fuscumol + *R*-fuscumol acetate	X			
R-fuscumol + *S*-fuscumol acetate	X			
S-fuscumol + *S*-fuscumol acetate	X			
S-fuscumol + *R*-fuscumol acetate	X			
Fuscumol acetate + geranylacetone				X
Sulcatone				X
Sulcatone + sulcatol			X	
Sulcatone + *R*-sulcatol			X	
Sulcatone + *S*-sulcatol			X	
Sulcatone + fuscumol acetate				X
Sulcatone + geranylacetone				X
Sulcatone + fuscumol acetate + geranylacetone				X
Solvent control	X	X	X	X

Table A.3. Total numbers of adult cerambycid beetles that were captured during four field experiments conducted in east-central Illinois that assessed attraction of beetles to synthesized pheromones

Taxonomy	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Total
Cerambycinae					
Clytini					
Clytoleptus albofasciatus (Laporte & Gory)	1				1
Neoclytus a. acuminatus (F.)		1	12	2	15
Neoclytus m. mucronatus (F.)	1		1		2
Neoclytus scutellaris (Olivier)		2			2
Xylotrechus colonus (F.)	6	5	14	8	33
Xylotrechus convergens LeConte				2	2
Eburiini					
Eburia quadrigeminata (Say)			1	1	2
Elaphidiini					
Anelaphus parallelus (Newman)				1	1
Anelaphus villosus (F.)			2	4	6
Elaphidion mucronatum (Say)	3	3	25	12	43
Parelaphidion aspersum (Haldeman)				5	5
Parelaphidion incertum (Newman)		1		3	4
Smodicini					
Smodicum cucujiforme (Say)	1		1		2
Lamiinae					
Acanthocinini					
Astyleiopus variegatus (Haldeman)	12	1	5	21	39
Astylidius parvus (LeConte)	2	17	4	8	31
Astylopsis macula (Say)	15	13	19	10	57
Graphisurus despectus (LeConte)	1	4	1	17	23
Graphisurus fasciatus (Degeer)	9	5		9	23
Hyperplatys maculata Haldeman		2			2
Leptostylus transversus (Gyllenhal)			10		10

Table A.3 (cont.)

Lepturges angulatus (LeConte)	1	7		108	16
Lepturges confluens (Haldeman)		1	1	26	28
Lepturges pictus (LeConte)		1			1
Lepturges symmetricus (Haldeman)		1		2	3
Sternidius alpha (Say)	1	7	6		14
Urgleptes querci (Fitch)	3	2	1	6	12
Urgleptes signatus (LeConte)		1		4	5
Acanthoderini					
Aegomorphus modestus (Gyllenhal)		2	1	45	48
Oplosia nubila (LeConte)				2	2
Dorcaschematini					
Dorcaschema cinereum (Olivier)		1			1
Monochamini					
Microgoes oculatus (LeConte)	1				1
Pogonocherini					
Ecyrus d. dasycerus (Say)	1	3		3	7
Lepturinae					
Lepturini					
Strangalia luteicornis (F.)		1			1
Typocerus v. velutinus (Olivier)				1	1
Parandrinae					
Parandrini					
Neandra brunnea (F.)	7	4		19	30
Prioninae					
Prionini					
Orthosoma brunneum (Forster)	3	6		5	14
Total	**68**	**91**	**104**	**323**	**586**

Table A.4. Mean (± 1 SE) number of cerambycid beetles captured per treatment and replicate during Experiments 1 to 4, and results of Friedman's *Q* analyses, for the cases in which overall treatment effects were statistically significant. Abbreviations for chemicals as in Table S-2. Bold text indicates treatments with means significantly greater than controls. Asterisks indicate significance level of Friedman's *Q*: * $P < 0.01$; ** $P < 0.001$; *** $P < 0.0001$ for overall treatment effects. Treatment means within species with the same letters are not significantly different (REGWQ means-separation test at $P < 0.05$).

Taxonomy and Expt.	Treatments	Mean ± SE	Friedman's Q (df)
ACANTHOCININI			
Astyleiopus variegatus – Expt. 1			
	R-fuscumol + *R*-fuscumol acetate	0b	38.1 (4,30)**
	R-fuscumol + *S*-fuscumol acetate	0b	
	S-fuscumol + *R*-fuscumol acetate	0.17 ± 0.1b	
	***S*-fuscumol + *S*-fuscumol acetate**	**1.8 ± 0.75a**	
	Control	0b	
Astylopsis macula – Expt. 3			
	Sulcatone + sulcatol	**0.9 ± 0.2a**	13.4 (3,40)*
	Sulcatone + *R*-sulcatol	0b	
	Sulcatone + *S*-sulcatol	**0.9 ± 0.4a**	
	Control	0.1 ± 0.1b	
Leptostylus transversus – Expt. 3			
	Sulcatone + sulcatol	**0.7 ± 0.3a**	13.1 (3,32)*
	Sulcatone + *R*-sulcatol	0b	
	Sulcatone + *S*-sulcatol	**0.7 ± 0.2a**	
	Control	0b	
Lepturges angulatus – Expt. 4			
	Fuscumol acetate	**2.3 ± 0.5a**	47.7 (7,96)***
	Geranylacetone	0.08 ± 0.08b	
	Sulcatone	0b	
	Fuscumol acetate + geranylacetone	**2.1 ± 0.4a**	
	Fuscumol acetate + sulcatone	1.3 ±. 5ab	

Table A.4 (cont.)

	Geranylacetone + sulcatone	0.08 ± 0.08b	
	Fuscumol acetate + geranylacetone + sulcatone	0.92 ± 0.2ab	
	Control	0.17 ± 0.17b	
Lepturges confluens – Expt. 4			
	Fuscumol acetate	0b	19.3 (7,104)*
	Geranylacetone	0.62 ± 0.3ab	
	Sulcatone	0b	
	Fuscumol acetate + geranylacetone	**0.9 ± 0.4a**	
	Fuscumol acetate + sulcatone	0b	
	Geranylacetone + sulcatone	0.23 ± 0.1b	
	Fuscumol acetate + geranylacetone + sulcatone	0.31 ± 0.2ab	
	Control	0b	
Sternidius alpha – Expt. 2			
	Fuscumol	**1.0 ± 0.3a**	20.6 (6,35)*
	R-Fuscumol	0.4 ± 0.2b	
	S-Fuscumol	0b	
	Fuscumol acetate	0b	
	R-fuscumol acetate	0b	
	S-fuscumol acetate	0b	
	Control	0b	
ACANTHODERINI			
Aegomorphus modestus – Expt. 4			
	Fuscumol acetate	0.5 ± 0.1bc	38.1 (7,96)***
	Geranylacetone	0c	
	Sulcatone	0c	
	Fuscumol acetate + geranylacetone	0.83 ± 0.2ab	

Table A.4 (cont.)

	Fuscumol acetate + sulcatone	**1.1 ± 0.3a**
	Geranylacetone + sulcatone	0.08 ± 0.08c
	Fuscumol acetate + geranylacetone + sulcatone	0.58 ± 0.2bc
	Control	0c

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