CHEMICAL COMMUNICATION IN CERAMBYCID BEETLES AND THE MOLECULAR BASIS OF OLFACTION

BY

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DISSERTATION

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ABSTRACT

Longhorned beetles of the insect family Cerambycidae are a highly diverse group of insects whose larvae bore into the stems of forbs and the wood and roots of trees, and the family contains numerous pests of agriculture, forestry, and structural timber. The reproductive biology of adult cerambycids is mediated by long-range volatile aggregation or sex pheromones, but the pheromone chemistry is highly conserved, and many species of beetles produce and respond to identical compounds. This parsimony may yield powerful and efficient means to control species that are pests, but the full extent of these pheromone "motifs" remains unknown, as are the mechanisms by which cerambycid species remain reproductively isolated in the face of identical pheromones.

In this dissertation, I present research that catalogs and defines new motifs of cerambycid pheromones. I identified (*E*)-6,10-dimethyl-5,9-undecadien-2-ol ("fuscumol") and its acetate as a conserved pheromone motif of the subfamily Lamiinae, and also as an attractant for some species in the subfamily Cerambycinae. I also described alkan-2-ones as a novel and potentially widespread motif of minor pheromone constituents in the subfamily Cerambycinae that may act to chemically separate cerambycid species.

I also present a comprehensive survey of volatile chemicals produced by *Megacyllene caryae* Gahan, the painted hickory borer, a common cerambycid of eastern North American that mimics vespid wasps. Male *M. caryae* produce an unusually complex aggregation pheromone of at least nine components, and I describe several behaviorally active components from this blend. I also report that agitated beetles of both sexes produce spiroacetals, a class of chemicals used as alarm pheromones by many species of vespid wasps, suggesting that beetles produce these

chemicals as a form of chemical mimicry to complement their physical resemblance to their models.

Finally, I present research on the odorant receptors (Ors) of cerambycids, which are members of a family of receptors responsible for detecting volatile chemicals at the molecular level. I sequenced 57 Ors from RNA extracted from antennae of male and female *M. caryae*, and tested several receptors against pheromone components in a heterologous expression system. I identified Ors tuned to the three pheromone components (*S*)-2-methyl-1-butanol, (2*S*,3*R*)-2,3-hexanediol, and 2-phenylethanol. McOr3 and McOr20 are also sensitive to structurally-related chemicals that are pheromones of other cerambycid beetles, suggesting that orthologous receptors may be present across many cerambycid species. These Ors are the first to be functionally characterized from any species of beetle and lay the groundwork for understanding the evolution of pheromones within the Cerambycidae.

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CHAPTER 1: INTRODUCTION

The longhorned beetles of the insect family Cerambycidae comprise one of the most diverse groups of insects, with a worldwide distribution of over 30,000 recognized species (Slipinski et al. 2011). Adults are named for their strikingly long antennae, which can be many times the length of the body (e.g., Lingafelter 2007). Cerambycid larvae are herbivorous, feeding on roots and within stems and twigs of forbs and trees (Solomon 1995). However, most cerambycid species feed within the woody tissues of trees, which can result in considerable economic damage when larvae feed in and kill living trees (Solomon 1995, Nowak et al. 2001, Haack et al. 2010) or in structural timber (Reddy 2007).

Exotic species in particular can cause extreme damage as they invade new habitats. For example, *Anoplophora glabripennis* Motschulsky, the Asian longhorned beetle, feeds in living trees and has been documented from over 10 genera including common urban plantings such as *Acer*, *Ulmus*, and *Fraxinus* (Haack et al. 2006). This species has been introduced into the United States multiple times through wooden packing material and is currently established in several cities (Haack et al. 2010). If unmanaged, the beetles are estimated to inflict damages of over 650 billion dollars in land value, including the loss of 1.2 billion trees and over 30% of urban canopy cover (Nowak et al. 2001). *Tetropium fuscum* (F.), the brown spruce beetle, is a European species that is now established in Nova Scotia, Canada, where it is infesting and killing multiple species of North American spruce, even though it does not infest living spruce in its native range (Silk et al. 2007). The citrus longhorned beetle, *A. chinensis* (Forster), has an even wider host range than its congener *A. glabripennis* and is frequently intercepted in shipments of live plants from east Asia to the United States (Haack et al. 2010). In 2001, five individual

beetles escaped from a bonsai shipment in Washington State, spurring a five-year eradication program costing over 2 million dollars (Haack et al. 2010).

The concealed larval habitat and often cryptic colors of adults makes detection of these insects difficult, and chemical attractants are a key focus of cerambycid research. Cuticular hydrocarbons that act as contact pheromones have been described from several cerambycid species (Ginzel et al. 2003, Ginzel and Hanks 2003, Ginzel et al. 2006), but volatile pheromones were poorly understood until the discovery of 2,3-hexanediol as the male-produced aggregation pheromone of the red-headed ash borer *Neoclytus acuminatus acuminatus* (Lacey et al. 2004). Subsequent field bioassays that tested 2,3-hexandiol and the related 3-hydroxyhexan-2-one quickly revealed that these compounds are attractants and pheromones for dozens of species in the subfamily Cerambycinae (Lacey et al. 2007, 2008, 2009, Ray et al. 2009). Cerambycids of other subfamilies and genera followed a similar theme of conserved pheromone "motifs," with (*E*)-6,10-dimethyl-5,9-undecadien-2-ol ("fuscumol") described as male-produced aggregation pheromones of the genus *Tetropium* (Aseminae; Silk et al. 2007, Sweeney et al. 2010), 2-(undecyloxy)-ethanol as the male-produced aggregation pheromone of the genus *Monochamus* (Lamiinae; Pajares et al. 2010, Teale et al. 2011) and 3,5-dimethyldodecanoic acid as a female-produced sex pheromone and attractant for males of many species in the genus *Prionus* (Prioninae; Barbour et al. 2011, Rodstein et al. 2009). The discovery of such widespread, highly conserved pheromones has yielded efficient and powerful means for monitoring cerambycids (Wong et al. 2012).

The goal of my dissertation research was to identify and catalog pheromones of cerambycid beetles, as well as to study the ecology and evolution of cerambycid pheromone systems. Despite considerable recent progress in the study of cerambycid chemical ecology,

large gaps remain in our knowledge of cerambycid pheromones, and few publications have explained how species might remain reproductively isolated in the face of seemingly identical pheromones (but see Striman 2010). The evolution of cerambycid pheromone systems is similarly unexplored, though recent research suggests the pheromones 2,3-hexanediol and 3-hydroxyhexan-2-one share a common evolutionary origin in the Cerambycinae (Ray 2009).

In Chapter 2, I report the discovery of fuscumol and its acetate as a new, widespread pheromone motif for many cerambycid beetles in the subfamily Lamiinae. Field bioassays testing these chemicals at field sites in Illinois, Indiana, and Texas captured beetles of a dozen species in four different tribes. Lures that blended fuscumol and fuscumol acetate were also highly attractive, suggesting that traps baited with both chemicals will be effective tools to monitor beetles of this subfamily.

In Chapter 3, I report the discovery that males of three species of the subfamily Cerambycinae produce the common cerambycid motif (*R*)-3-hydroxyhexan-2-one ("3*R*-ketone") as an aggregation pheromone, but also produce a secondary component of nonan-2-one or decan-2-one. Field bioassays targeting the species *Cyrtophorus verrucosus* (Olivier) demonstrated that nonan-2-one synergizes attraction to 3*R*-ketone, but only when used in concentrations that approximate the blend produced by males. Additionally, nonan-2-one inhibited the species *Euderces pini* (Olivier), which overlaps in daily activity period with *C. verrucosus* and also produces 3*R*-ketone as its pheromone. This is the first description of alkan-2-ones in the Cerambycidae and suggests these compounds may be produced as a mechanism to reinforce reproductive isolation and segregate the dozens of species that produce 3*R*-ketone as a pheromone.

In Chapters 4-6, I focus on the chemical ecology of the painted hickory borer, *Megacyllene caryae* (Gahan). Unusually, males of this species produce a highly complex pheromone blend that was originally described as eight different pheromone components (Lacey et al. 2008). In Chapter 4, I describe how the many components of this pheromone contribute to the attractive power of the blend. I identified (*S*)-2-methylbutan-1-ol ("2*S*-methylbutanol") as a ninth component of the aggregation pheromone produced by male beetles, and I demonstrated through field bioassays that a combination of the major components geranial and neral (together, citral) are attractive to adult beetles. Additional bioassays demonstrated that the complete blend of all pheromone components is more attractive than citral alone, but a combination of citral and 2*S*-methylbutanol is as attractive as the complete blend.

In Chapter 5, I report 22 new volatile compounds produced by *M. caryae*, including several bicyclic spiracetals that are released by both sexes when they are disturbed. I also identified similar spiroacetals produced by the congener *M. robiniae* (Forster). Both species are visual mimics of vespid wasps, and many vespids produce spiroacetals as alarm pheromones when disturbed (Bruschini et al. 2006). I identified spiroacetals from three vespid wasps that co-occur with *M. caryae* and *M. robiniae*, suggesting that the spiroacetals are released by the beetles as a chemical complement to visual mimicry.

In Chapter 6, I present research on odorant receptors ("Ors"), a large family of receptors used by insects to detect volatile chemicals (Rützler and Zwiebel 2005). I sequenced 57 odorant receptors from RNA extracted from the antennae of adult *M. caryae*, and characterized the function of receptors that are sensitive to 2*S*-methylbutanol (McOr3), 2-phenylethanol (McOr5), and (2*S*,3*R*)-2,3-hexanediol (McOr20). Additionally, McOr3 and McOr20 are sensitive to other enantiomers of these compounds, and McOr20 is also sensitive to 3*R*-ketone. The functional

flexibility of these receptors suggests that orthologs or similar receptors are used by other cerambycids of the subfamily Cerambycinae, and that the evolution of these pheromones might be reconstructed by studying the evolution of these lineages of pheromone receptors.

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CHAPTER 2: FUSCUMOL AND FUSCUMOL ACETATE ARE GENERAL ATTRACTANTS FOR MANY SPECIES OF CERAMBYCID BEETLES IN THE SUBFAMILY LAMIINAE[1]

Abstract

(*E*)-6,10-Dimethyl-5,9-undecadien-2-ol ('fuscumol') is an important component of male-produced aggregation pheromones for several species of cerambycid beetles in the genus *Tetropium* (subfamily Aseminae/Spondylidinae). Here, I describe experiments that tested the hypothesis that fuscumol and/or fuscumol acetate also are general attractants for species in the cerambycid subfamily Lamiinae. At field sites in northwestern Indiana and central Texas (USA), panel traps baited with fuscumol or its acetate captured 331 lamiine beetles, compared to 11 beetles captured in control traps. Three species were attracted to traps baited with fuscumol as a single component, whereas another four species were attracted to fuscumol acetate alone. Surprisingly, fuscumol acetate also attracted two species in the subfamily Cerambycinae: *Xylotrechus colonus* (Fabricius) (males of which produce a pheromone composed only of stereoisomers of 2,3-hexanediol and 3-hydroxyhexan-2-one), and *Obrium maculatum* (Olivier) (for which a pheromone has yet to be identified). In an independent field experiment in east-central Illinois (USA), traps baited with fuscumol and/or its acetate captured 136 beetles of eight lamiine species, all but one species of which were also captured in the other experiment. Blending fuscumol and its acetate did not inhibit responses of species to either of the individual compounds, but synergized their activity for one species. My results support the hypothesis that

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fuscumol and fuscumol acetate are widespread pheromone components or attractants for a variety of cerambycid species, especially lamiines in the tribe Acanthocinini.

Introduction

Volatile aggregation pheromones produced by males, and sex pheromones produced by females, recently have been described for several species of cerambycid beetles (e.g., Millar et al. 2009, Ray et al. 2009, 2011, Rodstein et al. 2011). Male beetles of the subfamily Cerambycinae emit pheromones that attract both sexes when on larval host plants (Lacey et al. 2004), suggesting that these pheromones may signal the presence of breeding material that is scarce and ephemeral (Hanks 1999). Similarly, beetles of the genus *Tetropium* (subfamily Aseminae/Spondylidinae; Monné & Hovore 2005, Bousquet et al. 2009) are only attracted to aggregation pheromones when odors of host plants are present (Silk et al. 2007).

There appears to be considerable parsimony within the family Cerambycidae in relation to pheromone biosynthesis and use: closely-related species of cerambycids often share pheromone components or even produce pheromones of identical composition. For example, (*R*)-3-hydroxyhexan-2-one is a common, and often the sole component of volatile pheromones of many species in the large subfamily Cerambycinae (Hanks et al. 2007, Millar et al. 2009, Ray et al. 2009). This similarity in pheromone composition across cerambycine species can result in simultaneous attraction of multiple species to traps baited with a single synthetic pheromone component. Thus, in field bioassays, racemic 3-hydroxyhexan-2-one and the structurally related 2,3-hexanediols serve as general attractants for many cerambycine species, and may even attract species that do not actually produce them (Lacey et al. 2004, Hanks et al. 2007). In a different subfamily, the Prioninae, the female-produced pheromone of *Prionus californicus* Motschulsky

(3,5-dimethyldodecanoic acid; Rodstein et al. 2011) has been shown to attract males of seven congeners (Barbour et al. 2011). Similarly, in the subfamily Lamiinae, 2-(undecyloxy)-ethanol serves as the male-produced aggregation pheromone for *Monochamus galloprovincialis* (Olivier) (Pajares et al., 2010), but also attracts both sexes of other congeners (Teale et al. 2011, L. M. Hanks, unpub. data).

(*E*)-6,10-Dimethyl-5,9-undecadien-2-ol ('fuscumol') has been identified as a male-produced aggregation pheromone for the invasive European cerambycid *Tetropium fuscum* (Fabricius) and its North American congener *Tetropium cinnamopterum* (Kirby) (Silk et al. 2007). It also attracts and is likely a pheromone component for another European species, *Tetropium castaneum* (L.) (Sweeney et al. 2010). In addition, this same compound has been identified as a pheromone component for two South American species in the subfamily Lamiinae: *Hedypathes betulinus* (Klug) (Fonseca et al. 2010) and *Steirastoma breve* (Sulzer) (Liendo et al. 2005, C Liendo, pers. comm.). Additionally, males of both species produce (*E*)-6,10-dimethyl-5,9-undecadien-2-one (common name: geranylacetone; Fonseca et al. 2010, C Liendo, pers. comm.) and *H. betulinus* produces (*E*)-6,10-dimethyl-5,9-undecadien-2-yl acetate ('fuscumol acetate'; Fonseca et al., 2010). These findings suggest that fuscumol and its analogs may be common pheromone components for a diversity of species across subfamilies of the Cerambycidae. In fact, in recent years I have consistently captured several lamiine species in traps baited with the blend of fuscumol and its acetate during field trials in east-central Illinois that targeted asemine species (unpub. data). Here, I describe experiments, conducted in Indiana, Illinois, and Texas, that specifically address the hypothesis that fuscumol and fuscumol acetate represent another widespread motif for cerambycid beetle pheromones, analogous to the 3-hydroxyalkan-2-one and 2,3-alkanediol motifs.

Materials and Methods

Synthesis of compounds

Geranylacetone (97 g, 0.5 mol; Aldrich Chemical, Flavor and Fragrance Division, Milwaukee WI, USA) was purchased as a 1:1.25 mixture of (*Z*)- and (*E*)-isomers due to the prohibitive cost of the pure (*E*)-isomer. Lithium aluminum hydride (6.3 g, 166 mmol) was added in portions over 20 min to 1 l of dry ether at 0 ºC under argon atmosphere. Geranylacetone in 100 ml dry ether was added dropwise to the resulting slurry over 40 min, and the mixture was warmed to room temperature overnight, during which time it became viscous. After checking that the reduction was complete using thin layer chromatography on silica gel [the reactant and product had virtually identical retention times by gas chromatography (GC) on a DB-5 column], the magnetic stir-bar was replaced with a mechanical stirrer, and the mixture was cooled in an ice-bath while adding, sequentially and dropwise, 6.64 ml water (caution: vigorous hydrogen evolution!), 5 ml aqueous 20% NaOH, and 23.5 ml water. The mixture was stirred for an additional 20 min, during which time the metal salts formed a granular white precipitate. The mixture was filtered with suction, rinsing the filter cake with ether. The filtrate was extracted with water and brine, then dried over anhydrous Na_2SO_4, and concentrated on a rotary evaporator at room temperature. The residue was purified by Kugelrohr distillation (oven temperature ca. 80 ºC, 0.2 mm Hg), yielding racemic fuscumol [hence, (*E*/*Z*)-fuscumol] as a clear, colorless oil [94.6 g, 97% yield, >98% chemically pure by GC, 1:1.25 mixture of racemic (*Z*)- and (*E*)-isomers]. The nuclear magnetic resonance (NMR) and mass spectra were analogous to those recently reported (Fonseca et al., 2010; Sweeney et al., 2010).

(*E*/*Z*)-Fuscumol acetate was synthesized by adding (*E*/*Z*)-fuscumol (44 g, 224 mmol) to a

solution of pyridine (20.2 ml, 250 mmol) and methylene chloride (400 ml), and the mixture was stirred and cooled in an ice-bath while acetyl chloride (17.8 ml, 250 mmol) was added dropwise. The mixture was then warmed to room temperature and stirred overnight. The small excess of acetyl chloride was destroyed by addition of ethanol (1.4 g, 30 mmol), followed by stirring for an additional 4 h. The mixture was then poured into water and the layers were separated. The aqueous layer was extracted with ether and the combined organic extracts were washed successively with water, 1M HCl, saturated aqueous $NaHCO_3$, and brine. After drying over anhydrous Na_2SO_4, the solution was concentrated by rotary evaporation followed by Kugelrohr distillation of the residue (oven temperature ca. 80 ºC, 0.15 mm Hg), yielding (*E*/*Z*)-fuscumol acetate (52.4 g, 98%, >98% pure by GC). Mass and NMR spectra were analogous to those reported in Fonseca et al. (2010).

Field bioassays

Two experiments were conducted to test for attraction of lamiine species to (*E*/*Z*)-fuscumol and its acetate. Experiment 1 tested for attraction of beetles to each compound separately, and was replicated at study sites in northwestern Indiana and central Texas. Experiment 2 was similar in design but included a 1:1 blend of (*E*/*Z*)-fuscumol and its acetate to test whether the combination inhibited or enhanced attraction, and was conducted in east-central Illinois. I used black cross-vane flight intercept traps (Panel Trap model; AlphaScents, Portland, OR, USA) that were coated with Fluon® PTFE (AGC Chemicals Americas, Exton, PA, USA) to enhance trapping efficiency (Graham et al. 2010). Traps were suspended from frames constructed of PVC pipe (see Graham et al. 2010). Pheromone lures were polyethylene sachets (Bagettes™ model 14770, 5.1 × 7.6 cm; Cousin, Largo, FL, USA) loaded with 25 mg of

synthetic pheromone diluted in 1 ml of 95% ethanol, or 1 ml of neat ethanol (control). Ethanol is an efficient carrier for these compounds, and does not attract cerambycid beetles in these quantities (e.g., Hanks et al. 2007). I collected beetles from traps at intervals of 1-3 days, at which time treatments were rotated within transects. Lures were examined when traps were checked and replaced when visibly depleted, usually after ca. 1 week. Captured beetles were sexed, usually by the relative length of the antennae to the body, and by the length of the fifth abdominal sternite (see volumes indexed in Linsley & Chemsak 1997). Representative specimens of all species were pinned and retained for further study and voucher specimens were deposited when necessary with the collection of the Illinois Natural History Survey, Champaign, IL, USA.

Experiment 1 consisted of a single block of three traps (10 m apart) at each field site that were baited with (*E*/*Z*)-fuscumol, (*E*/*Z*)-fuscumol acetate, or with a control lure of 95% ethanol. There were two study sites in Indiana (Tippecanoe County), both in Martell Forest, a mixed hardwood forest of 150 ha (site 1: 40º26’31.38”N, 87º2’1.37”W; site 2: 40º26’10.69”N, 87º2’18.20”W). The experiment was conducted from 25 May to 17 September 2010 (air temperature 8.3-35 ^{o}C, 44 cm total precipitation; Weather Underground, Ann Arbor, MI, USA). In Texas (Erath County), there were three study sites: 1) a residential area in Stephenville (32°13'26.40"N, 98°13'15.10"W) that was dominated by ornamental pecan trees [*Carya illinoinensis* (Wangenh.) K. Koch], 2) a lumber yard surrounded by pasture (32°9'35.18"N, 98°11'21.09"W), and 3) a wetlands with stands of *Quercus stellata* Wangenh., *Celtis laevigata* Willd., and *Prosopis glandulosa* Torr., that was the property of Texas AgriLife Research and Extension Center (32°14'27.7965"N, 98°11'18.7731"W). The experiment was conducted from 24 May to 8 June 2010 (air temperature 21-34 °C, skies usually clear, 2 cm total precipitation;

Weather Underground).

Experiment 2 included the same treatments as Experiment 1, but lures were loaded with 100 mg of synthetic pheromone and included a treatment that was a blend of (*E*/*Z*)-fuscumol and its acetate (100 mg of each pheromone component in 95% ethanol). There were two study sites in Illinois: 1) Allerton Park (Piatt County: 39°59'11.01"N, 88°39'3.75"W), a mixed-hardwood forest of 600 ha, and 2) a residential neighborhood in Urbana (Champaign County; 40°5'49.30"N, 88°12'11.33"W). There were two blocks of treatments at the Allerton site and one block at the residential site. The experiment was conducted from 26 May to 16 August 2010 (air temperature 13-35 °C, 35.2 cm total precipitation; Weather Underground).

Statistical analysis

I tested differences between treatments, separately for each state and blocked by site and date, using the nonparametric Friedman's test (Proc FREQ, option CMH; SAS Institute, 2001) because data violated the equal variances assumption of ANOVA (Sokal & Rohlf, 1995). Differences between pairs of means were tested with the REGWQ means-separation test to control maximum experiment-wise error rates (Proc GLM; SAS Institute, 2001). Data for site and date replicates were included in the analysis based on a threshold number of specimens (1-4, depending on abundance of the species) so as to optimize sample size per replicate while maintaining sufficient replication for a robust analysis. I also tested for deviations from a 1:1 sex ratio using χ^2 tests.

Results

In Experiment 1, traps in Indiana and Texas caught a total of 336 beetles of 12 lamiine

species in four tribes (Table 2.1), with 87% of the trapped beetles being in the tribe Acanthocinini. For each of the seven species that were best represented (Table 2.1), traps baited with (*E*/*Z*)-fuscumol or (*E*/*Z*)-fuscumol acetate captured significantly more beetles than did control traps. I include trap catch data for the remaining five species, even though treatment means were not significantly different, because they suggest leads for follow-up investigations.

Of the best represented species captured in Indiana (Fig. 2.1A), three were attracted to (*E*/*Z*)-fuscumol (*Astylidius parvus*, *Leptostylus transversus*, *Sternidius alpha*), and two species were attracted to (*E*/*Z*)-fuscumol acetate (*Graphisurus fasciatus*, *Aegomorphus modestus*). *Sternidius alpha* also was captured in Texas, but there it was attracted equally to (*E*/*Z*)-fuscumol and the acetate (Fig. 2.1B). Two other species that were captured in significant numbers in Texas responded only to (*E*/*Z*)-fuscumol acetate (Fig. 2.1B; *Astyleiopus variegatus*, *Lepturges angulatus*). For most of the species, males and females were attracted in similar numbers to (*E*/*Z*)-fuscumol or its acetate (Table 2.1), but the sex ratio was significantly female biased for *A. variegatus* and *L. angulatus*.

Unexpectedly, two species of the subfamily Cerambycinae also were attracted in large numbers to traps baited with these compounds. In Indiana, I caught 70 *Xylotrechus colonus* (Fabricius) (sex ratio = 43% female, not significantly different from 1:1; $\chi^2 = 1.29$, d.f. = 1, $P = 0.26$), with significantly greater numbers in the (*E*/*Z*)-fuscumol acetate treatment (Fig. 2.2; Friedman's $Q_{2,18} = 10.0$, $P = 0.0067$). In Texas, I captured 31 *Obrium maculatum* (Olivier) (sex ratio = 43% female, not significantly different from 1:1; $\chi^2 = 0.14$, d.f. = 1, $P = 0.71$), with significantly greater numbers in the (*E*/*Z*)-fuscumol acetate treatment, and intermediate numbers in the (*E*/*Z*)-fuscumol treatment (Fig. 2.2; Friedman's $Q_{2,21} = 10.1$, $P = 0.006$).

During Experiment 2, traps in Illinois captured 145 beetles of 9 species (Table 2.2), all

but one of which [*Oplosia nubila* (LeConte)] also were captured during Experiment 1. The data were consistent with Experiment 1 in indicating a preference for (*E*/*Z*)-fuscumol acetate by *A. variegatus*, *G. fasciatus*, and *L. angulatus*, and a preference for (*E*/*Z*)-fuscumol by *A. parvus* (Fig. 2.3; compare with Fig. 2.1). The addition of the blend of (*E*/*Z*)-fuscumol and its acetate to the bioassay revealed that *A. variegatus* was more attracted to the blend than to (*E*/*Z*)-fuscumol acetate alone (Fig 2.3). However, blending the two compounds did not influence the responses of *G. fasciatus* and *L. angulatus* compared to (*E*/*Z*)-fuscumol acetate, nor the response of *A. parvus* compared to (*E*/*Z*)-fuscumol (Fig. 2.3; compare with Fig. 2.1). My findings suggest that these species are attracted despite the presence of these other compounds, and in some cases may be more attracted. Sexes of the four species responded equally to all treatments (Table 2.2).

Finally, the fact that I caught 14 of the cerambycine *X. colonus* in Experiment 2 provided further evidence that it is attracted to (*E*/*Z*)-fuscumol and its acetate: eight were caught in traps baited with (*E*/*Z*)-fuscumol, three with the acetate, and two with the blend, compared to a single beetle in control traps ($\chi^2 = 12.1$, d.f. = 1, $P < 0.001$).

Discussion

My hypothesis was supported by the significant attraction of beetles of seven species in four tribes of the Lamiinae to ethanol solutions of (*E*/*Z*)-fuscumol, (*E*/*Z*)-fuscumol acetate, or a mixture of the two compounds. Few beetles were captured by control traps baited with ethanol, but ethanol may still have enhanced the attractiveness of the other lures (e.g., Sweeney et al. 2010). In most cases, both sexes responded in approximately equal numbers, suggesting that these compounds serve as aggregation attractants, if not pheromones. Whether there was indeed a sex bias in the responses of two species at some field sites cannot be determined because I had

no independent measure of the sex ratio of the local populations at the times that bioassays were conducted.

Although fuscumol is an aggregation pheromone for some species in the subfamily Aseminae/Spondylidinae, I did not trap any beetles of that subfamily in Texas, Indiana, or Illinois, even though one species of *Tetropium* and other asemine/spondylidine genera have been recorded from these regions (volumes indexed in Linsley & Chemsak 1997). However, the activity of fuscumol for species in this subfamily may be strongly synergized by volatile terpenoids produced by host plants (which were not included in my trap lures), as shown for *Tetropium* species (Silk et al. 2007, Sweeney et al. 2010). Additionally, the presence of (*Z*)-fuscumol in the inexpensive blend of fuscumol stereoisomers used in the trials may have affected the response to the (*E*)-isomer.

Both sexes of *A. modestus* responded to (*E*/*Z*)-fuscumol acetate. This species is in the same tribe as *H. betulinus* and *S. breve* (Acanthoderini; Yanega, 1996), males of which produce fuscumol and, in the case of *H. betulinus*, fuscumol acetate (Fonseca et al. 2010, C Liendo, pers. comm.). This suggests that fuscumol or its acetate may be pheromone components for other species in that tribe as well. However, it must be noted that I have yet to confirm that fuscumol or related compounds are produced by any of the species captured during the study. Nevertheless, the generalized activity of (*E*/*Z*)-fuscumol and (*E*/*Z*)-fuscumol acetate across many lamiine species in several tribes, and in three regions of the USA, suggests that the basic structure of fuscumol represents another shared motif of pheromones in the Cerambycidae. It seems likely that fuscumol and its acetate serve as pheromones that signal the presence of larval hosts as well as serving a sexual function, as appears to be the case for cerambycine species (Lacey et al. 2004).

Attraction of the cerambycines *X. colonus* and *O. maculatum* to (*E*/*Z*)-fuscumol and its acetate provides further evidence that the biological activity of these compounds extends across subfamily lines. The same is true of stereoisomers of 2,3-hexanediol, which are common pheromone components of male cerambycines (Lacey et al. 2004, 2009) but which also serve as female-produced sex pheromones for several *Tragosoma* species in the subfamily Prioninae (A. M. Ray, unpub. data). At present, the pheromone of *O. maculatum* remains uncharacterized, but may include fuscumol or related compounds. However, the pheromone of male *X. colonus* appears to be composed of (*R*)- and (*S*)-3-hydroxyhexan-2-one, and (2*S*,3*S*)- and (2*R*,3*R*)-2,3-hexanediol, with no trace of fuscumol or related compounds (Lacey et al. 2009). This suggests that fuscumol and its acetate mediate a variety of interactions within and among multiple sympatric species. For example, fuscumol acetate may act as a kairomone that is exploited by *X. colonus* to find suitable host plants for oviposition, which would be consistent with its highly polyphagous nature (volumes indexed in Linsley & Chemsak 1997).

Pheromones of cerambycid beetles currently are being developed as management tools for endemic species that are important pests (Maki et al. 2011), as well as for quarantine applications (e.g., Nehme et al. 2010, Barbour et al. 2011, Teale et al. 2011). Furthermore, synthetic pheromones of cerambycids will be deployed by USDA-APHIS for monitoring of exotic species nationwide in 2012 (V. Mastro, pers. comm.). Fuscumol and its acetate will be included in these surveillance efforts because they will attract species that do not respond to the synthetic pheromones that target cerambycines (e.g., 3-hydroxyalkan-2-ones and corresponding diols) or species of lamiines other than those in the tribe Monochamini that use 2-(undecyloxy)-ethanol as a pheromone (Millar et al. 2009, Pajares et al. 2010, Teale et al., 2011). These results confirm that lures containing the inexpensive blend of fuscumol stereoisomers would be

effective for monitoring a number of lamiine species, as well as some cerambycine species. Preliminary experiments have indicated that the presence of the (*Z*)-isomer does not influence the attraction of lamiine species to the (*E*)-isomer (L. M. Hanks, unpub. data).

In summary, this study has provided the key initial data to drive more comprehensive follow-up studies by demonstrating that fuscumol, fuscumol acetate, and related isomers attract multiple cerambycid species from different tribes and subfamilies. Traps baited with these compounds can be used to capture live beetles of both sexes, providing a means to determine whether these compounds are part of the pheromone, or whether the beetles are instead exploiting the signals of other species. This research will lay the groundwork for identifying the multiple functions of these compounds in structuring cerambycid communities.

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Table 2.1. Total number of cerambycid beetles in the subfamily Lamiinae that were captured in northwestern Indiana and central Texas during Experiment 1. Fuscumol (Fusc.) and fuscumol acetate (Fusc. acetate) consist of racemic blends of their (*E*)- and (*Z*)-isomers. Treatment means for the best represented species are presented in Fig. 2.1. Sex ratio calculated across treatments, exclusive of control.

Tribe	Species	Site	Fusc.	Fusc. acetate	Control	Total (%♀)	Friedman's Q (*P*)
Acanthocinini	*Astyleiopus variegatus* (Haldeman)	TX	1	22	0	23 (73[a])	15.3 (<0.001)
	Astylidius parvus (LeConte)	IN	59	0	0	59 (40)	50.3 (<0.001)
	Graphisurus fasciatus (DeGeer)	IN	10	33	1	44 (58)	28.6 (<0.001)
	Graphisurus despectus (LeConte)	IN	2	10	2	14 (58)	ns
	Leptostylus transversus (Gyllenhal in Schoenherr)	IN	8	1	2	11 (22)	10.9 (0.004)
	Lepturges angulatus (LeConte)	IN	0	4	1	5 (50)	ns
		TX	1	79	0	80 (91[a])	37.3 (<0.001)
	Lepturges confluens (Haldeman)	IN	1	5	0	6[b]	ns
	Sternidius alpha (Say)	IN	23	2	0	25 (40)	35.4 (<0.001)
		TX	8	8	0	16[b]	6.8 (0.033)
	Urgleptes facetus (Say)	IN	0	8	0	8[b]	ns
Acanthoderini	*Aegomorphus modestus* (Gyllenhal in Schoenherr)	IN	0	29	2	31 (37)	22.6 (<0.001)
Pogonocherini	*Ecyrus arcuatus* Gahan	TX	6	1	0	7[b]	ns
Pteropliini	*Ataxia crypta* (Say)	TX	3	4	0	7[b]	ns
	Total		122	206	8	336	

[a]Significantly different from 1:1 (χ^2 test, $P < 0.05$).
[b]Sex of beetles indeterminate or not recorded.
ns, not significant ($P > 0.05$).

Table 2.2. Total number of cerambycid beetles in the subfamily Lamiinae that were captured in east-central Illinois during Experiment 2. Fuscumol and fuscumol acetate consist of racemic blends of their (*E*)- and (*Z*)-isomers, and the 'Blend' treatment consists of equal parts of (*E*/*Z*)-fuscumol and (*E*/*Z*)-fuscumol acetate. Treatment means for the best represented species are presented in Fig. 2.3. Sex ratio calculated across treatments, exclusive of control

Tribe	Species	Fuscumol	Fuscumol acetate	Blend	Control	Total (%♀)	Friedman's Q (*P*)
Acanthocinini	*Astyleiopus variegatus*	1	5	12	1	19 (64)	14.0 (0.003)
	Astylidius parvus	5	1	9	0	15 (43)	20.8 (<0.001)
	Graphisurus fasciatus	4	26	15	5	50 (50)	22.3 (<0.001)
	Leptostylus transversus	1	1	0	2	4[a]	ns
	Lepturges angulatus	3	19	16	0	38 (48)	21.2 (<0.001)
	Lepturges confluens	2	2	1	0	5[a]	ns
	Sternidius alpha	2	0	6	1	9[a]	ns
Acanthoderini	*Aegomorphus modestus*	0	5	0	0	5 (80)	ns
Total		18	59	59	9	145	

[a]Sex of beetles indeterminate or not recorded.
ns, not significant ($P > 0.05$).

Fig. 2.1. Mean (± SE) number of beetles captured (per trap and count period) during Experiment 1 in: A) northwestern Indiana, and B) central Texas. Fuscumol and fuscumol acetate consist of racemic blends of their (*E*)- and (*Z*)-isomers. Means with different letters within species are significantly different (REGWQ means-separation test: $P < 0.05$).

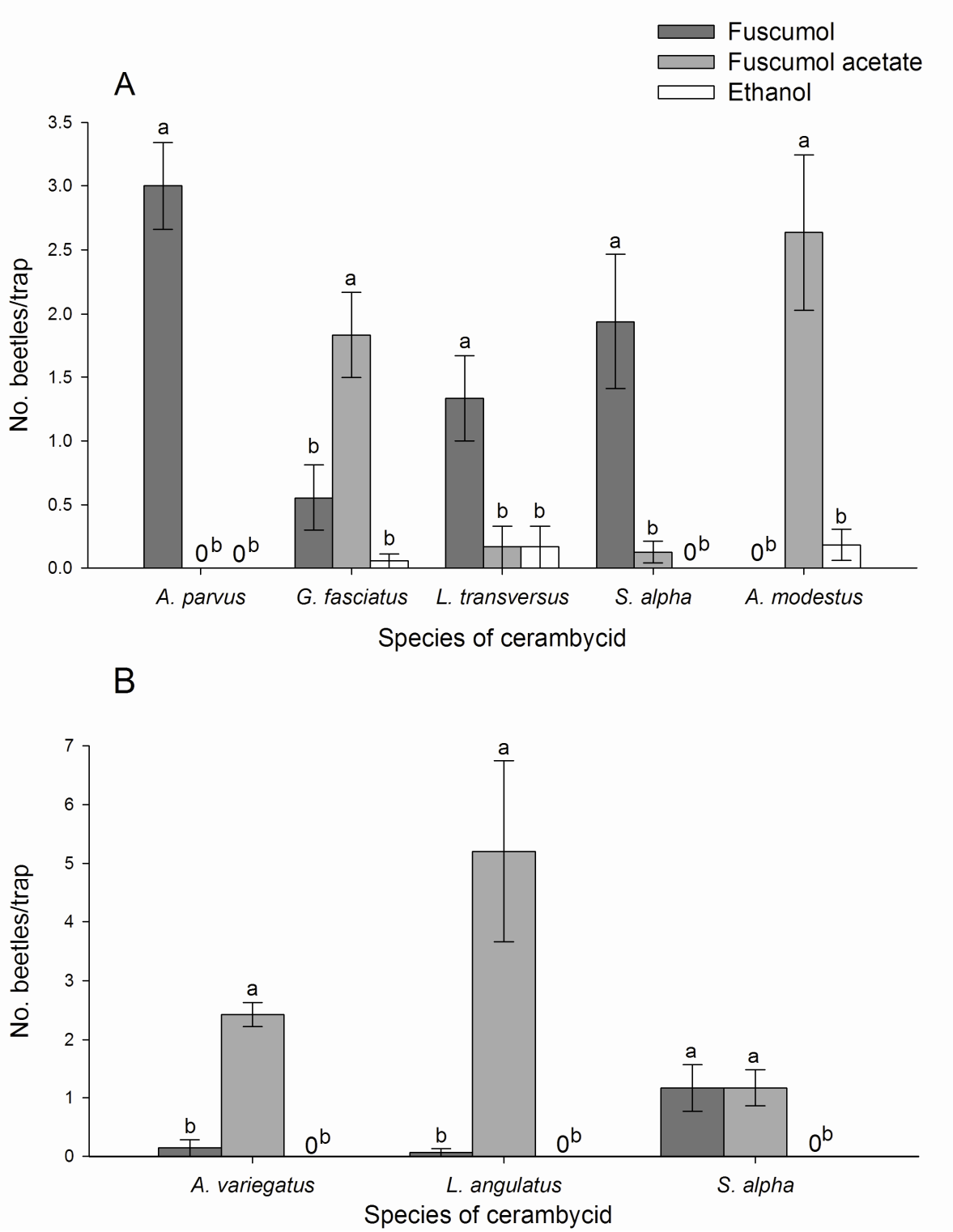


Fig. 2.2. Mean (± SE) number of beetles captured (per trap and count period) in northwestern Indiana (IN) and central Texas (TX) during Experiment 1 for two species in the subfamily Cerambycinae. Fuscumol and fuscumol acetate consist of racemic blends of their (*E*)- and (*Z*)-isomers. Means with different letters within species are significantly different (REGWQ means-separation test: $P < 0.05$).

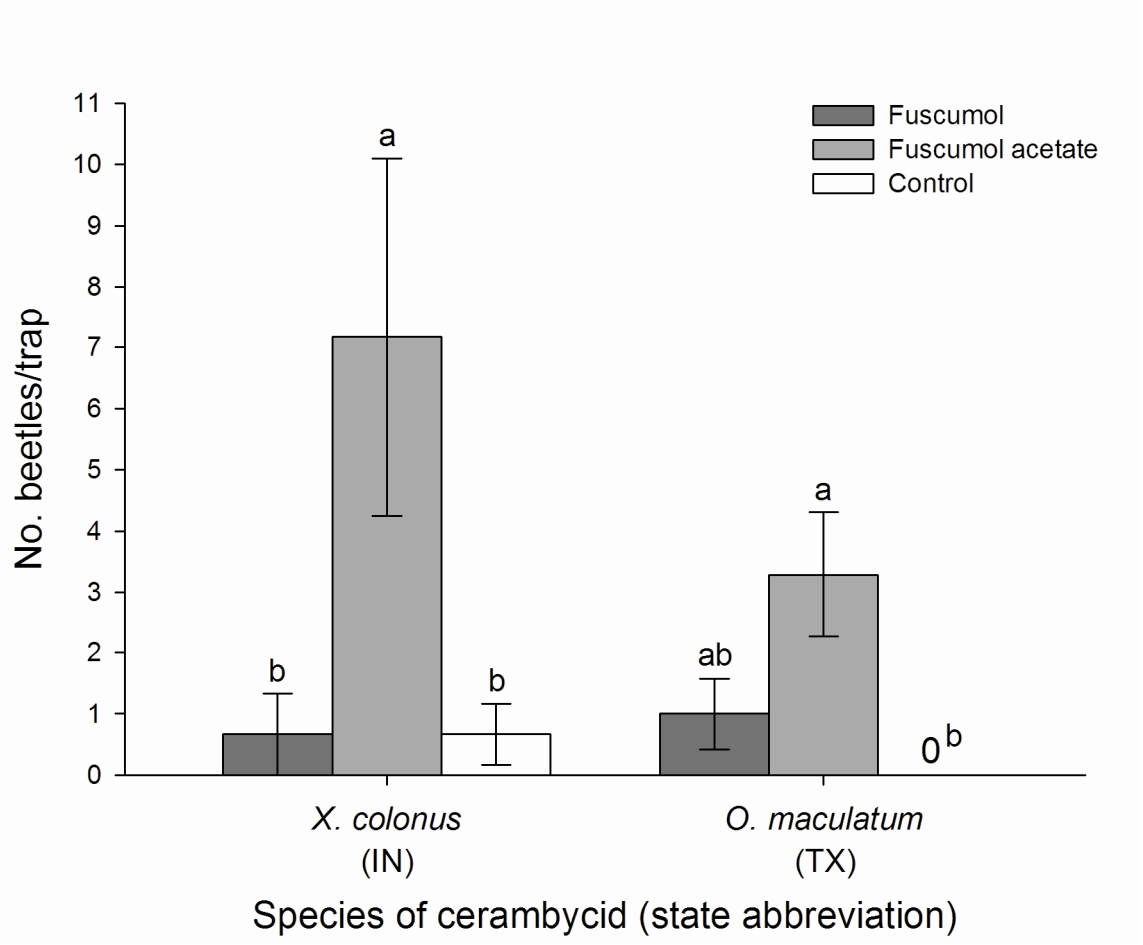
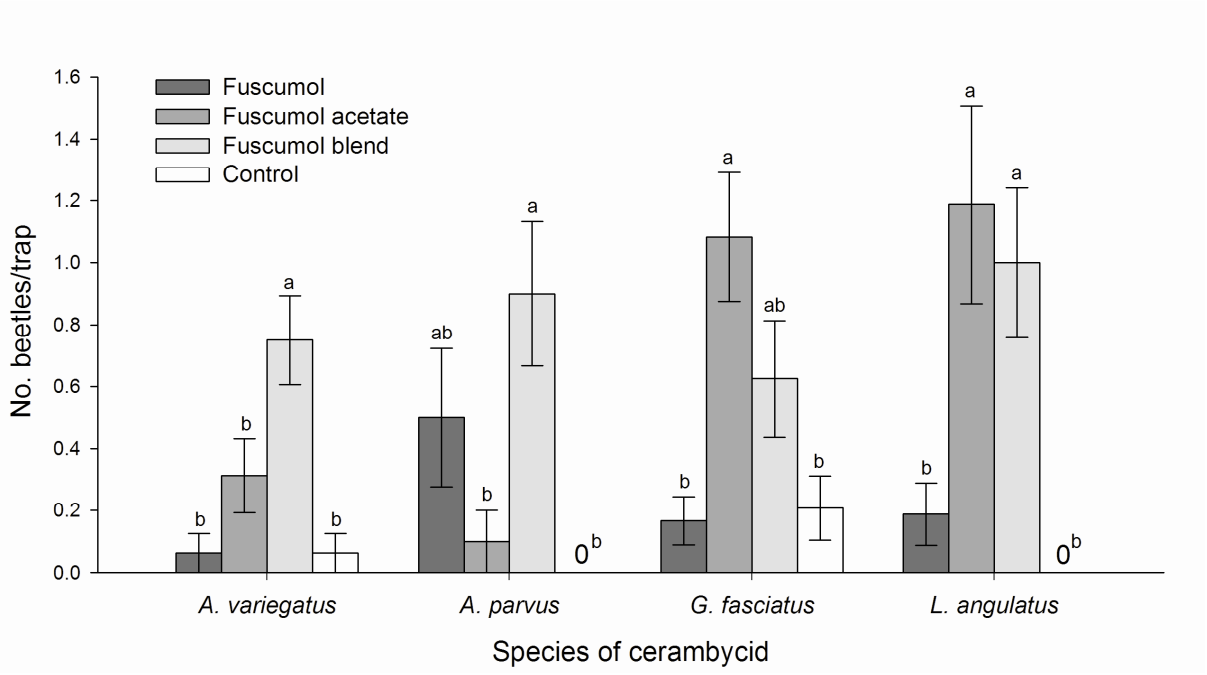


Fig. 2.3. Mean (± SE) number of beetles captured (per trap and count period) in east-central Illinois during Experiment 2 for four lamiine species (see Table 2.2) that also were captured in Experiment 1 (see Fig. 2.1). Fuscumol and fuscumol acetate consist of racemic blends of their (*E*)- and (*Z*)-isomers, and the fuscumol blend consists of equal parts (*E*/*Z*)-fuscumol and (*E*/*Z*)-fuscumol acetate. Means with different letters within species are significantly different (REGWQ means-separation test: $P < 0.05$).



CHAPTER 3: ALKAN-2-ONES: A NEW STUCTURAL MOTIF FOR PHEROMONES OF CERAMBYCID BEETLES

Abstract

Males of many species of cerambycid beetles in the subfamily Cerambycinae produce identical pheromones, bringing into question how these species avoid cross attraction. In this article, I summarize data on three such species, each of which produces the common cerambycine pheromone component (*R*)-3-hydroxyhexan-2-one, but also a novel motif of cerambycid pheromone components, alkan-2-ones. Analysis by gas chromatography-mass spectrometry of the volatiles produced by male beetles indicated that *Cyrtophorus verrucosus* (Olivier) produced nonan-2-one at ~18% of the ketone component, while *Orwellion gibbulum arizonense* (Casey) and *Parelaphidion aspersum* (Haldeman) produced decan-2-one at ~40% and 7%, respectively. In field bioassays, adult *C. verrucosus* were attracted by (*R*)-3-hydroxyhexan-2-one alone, but attraction was significant enhanced by nonan-2-one. Attraction to the blend was reduced if the quantity of nonan-2-one exceeded 100% of the quantity of the ketone, suggesting beetles could perceive ratios of the two chemicals and were most strongly attracted to those approximating the blend produced by males. It is likely that decan-2-one plays a similar role in mate location for *O. g. arizonense* and *P. aspersum* and that the alkan-2-ones serve to minimize cross-attraction among cerambycid species.

Introduction

Species of cerambycid beetles produce pheromones with a remarkably conserved chemical structure, and beetles often produce a single chemical that potentially could attract

many different species (e.g., Barbour et al. 2011, Hanks et al. 2007; Chapter 2). An example of this parsimony is the male-produced aggregation pheromone (*R*)-3-hydroxyhexan-2-one (hereafter "3*R*-ketone"), which is produced by many species in the subfamily Cerambycinae (e.g., Fettköther et al. 1995; Hanks et al. 2007, 2012; Lacey et al. 2007, 2009; Leal et al. 1995 Ray et al. 2009). Several of these species overlap in geographic range and phenology, and traps baited with 3*R*-ketone capture multiple species in field bioassays (e.g., Hanks et al. 2007, Wong et al. 2012).

Sympatric cerambycine species that produce 3*R*-ketone as their primary pheromone component may avoid cross-attraction by differential response to certain host plant volatiles (e.g., Hanks et al. 2012), or by differing in seasonal or circadian periods of activity (Striman 2011). Species specificity of pheromone signals may also be maintained by the presence of minor pheromone components. For example, males of the cerambycid *Phymatodes lengi* Joutel produce 3*R*-ketone, but neither sex is attracted to the synthetic compound unless it is released along with a second component of the pheromone, (*R*)-2-methyl-1-butanol (Striman 2011). Similarly, the cerambycids *Xylotrechus colonus* (F.) and *Sarosesthes fulminans* (F.) produce 3*R*-ketone and are attracted to traps baited with the synthetic compound, but species specificity may be imparted by the different isomers of 2,3-hexanediol that males produce in lesser quantities (Lacey et al. 2009).

In this article, I summarize research on the role of minor pheromone components for the native cerambycid species in the subfamily Cerambycinae: *Cyrtophorus verrucosus* (Olivier), *Orwellion gibbulum arizonense* (Casey), and *Parelaphidion aspersum* (Haldeman). The males of all three species produce 3*R*-ketone as a primary component, similar to other species in the

subfamily, but these three species also produce an alkan-2-one (nonan-2-one or decan-2-one) as a minor component in the blend.

Materials and Methods

Source of synthetic pheromones

Racemic 3*R*-ketone and (*S*)-3-hydroxyhexan-2-one ("3*S*-ketone") were synthesized as described by Lacey et al. (2007). Nonan-2-one (99% purity) was purchased from Sigma-Aldrich (St. Louis, MO) and decan-2-one (>99%) was purchased from TCI America (Portland, OR).

Pheromone identification

I collected live males and females of the three species for aeration and pheromone identification with pheromone-baited traps. Traps were black cross-vane flight intercept traps (Panel Trap model; AlphaScents, Portland, OR) suspended from frames of PVC pipe (for details, see Graham et al. 2010). Pheromone lures were suspended in the center of traps and consisted of polyethylene sachets (Bagettes™ model 14770, 5.1 × 7.6 cm; Cousin Corp., Largo, FL, USA) loaded with racemic 3-hydroxyhexan-2-one ("3*R**-ketone") diluted in 1 ml ethanol. Adult *P. aspersum* were trapped during August 2008 at the municipal Landscape Recycling Center in Urbana, IL (Champaign Co.; 40° 7'19.23"N, 88° 10'44.79"W), an open-air processing facility where woody plant material is recycled into mulch and compost. Adult *C. verrucosus* were trapped at Forest Glen Preserve, IL (Vermilion Co.; 40° 0'51.97"N, 87° 34'0.74"W) during May 2009. A single male *O. g. arizonense* was trapped during June 2009 in a private lumber yard south of Stephenville, TX (Erath Co., 32° 9'35.18"N, 98° 11'21.09"W), which contained stacked trunks from many species of trees native to the region.

Specimens of *P. aspersum* and *O. g. arizonense* were sexed by the relative length of the antennae (Linsley, 1963; under former genus *Elaphidionoides*). Male and female *C. verrucosus* are very similar in morphology, so beetles were sexed by briefly holding them in common cages and observing mating behavior (i.e., males mount females). The sexes were otherwise housed separately in the laboratory in cylinders of aluminum window screen (9 cm diameter, ~10 cm height) under laboratory conditions (12:12h L:D, ~20 °C) and provided with 10% aqueous sucrose solution. Beetles were allowed at least 3 d to acclimate before being aerated to collect volatiles.

Volatiles produced by beetles were collected by placing them in ~0.5 l glass canning jars (Ball ®, Jarden Home Brands, Daleville, IN) that were lined with aluminum window screen to allow beetles to move freely. Jar lids were fitted with two threaded brass pipes seated with brass nuts and Teflon® washers. One pipe extended ~2 cm into the jar and was connected to a charcoal scrubber through which air was drawn into the chamber. The other pipe extended ~6 cm into the jar and was connected to a collector (10-cm glass tube packed with ~150 mg HayeSepQ [Sigma-Aldrich], between plugs of silanized glass wool) through which air was drawn by vacuum (1 l/min). Headspace volatiles were sampled for 24 h under natural lighting (~14:10h L:D, ~20 °C). Insects usually were sampled individually, but in some cases they were sampled in groups of two to three of a single sex to increase the chances of collecting quantities of volatiles sufficient for analysis. Control aerations, with identical chambers that lacked insects, were conducted at the same time to check for external contaminants. Collectors were extracted with 1.5 ml dichloromethane into silanized glass vials that were stored at -20 °C until analysis. Aerated beetles were returned to cages and left to recover for at least 24 h before any additional

aerations. Specimens were pinned at the conclusion of the study and voucher specimens have been deposited in the collection of the Illinois Natural History Survey, Champaign, IL, USA.

Extracts were analyzed by gas chromatography-mass spectrometry (GCMS) with a HP 6890 gas chromatograph (Hewlett-Packard, Palo Alto, CA) fitted with an AT-5ms column (30 m, 0.25 mm i.d., 0.25 μm film; Alltech Associates, Inc., Deerfield, IL) and coupled to an HP 5973 mass selective detector. Injector temperature was 250 °C, and oven temperature was held at 40 °C for 1 min, increased at 10 °C/min to 210 °C, and held 3 min. Sex-specific peaks were tentatively identified by matching mass spectra of samples to a library of known spectra (National Institute of Standards and Technology, Gaithersburg, MD) and confirmed by comparing spectra and retention times with authentic standards.

Chirality of 3-hydroxyhexan-2-one was determined on a HP 5890 fitted with a Cyclodex-B column (30 m, 0.25 mm i.d., 0.25 μm film; Agilent Technologies, Inc., Santa Clara, CA) and a flame ionization detector. Injector temperature was 250 °C, and oven temperature was held at 50 °C for 1 min, increased at 5 °C/min to 130 °C, and held 10 min. 3*R*- and 3*S*-ketone were separated to baseline resolution and identity of enantiomers was confirmed by co-injection of 3*S*-ketone and observing for changes in peak area.

Field bioassays of synthetic compounds

I conducted field bioassays to evaluate the influence of nonan-2-nonone, but was unable to conduct similar experiments with *O. g. arizonense* and *P. aspersum* in IL or TX because they were too rarely collected. The same flight intercept traps were used but coated with Fluon® PTFE to improve trapping efficiency (AGC Chemicals Americas, Inc., Exton, PA; for details, see Graham et al. 2010). Pheromone lures were again polyethylene sachets loaded with synthetic

pheromone, but diluted in 1 ml isopropanol, or containing 1 ml of neat isopropanol as a control. Traps were positioned 10 m apart in linear transects, and treatments were assigned randomly to traps on the first day, 1 trap per treatment. I collected beetles from traps at intervals of 1-3 d, at which time treatments were rotated within transects to control for location effects. Lures were replaced as needed, usually every 5-7 d.

I confirmed activity of male-produced compounds of *C. verrucosus* by testing synthetic blends in two independent field bioassays. Experiment 1, conducted at Forest Glen Preserve, tested for synergism of 3*R*-ketone by nonan-2-one, and simultaneously tested for inhibition by the unnatural 3*S*-ketone isomer. The experimental treatments represented different combinations of 3*R*-ketone (25 mg per lure), 3*R**-ketone (50 mg), and nonan-2-one (5 mg per lure), that simulated the relative amount of nonan-2-one in aeration extracts of males (~20% of the amount of 3*R*-ketone), as follows: 1) 3*R*-ketone alone, 2) 3*R**-ketone alone, 3) nonan-2-one alone, 4) 3*R*-ketone + nonan-2-one, 5) 3*R**-ketone + nonan-2-one, and 6) neat isopropanol. The experiment was conducted during 29 April – 1 June 2011 (average low temperature 11.3 °C, average high temperature 23.1 °C, total precipitation 11 cm; Weather Underground, Inc., Ann Arbor, MI).

Experiment 2 tested the relationship between the relative amount of nonan-2-one and the degree to which it synergized attraction to 3*R*-ketone, and was conducted at Allerton Park near Monticello, IL (Piatt County; 39° 59’11.01”N, 88° 39’3.75”W), a 600 ha riparian forest. The five treatments represented varying concentrations of nonan-2-one relative to the amount of 3*R**-ketone (at 50 mg per lure), including 0, 10, 20, 100, 500, and 1,000% nonan-2-one (i.e., 0, 2.5, 5, 25, 125, and 250 mg). I used 3*R**-ketone because it was more economical than the chiral material, and because the (*S*)-enantiomer usually does not affect attraction of cerambycids to 3*R*-ketone (Hanks et al. 2007, Wong et al. 2012), as was confirmed for *C. verrucosus* by Experiment

1 (see Results). Experiment 2 was conducted during 2 May – 31 May 2011 (average low temperature 11.5 °C, average high temperature 23.2 °C, total precipitation 10.6 cm).

Statistical analysis

Differences between treatment means were tested by fitting the data to a negative binomial distribution with a log-link function (PROC GENMOD; SAS Institute, 2010) and using a Type 3 analysis with likelihood ratio statistics (TYPE3 option). Date-site combinations were dropped from the analysis when two or fewer specimens were collected across all traps, so as to exclude periods of low beetle activity (N = 10 replicates per experiment). I measured specific differences between treatments with a set of a priori contrasts that tested specific hypotheses within each experiment (CONTRAST option). Contrasts were non-orthogonal, so significant *P*-values were determined by a Šidák correction that controlled family-wise error to $\alpha = 0.05$, based on the total number of contrasts in each experiment (Abdi and Williams 2010).

I also measured the effect of nonan-2-one on additional species captured during Experiment I. Total specimen number was pooled within treatments containing 3*R*-ketone and nonan-2-one (3*R*-ketone + nonan-2-one, 3*R**-ketone + nonan-2-one) and traps that contained only 3*R*-ketone (3*R*-ketone and 3*R**-ketone). Chi-square analysis was used to determine deviation from an expected 1:1 distribution between these traps.

Results

Pheromone identification

Aeration extracts of male *C. verrucosus*, *O. g. arizonense,* and *P. aspersum* all contained two peaks (Fig. 3.1) that were absent in control aerations, and also were absent in extracts of female *C. verrucosus* and *P. aspersum* (no female *O. g. arizonense* were available for aeration).

Mass spectra and retention times of the early-eluting components matched that of 3-hydroxyhexan-2-one for all three species, consistent with the collection of these insects from traps baited with this chemical. Co-injection with a pure standard of 3*S*-ketone resulted in a novel peak when analyzed in the Cyclodex B column, suggesting that all three species produce the 3*R*- enantiomer. Aeration extracts of *C. verrucosus* also contained nonan-2-one, whereas those of both *O. g. arizonense* and *P. aspersum* contained decan-2-one (confirmed by matching spectra and retention times with authentic standards). Trace amounts of 2,3-hexanedione also were recovered from aerations of male beetles, but I have found that similar amounts of that chemical often are detected in synthetic standards of 3*R**-ketone (unpub. data), suggesting that may be a product of isomerization, or an analytical artifact (Fettköther et al. 1995).

For each species, the amount of the alkan-2-one component was less than half that of 3*R*-ketone. Separate aerations of male *C. verrucosus* contained nonan-2-one in quantities of 18.8, 16.8, and 17.4% (mean 17.7 ± 0.59%) relative to 3*R*-ketone. The amount of decan-2-one (relative to 3*R*-ketone) was 39.4% in an aeration extract from the one male *O. g. arizonense* and 6.6% in an aeration extract from two male *P. aspersum*.

Field bioassays of synthetic compounds

I collected 105 adult *C. verrucosus* during Experiment 1 and 174 adults during Experiment 2 (total 279). In Experiment 1, treatment means were significantly different (χ^2 = 21.7, d.f. = 5, P = 0.0006) and two of six a priori contrasts were significant ($P < 0.008$; Table 3.1). Traps baited with 3*R*-ketone and 3*R**-ketone (alone or combined with nonan-2-one) captured similar numbers of beetles (Fig. 3.2, Table 3.1), indicating that the 3*S*-enantiomer had no significant effect on attraction. 3*R*-ketone and 3*R*-ketone+nonan-2-one were both

significantly different from the negative control (Fig. 3.2, Table 3.1). Nonan-2-one alone was not significantly attractive and in this experiment did not enhance attraction to 3*R*-ketone and 3*R**-ketone (Table 3.1). In Experiment 2, nonan-2-one enhanced attraction of *C. verrucosus* to 3*R**-ketone when present at 10%, 20%, or 100% of the quantity of ketone but had no effect at 500% or 1000% (Fig. 3.3, Table 3.1; treatment means significantly different, $\chi^2 = 11.02$, d.f. = 5, $P = 0.05$).

I also collected 239 adult *Anelaphus pumilus* (Newman) and 69 *Phymatodes aereus* (Newman) during Experiment I, and only in traps that included 3*R*-ketone. The presence of nonan-2-one did not affect the capture of these species (*A. pumilus*: 3*R*/3*R**-ketone: 116, 3*R*/3*R**-ketone+nonan-2-one: 97, $\chi^2 = 1.69$, $P = 0.19$; *P. aereus*: 3*R*/3*R**-ketone: 30, 3*R*/3*R**-ketone+nonan-2-one: 39, $\chi^2 = 1.17$, $P = 0.28$).

Discussion

Verification that 3*R*-ketone is an important pheromone component of *C. verrucosus*, and evidence that it also is produced by male *O. g. arizonense* and *P. aspersum*, adds to the growing list of cerambycid species that rely on these compounds for mate location (e.g., Hanks et al. 2012). The alkan-2-ones reported here are the first to be described as pheromone components of cerambycid beetles, although they are structurally similar to the 3-hydroxyalkan-2-ones produced by many species in the subfamily Cerambycinae (Millar et al. 2009). The fact that alkan-2-ones are produced by three cerambycine species of two different tribes (Elaphidiini and Anaglyptini) suggests that the alkanon-2-one motif may be common in species that produce 3*R*-ketone and other 3-hydroxyalkan-2-ones. Alkan-2-ones are common floral volatiles of plants, and nonan-2-one in particular is recorded from more than 200 species of plants (El-Sayed, 2012).

Nonan-2-one is also a pheromone component of many other types of arthropods, especially Trichoptera (e.g., Bergmann 2002, Löfstedt et al. 1994), but for only a few species of beetles, as is also the case for decan-2-one (Bartelt et al. 2009, Francke et al. 1979).

Results of the field bioassays supported the hypothesis that nonan-2-one synergizes attraction of *C. verrucosus* to the major component of its pheromone, 3*R*-ketone. Nevertheless, strong attraction of this species to 3*R*-ketone alone would seem to obviate the minor component. The role of nonan-2-one may be to lend species specificity to the chemical signal by deterring attraction to calling males of other cerambycid species that use 3*R*-ketone as a pheromone. Support for this function was provided by the cerambycid *Euderces pini* (Olivier), which also was trapped during the present study. Male *E. pini* produce only 3*R*-ketone (unpub. data), and 23 specimens were captured in a sentinel trap baited with 3*R*-ketone that was situated ~50 m from the study site. The lack of specimens in any trap near a source of nonan-2-one strongly suggests that this species is inhibited by calling male *C. verrucosus*. Furthermore, the co-occurring species *A. pumilus* and *P. aereus* are likely not affected by nonan-2-one because they are nocturnal and thus do not overlap in activity period with *C. verrucosus* (unpub. data). The dual role of the minor components, as synergists for conspecifics and repellents for other species, may serve to minimize cross-attraction between *C. verrucosus* and other species that overlap in activity period and for which 3*R*-ketone is the primary pheromone component. It seems likely the decane-2-one serves the same function for *O. g. arizonense* and *P. aspersum*, but further research is required to confirm that relationship.

The data from Experiment 2 indicated that nonan-2-one synergized attraction of *C. verrucosus* to 3*R*-ketone in quantities that approximated the natural blend produced by males. This finding confirms that the beetles were able to distinguish between different ratios of the two

components, a behavior common in other groups of insects such as moths (e.g., Allison and Cardé, 2008) and bark beetles (e.g., Aukema et al. 2010), but has yet to be reported for cerambycid beetles. This behavior may be adaptive by circumventing cross attraction to some as yet unidentified species that produces a different ratio of nonan-2-one to 3*R*-ketone.

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Table 3.1. A priori linear contrasts of treatment effects for field bioassays of *Cyrtophorus verrucosus* during Experiments 1 (means presented in Figs. 3.1 and 3.2). Significance levels were $P = 0.008$ in Experiment 1 and $P = 0.01$ in Experiment 2, based on a Šidák correction for the number of comparisons. D.f. = 1 for all contrasts. Compound abbreviations: 3*R*-ketone = (*R*)-3-hydroxy-2-hexanone, 3*R**-ketone = racemic 3-hydroxyhexan-2-one. Percentages in Experiment 2 refer to an amount of nonan-2-one in each trap that is expressed as a percentage of 50 mg of 3*R**-ketone.

Contrast	χ^2	*P*
Experiment 1		
3*R*-ketone versus 3*R**-ketone	0.08	0.78
3*R*-ketone+nonan-2-one versus 3*R**-ketone+nonan-2-one	0.11	0.74
nonan-2-one versus control	1.12	0.29
3*R*-ketone versus control	8.4	0.004
3*R*-ketone+nonan-2-one versus control	16.3	<0.001
3*R*-ketone versus 3*R*-ketone+nonan-2-one	4.18	0.13
Experiment 2		
10% versus 3*R**-ketone	7.85	0.005
20% versus 3*R**-ketone	6.41	0.01
100% versus 3*R**-ketone	7.85	0.005
500% versus 3*R**-ketone	1.89	0.17
1000% versus 3*R**-ketone	2.85	0.09

Fig. 3.1. Representative total ion chromatograms of aeration extracts from male **A**) *Cyrtophorus verrucosus*, **B**) *Orwellion gibbulum arizonense*, and **C**) *Parelaphidion aspersum*. Asterisks signify peaks that were not present in control aerations. AT-5ms column, 40 °C/1 min, increasing at 10 °C/min, end at 210 °C/1 min.

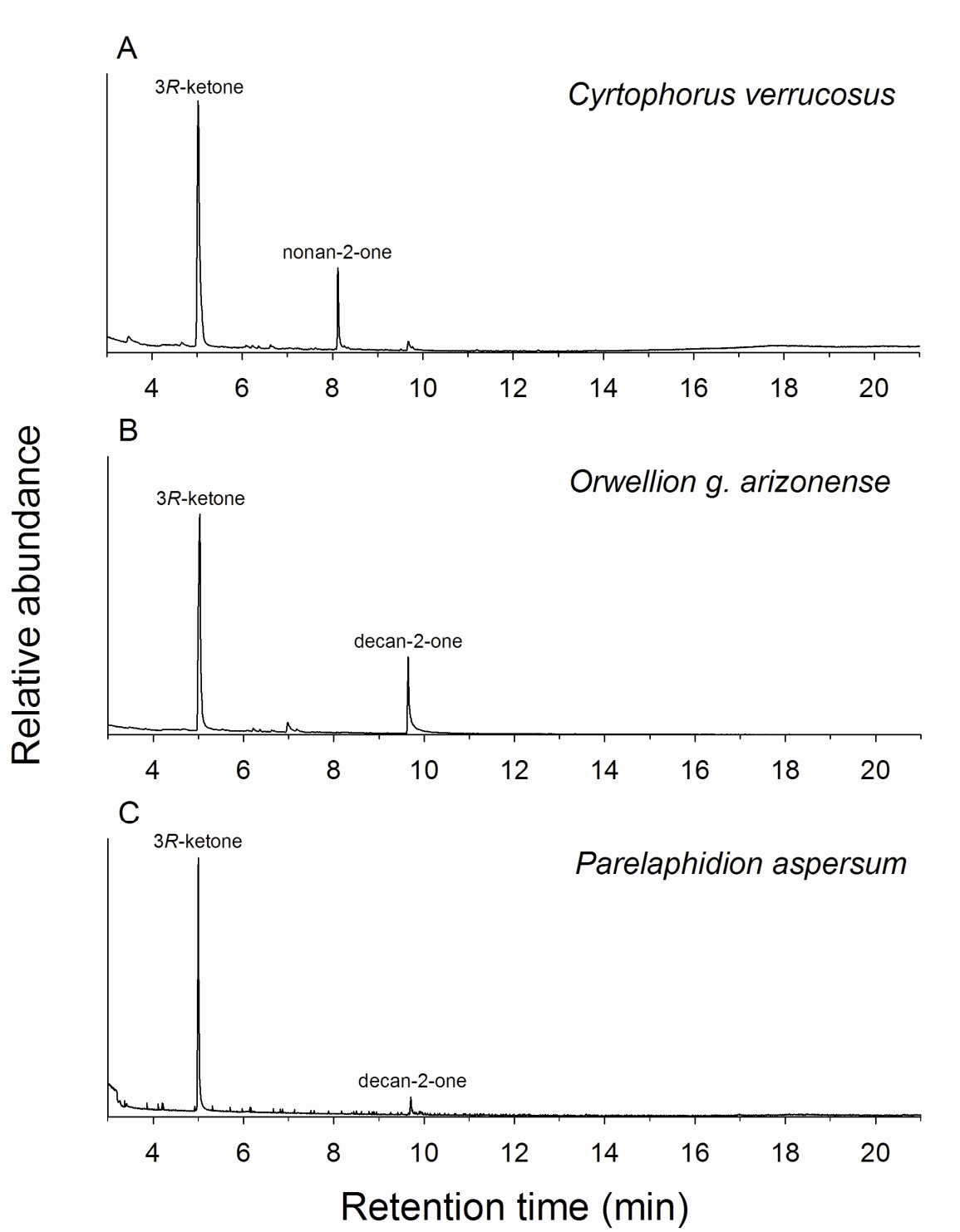


Fig. 3.2. Mean (±SE) number of *Cyrtophorus verrucosus* captured during Experiment 1 by traps baited with pheromone components. Compound abbreviations as in Table 3.1.

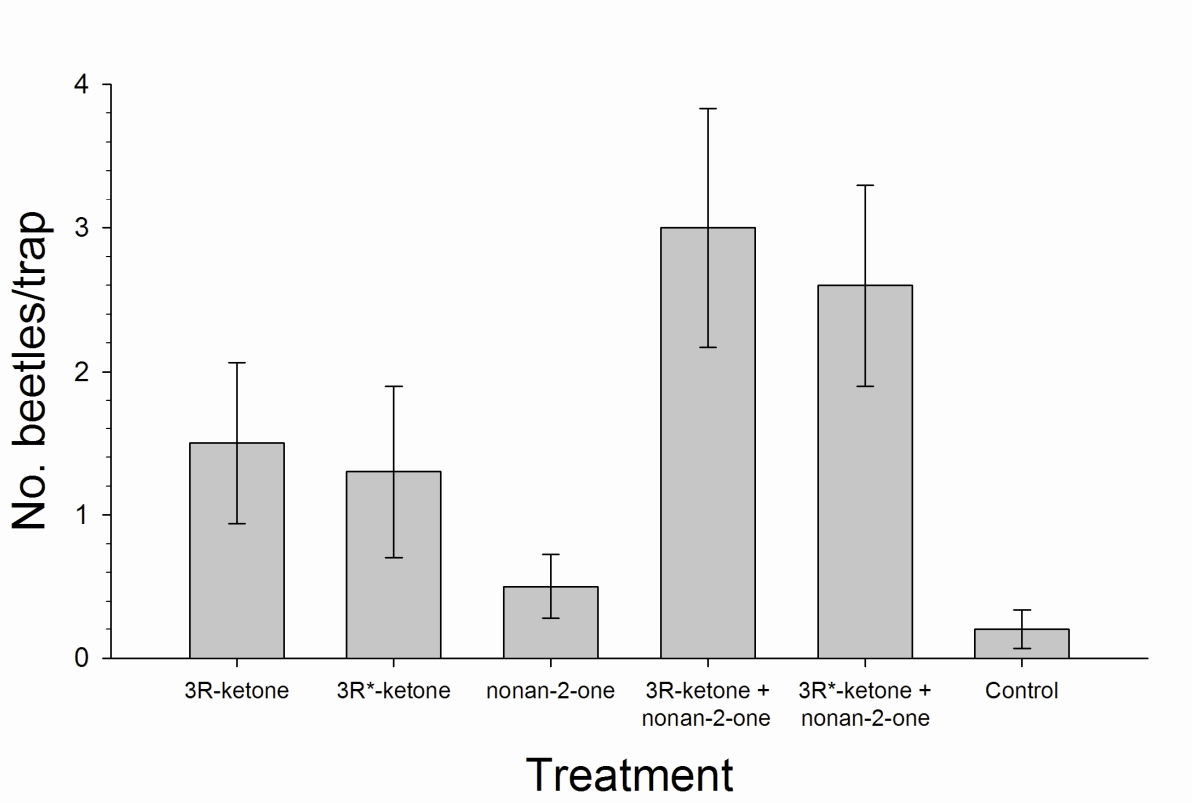
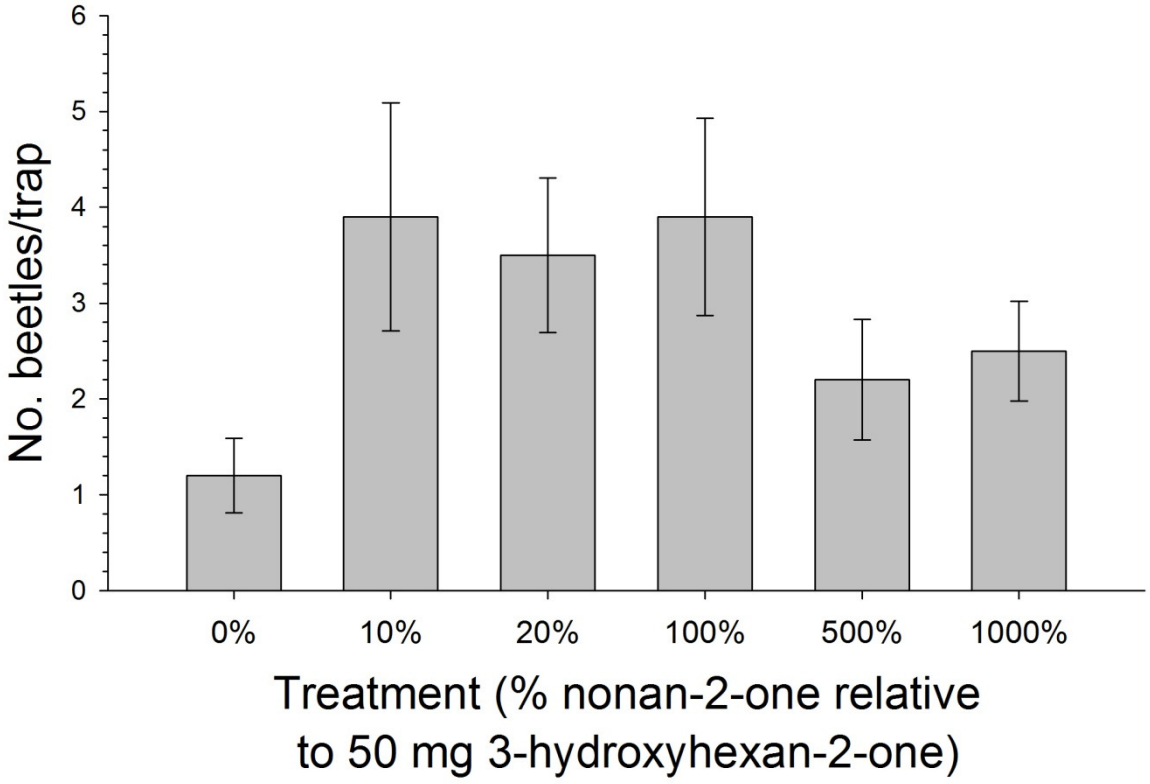


Fig. 3.3. Mean (±SE) number of *Cyrtophorus verrucosus* captured during Experiment 2 by traps baited with 3*R**-ketone combined with varying concentrations of nonan-2-one. The amount of nonan-2-one in each trap is expressed as a percentage of 50 mg 3*R**-ketone.



CHAPTER 4: BEHAVIORALLY ACTIVE COMPONENTS OF THE NINE-PART VOLATILE PHEROMONE OF *MEGACYLLENE CARYAE* (GAHAN)

Abstract

Adult males of the cerambycid beetle *Megacyllene caryae* (Gahan), the painted hickory borer, produce a volatile aggregation pheromone that ranks among the most complex of insects, consisting of eight components that include several terpenoids, isomers of 2,3-hexanediol, and 2-phenylethanol. I report here a previously unrecognized ninth component, (*S*)-2-methylbutan-1-ol. Gas chromatography-electroantennography revealed that antennae of adult beetles are sensitive to all components of the blend except (*S*)-(–)-limonene, but no components other than geranial and neral (tested together as "citral") attracted beetles when tested singly in field bioassays. Citral was an effective attractant when used alone, but beetles strongly preferred traps baited with the blend of citral and (*S*)-2-methylbutan-1-ol, which was equally as attractive as the complete blend of pheromone components.

Introduction

The longhorned beetles (Cerambycidae) are well-known for producing pheromones conforming to certain motifs that are conserved within genera or subfamilies (e.g., Barbour et al. 2011, Fierke et al. 2012, Millar et al. 2009, Teale et al. 2011; Chapter 2). For example, males of many species in the subfamily Cerambycinae, especially in the tribe Clytini, produce aggregation pheromones that are 6-10 carbon alkanes with ketones and hydroxyl groups at carbons 2 and 3 (Millar et al. 2009). These pheromones usually consist of single components (Lacey et al. 2004, 2009, Ray et al. 2009) or blends of a few structurally related chemicals (Lacey et al. 2007). As

discussed earlier (Chapter 3), traps baited with a few of these compounds will capture many individuals of diverse cerambycid species (Wong et al. 2012).

Species in the clytine genus *Megacyllene* stand as exceptions to the rule, in that adult males produce extensive and unusual blends of components. For instance, *M. robiniae* (Forster), the locust borer of eastern North America, produces several of the common ketones and diols, but also the aromatic alcohol 1-phenylethanol and a furan structure that is unusual among cerambycid beetles (Wheeler et al. 1988, RFM, unpub. data). The western congener *M. antennata* (White) produces a variety of volatile terpenoids as its presumed pheromone (A. M. Ray, pers. comm.). The pheromone of the eastern species *M. caryae* (Gahan), the painted hickory borer, consists of the common (*R*,*S*)- and (*S*, *R*)-2,3-hexanediols, but also 2-phenylethanol and the terpenoids (–)-α-terpineol, (*S*)-(–)-limonene, nerol, neral, and geranial (Lacey et al., 2008). This pheromone ranks among the most complex among insects, comparable to those produced by social insects (Keeling et al. 2003), but the role and necessity of each component remain uncertain.

Adult *Megacyllene caryae* are not attracted to traps baited with the ketone and diol components of its pheromones (RFM, unpub. data), unlike other clytines that produce the same components (e.g., Wong et al. 2012. Previous research has suggested that adult *M. caryae* require all eight pheromone components for attraction (Lacey et al. 2008). More recent bioassays, however, have suggested that a combination of neral and geranial (together, "citral") can evoke attraction (RFM, unpub. data). The critical role for citral is consistent with its being the dominant pheromone component (>70% of the blend; Lacey et al. 2008). In fact, calling males emit a powerful citrus smell that is easily detectable by humans (RFM, pers. obs.).

In this chapter, I document which components of the pheromone blend of *M. caryae* cue attraction, and test the hypothesis that citral alone is a sufficient attractant. I also present evidence for a ninth pheromone component, (*S*)-2-methylbutan-1-ol, which strongly synergizes the attractive power of citral.

Methods

Sources of insects and synthetic pheromones

Adult *M. caryae* were captured alive with black cross-vane flight intercept traps (Panel Trap model; AlphaScents, Portland, OR) baited with lures containing 50 mg citral in 1 ml ethanol (for details, see Chapters 2 and 3). Adult *M. caryae* used in aerations were trapped in May 2008 at Trelease Woods, IL (Champaign Co.; 40° 7'55.68"N, 88° 8'29.43"W), a 24-ha prairie grove remnant of mixed hardwoods northeast of Urbana, IL. Specimens used for electroantennography were collected in May 2011 with traps at Trelease and Forest Glen Preserve, IL (Vermilion Co.; 40° 0'51.97"N, 87° 34'0.74"W), a 730 ha preserve of mixed prairie and beech-maple/oak-hickory forests.

Stocks of racemic and homochiral 2,3-hexanediol were synthesized as described in Appendix A. (*S*)-2-methybutan-1-ol was purchased from TCI America (Portland, OR), and (*S*)-(–)-limonene, 2-phenylethanol, nerol, and (–)-α-terpineol from Sigma-Aldrich (St. Louis, MO). Neral and geranial readily isomerize, so citral (a ~1:1 mixture) was used as a substitute (Sigma-Aldrich).

Collection and identification of volatiles

I collected headspace volatiles from adult *M. caryae* in custom-built aeration equipment with the protocols described in Chapter 3. Sexes were aerated separately, in groups of five adults, and assays always included an empty aeration chamber as a negative control. Volatiles were extracted with 1.5 ml dichloromethane and 1 µl of each sample was injected into a Hewlett-Packard 6890 (Hewlett Packard, Palo Alto, CA) with an AT-5ms column (30 m, 0.25 mm i.d., 0.25 µm film; Alltech Associates, Inc., Deerfield, IL) coupled to a 5973 mass selective detector (Hewlett-Packard). Samples were injected at 250 °C with oven temperature 40 °C, held 1 min, increased at 10 °C/min to 210 °C, and held 1 min. Peaks were identified by matching retention times and mass spectra with those of known pheromone components of *M. caryae* (Lacey et al. 2008). I matched mass spectra of unknown chemicals to a library of standards (National Institute of Standards and Technology, Gaithersburg, MD) and confirmed identifications by comparison with retention times and spectra of authentic standards.

The enantiomer of 2-methyl-1-butanol was resolved by gas chromatograph with a Cyclodex B column (30 m, 0.25 mm i.d., 0.25 µm film; Agilent Technologies, Inc., Santa Clara, CA) at an isothermal oven temperature of 35 °C. Under these conditions, the (*R*)-enantiomer eluted at 12 min, whereas the (*S*)-enantiomer eluted at 12.3 min. The retention times and spectra matched those of a racemic standard (Sigma-Aldrich).

Gas Chromatography-Electroantennography (GC-EAD)

I evaluated the sensitivity of *M. caryae* to each chemical by testing them separately against antennae in a coupled GC-EAD. Antennae were excised from live, field-collected insects at the third antennomere, also removing the terminal antennomere. Antennal sections were suspended between glass capillary tubes (~1 mm i.d.) filled with a saline solution (7.5 g

NaCl, 0.21 g $CaCl_2$, 0.35 g KCl, 0.2 g $NaHCO_3$ in 1 l distilled water) that were placed over gold wires (7 cm x 0.25 mm; Alfa Aesar, Ward Hill, MA) mounted on micromanipulators (Model S-725-CRM; Signatone Corporation, Gilroy, CA). The gold wires were soldered to shielded coaxial cables that connected to an amplifier (Model P15D, Grass Technologies-Astro-Med, Inc., West Warwick, RI) set at 100X, high-pass filter at 1 kHz, low-pass filter at 0.1 Hz. Antennal preparations were fairly robust and withstood 3-4 trials.

The EAD was interfaced with a HP 5890 Series II gas chromatograph (Hewlett Packard) with an AT-5ms column (30 m, 0.32 mm i.d., 0.25 μm film; Alltech Associates, Inc.). Injector temperature was 250 °C, and oven temperature was 40 °C, held 1 min. (3 min at 30°C for [*S*]-2-methylbutan-1-ol), and increased at 10 °C/min to 210 °C, but usually stopped 1-2 min after the sample eluted. The column terminated at an untreated 3-way glass splitter (0.32 x 0.25 x 0.25 mm; Chromatographic Specialties Inc., Brockville, Ontario, Canada) that was connected to two lengths of deactivated fused silica capillary column (1 m, 0.25 mm i.d.; W. R. Grace & Co., Columbia, MD). One column length went to the flame ionization detector, and the other passed through a heated transfer line (220 °C; Type EC-03-300 Effluent Conditioner Assembly; Syntech, Kirchzarten, Germany) into a glass tube of 1 cm i.d. through which flowed clean, humidified air (500 ml/min) that discharged onto the antennal preparation. Signal output from the GC and EAD were digitized and synchronized, and output was recorded (model 302 Six Channel Data System, PeakSimple 3.93 software, SRI Instruments, Torrance, CA).

I tested each component of the pheromone blend against antennae of three beetles of each sex. Standards of each component were diluted in dichloromethane to 25 ng/μl and 1 μl was injected into the GC, thus exposing the antenna to ~12.5 ng in a volume of a few cm^3.

Field bioassays

I tested the response of *M. caryae* to pheromone components in three independent experiments that compared attraction to individual components as well as to combinations of the components that were most active in GC-EAD tests. Protocols for trapping beetles were as described in Chapters 2 and 3. Lures contained 25 ng of each isomer dissolved in 1 ml solvent (ethanol in Experiment I, isopropanol in Experiments II and III). Treatments were randomized at the start of the study and subsequently rotated within transects at the time that beetles were collected (every 1-3 d). Lures were replaced when depleted, usually every 5-7 d.

The goal of Experiment I was to confirm that citral is the primary attractant of adult *M. caryae* rather than the 2,3-hexanediol components. I compared attraction to citral and (*R**,*S**)-2,3-hexanediol (hereafter "*R***S**-diol") with two blocks of four traps, baited either with citral, (*R**,*S**)-diol, the two combined, or a negative control (1 ml ethanol). Blocks occasionally contained 1-2 additional traps baited with *R***S**-diol that were used to collect specimens for separate studies, and data from these traps were pooled in the *R***S**-diol treatment. I conducted this experiment from 11 April – 22 April 2010 at Allerton Park (Piatt County; 39° 59'11.01"N, 88° 39'3.75"W), a 600 ha riparian forest of primarily of oaks and hickories that is owned by the University of Illinois. Weather conditions were unusually warm, yielding an unexpectedly early emergence of adult *M. caryae* (average low temperature 5.8 °C, average high 23.3 °C, total precipitation 0.15 cm; Weather Underground, Inc., Ann Arbor, MI).

Experiment II was designed to identify any additional pheromone components of *M. caryae* that individually were attractive to adult beetles. The experiment consisted of seven traps, each baited with a single component: chirally pure (*R*,*S*)-2,3-hexanediol ("*RS*-diol"), (*S*,*R*)-2,3-hexanediol ("*SR*-diol"), (*S*)-(–)-limonene, 2-phenylethanol, nerol, (–)-α-terpineol, (*S*)-2-

methylbutan-1-ol ("2*S*-methylbutanol"), and a negative control (1 ml isopropanol). Citral was not included so that it would not compete with other treatments for beetles, but a separate sentinel trap baited with citral was used to confirm that *M. caryae* adults were active during the study. The sentinel trap was positioned 100 m from the transect to avoid interference with the experimental treatments. The experiment was conducted during 13 April – 10 May 2011 at Trelease Woods (average low temperature 7.1 °C, average high 18.4 °C, total precipitation 15.5 cm), and 28 April – 18 May 2011 at Forest Glen Preserve (average low temperature 8.8 °C, average high 19.0 °C, total precipitation 7.3 cm).

Experiment III was designed to identify pheromone components that could synergize attraction to citral, and tested *R***S**-diol and 2*S*-methylbutanol because antennae appeared most sensitive to these compounds in EAD assays. I compared these blends to citral alone and to the complete suite of pheromone components produced by male *M. caryae*, as well as a complete blend that lacked *R***S**-diol and 2*S*-methylbutanol. Thus, the experiment consisted of five treatments: the complete blend of pheromone components ([*R*,*S*]- and [*S*, *R*]-diol, 2-phenylethanol, [–]-α-terpineol, [*S*]-[–]-limonene, nerol, neral, geranial, and 2*S*-methylbutanol), the complete blend minus *R***S**-diol and 2*S*-methylbutanol, citral plus *R***S**-diol, citral plus 2*S*-methylbutanol, citral alone, and a negative control (1 ml isopropanol). This experiment was conducted from 10-13 May 2011 at Allerton Park during a period of hot, dry weather when adult *M. caryae* were abundant (average low temperature 16.8 °C, average high 30.8 °C, no precipitation) and Forest Glen from 20-30 May 2011 (average low temperature 13.8 °C, average high 24.8 °C, total precipitation 3.65 cm).

Statistical Analysis

Data from field bioassays were fit to a negative binomial distribution with a log-link function (PROC GLIMMIX; SAS Institute, 2010) and overall treatment effects were identified through a Type III test of fixed effects (TYPE3 option). For experiments occurring at multiple field sites, location and location × treatment terms were added to models. Date-location replicates were dropped from the analysis if no specimens were collected across all traps. This threshold was adjusted in Experiment III to exclude date-location replicates with fewer than five beetles because beetles were very abundant, allowing higher sample sizes for more robust analyses. Pairwise comparisons of treatments within an experiment were tested with a Tukey-Kramer adjustment to control family-wise error rates (LSMEANS statement). Deviations from a 1:1 sex ratio were tested using chi-square tests.

Results

Identification of pheromones

Aeration extracts of male *M. caryae* contained the same components reported by Lacey et al. (2008), but also a novel component (Fig. 4.1) that matched the mass spectrum of 2-methylbutan-1-ol. Analysis on the chiral column determined the enantiomer to be (*S*)-2-methylbutan-1-ol. None of these chemicals were present in aeration extracts of female beetles or controls.

Response of antenna to volatiles

Coupled GC-EAD indicated that the antennae of male and female *M. caryae* are sensitive to most components of the pheromone blend (Fig. 4.2), with the strongest responses to 2*S*-methylbutanol, *RS*-diol, *SR*-diol, neral, and geranial. Antennae also appeared to respond to

nerol, but that test was confounded by trace quantities of geranial and neral in the standard. Antennae did not respond to limonene at the quantities tested. Responses to components were consistent among the three samples tested for each sex and no differences were observed between sexes.

Field Bioassays

I captured 1,801 adult *M. caryae* across the three experiments, 1,096 during Experiment I, 18 during Experiment II, and 687 during Experiment III. Males outnumbered females in Experiment I (60.4% male; $\chi^2 = 47.2$, d. f. = 1, $P < 0.001$), but sexes were captured in similar numbers in Experiment III (52.9% male; $\chi^2 = 2.43$, d. f. = 1, $P = 0.12$), and insufficient numbers of beetles were captured in Experiment II for a robust test of sex ratio.

Experiment I confirmed that citral (geranial+neral) is sufficient to attract adult *M. caryae* in significant numbers, and attraction was not effected by *R***S**-diol (Fig. 4.3A; overall treatment effect $F_{3,41} = 52.9$, $P = 0.004$). Traps baited with *R***S**-diol alone did not capture significant numbers of beetles. Experiment II further demonstrated that no component other than citral was significantly attractive when tested alone (overall $F_{7,16} = 0.31$, $P = 0.94$). Sentinel traps baited with citral had captured 99 and 105 beetles at the Trelease and Forest Glen locations, respectively, but no beetles were trapped with the experimental treatments at Trelease, and only 18 beetles were trapped at Forest Glen (mean ± SE: *RS*-diol = 2.33±1.2; *SR*-diol = 2±1.53; (*S*)-(–)-limonene = 0; 2-phenylethanol = 0; nerol = 1±0.58; (–)-α-terpineol = 0; 2*S*-methylbutanol = 0; isopropanol = 0.67±0.67).

In Experiment III, the greatest numbers of beetles were captured by traps baited with the complete blend and citral combined with 2*S*-methylbutanol (Fig. 4.3B, overall treatment effect

$F_{4,19} = 9.04$, $P = 0.0003$). Attraction to the full blend was significantly reduced with omission of *R*S**-diol and 2*S*-methylbutanol. The location effect was highly significant due to differences in numbers of insects captured ($F_{1,19} = 57.63$, $P < 0.0001$; interaction term not significant, $F_{4,15} = 0.64$, $P = 0.64$). No beetles were captured by negative control traps, and this treatment was not included in the analysis.

Discussion

Results of the experiments supported the hypothesis that citral is a powerful and effective attractant for *M. caryae*. No other individual component was attractive, although it was impossible to determine the individual effects of geranial and neral because they readily isomerize under natural conditions. Nevertheless, the beetle antennae were similarly sensitive to both compounds in GC-EAD assays and it is possible that both trigger the same odorant receptor, as those receptors may not be enantioselective (see Chapter 6). In fact, antennae were so sensitive to both enantiomers that picogram quantities, undetectable in FID readings, elicited strong EAD signals. Another indication of the potency of citral was attraction of adult *M. caryae* to field vehicles that presumably were lightly contaminated with the compound, even in urban neighborhoods where the beetles would be expected to be rare (RFM, pers. obs.). It should be noted that citral also is an aggregation pheromone of honey bees (Shearer and Boch 1966), suggesting that it may play a role in the Batesian mimicry of *M. caryae*. The convergence of chemical signals between *Megacyllene* and its hymenopteran models is more fully explored in Chapter 5.

Even though citral alone was strongly attractive to adult *M. caryae* in Experiment I, traps baited with the combination of citral and 2*S*-methylbutanol in Experiment III captured

significantly more beetles than citral alone. This finding contrasts with the earlier study by Lacey et al. (2008), who concluded that the complete blend of components was necessary to achieve the highest level of attraction. However, that study had not identified 2*S*-methylbutanol as a pheromone component, due to its very early elution time, and thus had missed its strong synergism of citral. The results of the present study emphasize the importance of testing the activity of different combinations of pheromone components, rather than merely testing the complete blend versus individual components.

Strong attraction of adult *M. caryae* to citral alone is consistent with other cerambycid species that are drawn to the dominant component of their pheromone blends. For example, male *Xylotrechus colonus* (F.), *Sarosesthes fulminans* (F.), and *Cyrtophorus verrucosus* (Olivier) all produce multi-component pheromones, but both sexes are readily captured by traps baited with the dominant component, (*R*)-3-hydroxyhexan-2-one (Lacey et al. 2009, Hanks and Millar 2012; and see Chapter 3). In fact, only a single species of the Cerambycinae reported to date is known to require multiple pheromone components for attraction: adult *Phymatodes lengi* Joutel are not at all attracted by either of the two components of its pheromone when they are tested individually (Hanks et al. 2012).

The research presented here documents the behavioral interplay among components of the pheromone produced by male *M. caryae*, and this information may inform future study of the adaptive explanations for the evolution of such complicated pheromones, and perhaps on the molecular and neural systems for processing of pheromone signals in the insect brain.

Acknowledgments

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Fig. 4.1. Representative total ion chromatogram of an aeration extract from male *Megacyllene caryae*. Asterisk indicates (*S*)-2-methyl-1-butanol, and other pheromone components are as follows: (1) (*S*)-(–)-limonene, (2) 2-phenylethanol, (3) (–)-α-terpineol, (4) nerol (5) neral, and (6) geranial. AT-5ms column, 40 °C/1 min, increasing at 10 °C/min, terminating at 210 °C/1 min.

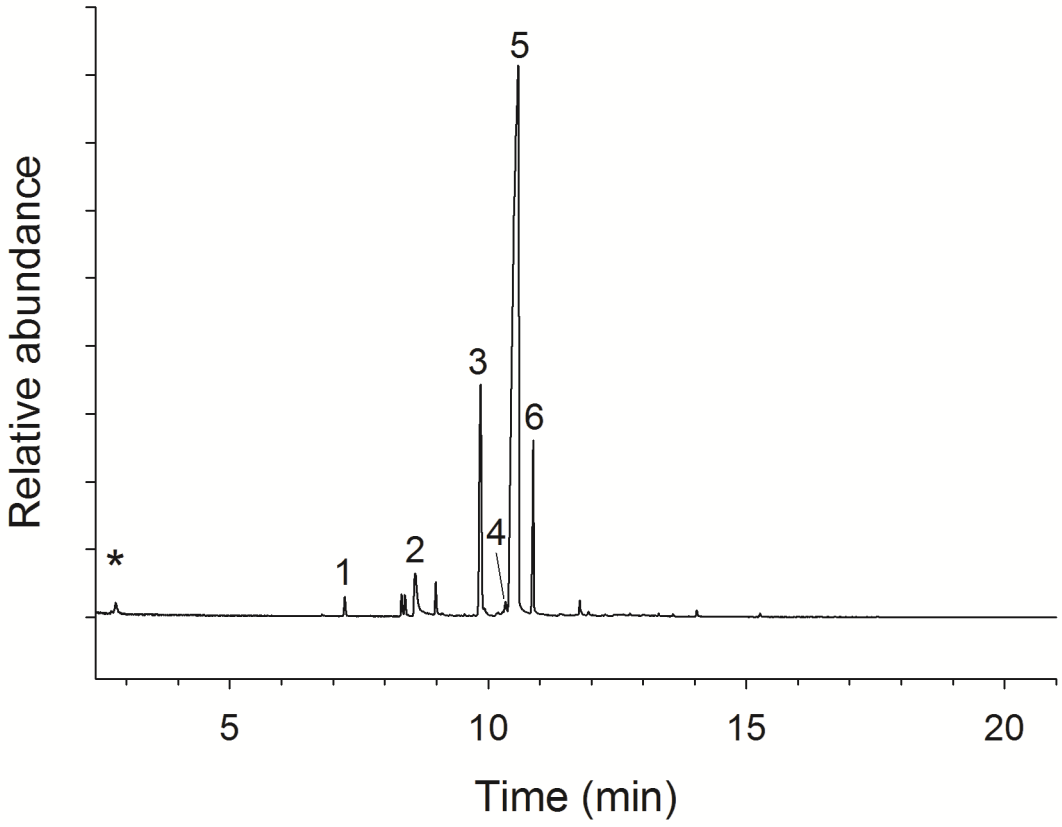


Fig. 4.2. Representative coupled gas chromatography-electroantennographs for the response of antennae of adult *Megacyllene caryae* to synthetic components of the male-produced pheromone. The response to nerol is questionable because the standard was contaminated by trace quantities of neral and geranial. Scale bars represent 25 mV for the flame ionization detector (FID) and 2.5 mV for the electroantennographic detector (EAD; 100X amplification). AT-5ms column, 40 °C/1 min (30 °C/3 min for (*S*)-2-methylbutan-1-ol), then 10°C/min until chemical standard elutes (maximum 210 °C).

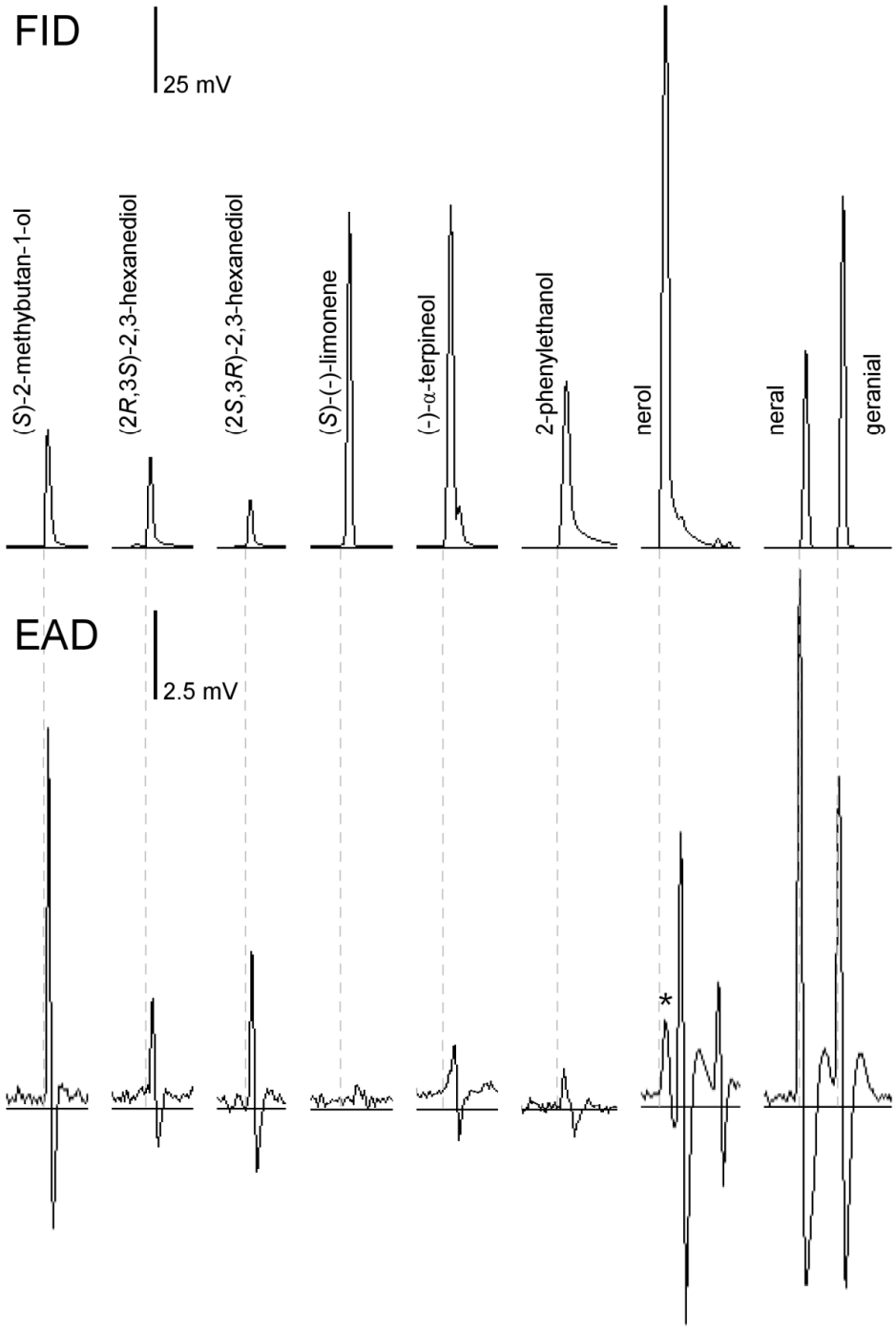
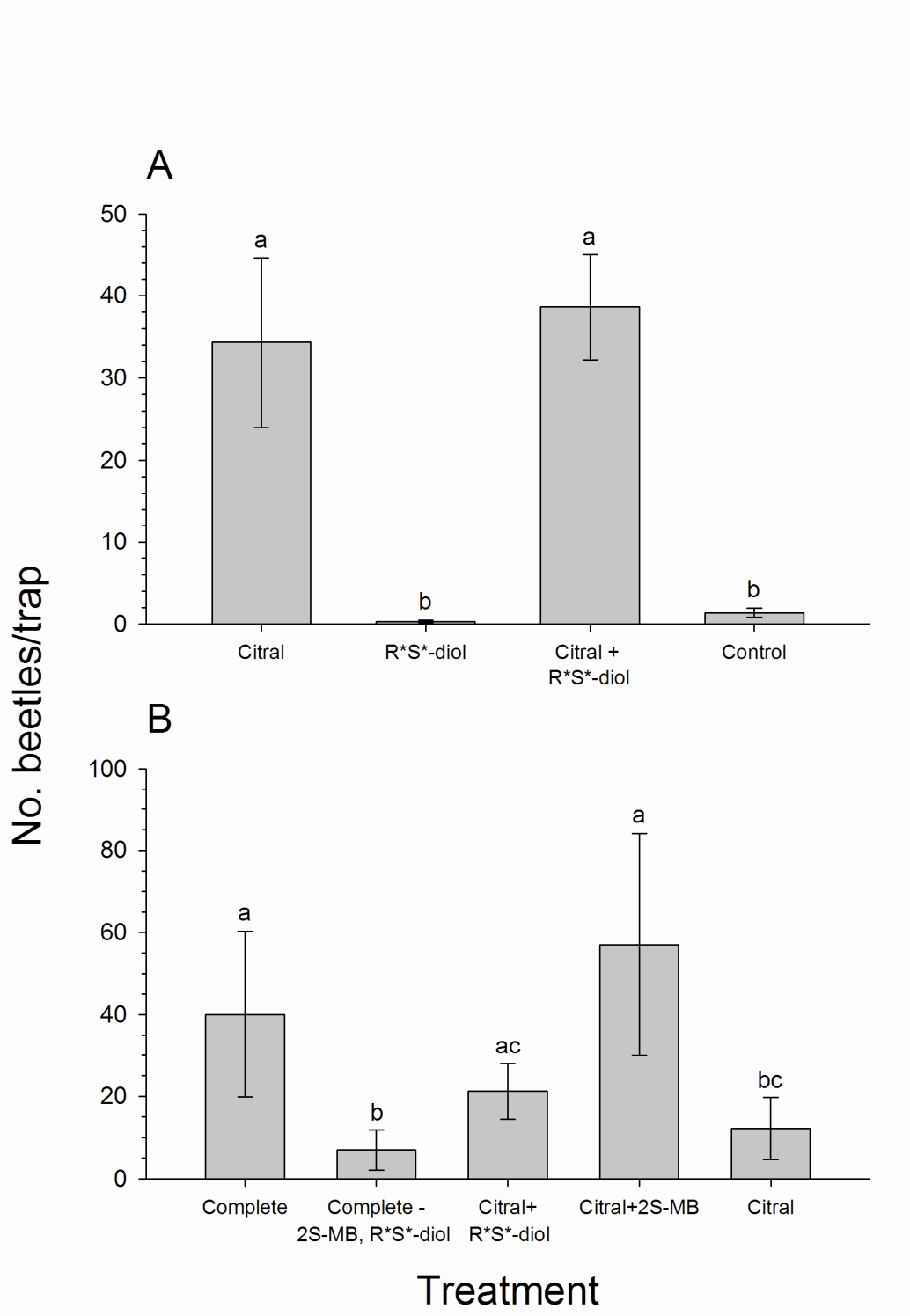


Fig. 4.3. Mean (±SE) number of adult *Megacyllene caryae* (sexes combined) that were captured by traps baited with synthetic components of the male-produced pheromone during Experiments I (A) and III (B). Treatment abbreviations: "Complete" = blend of (*R**,*S**)-2,3-hexanediol, (*S*)-(–)-limonene, 2-phenylethanol, nerol, (–)-α-terpineol, (*S*)-2-methylbutan-1-ol; "2S-MB" = (*S*)-2-methylbutan-1-ol; R*S*-diol = (*R**,*S**)-2,3-hexanediol. Means with different letters within experiments are significantly different (Tukey-Kramer adjustment, $P < 0.05$).



CHAPTER 5: MULTIMODAL MIMICRY IN THE CERAMBYCID GENUS *MEGACYLLENE*

Abstract

Many species of cerambycid beetles in the New World genus *Megacyllene* appear to be Batesian mimics of vespid wasps, such as yellowjackets (*Vespula* or *Dolichovespula*) and paper wasps (*Polistes*), resembling them in body size, color pattern, and their manner of walking and flying. Adult beetles of two *Megacyllene* species that are common in eastern North America, *M. caryae* (Gahan), the painted hickory borer, and *M. robiniae* (Forster), the locust borer, produce a conspicuous odor when handled or otherwise disturbed. Headspace sampling of volatiles from agitated individuals of both species revealed a complex blend of spiracetals, the same class of compounds that serve as alarm pheromones of many species of vespid wasps. Female *M. caryae* produce at least fifteen structurally distinct spiroacetals, only five of which are produced by males. Female *M. robiniae* produce six different spiroacetals, five of which are also produced by female *M. caryae*. Spiroacetals of similar or identical structure were found to be released by the co-occurring vespids *Vespula maculifrons* (du Buysson), *Polistes fuscatus* (F.), and *Eumenes fraternus* Say. I propose that spiroacetals produced by the beetle species represent an added dimension of chemical mimicry to complement their physical resemblance to their models. Males also produced six different compounds, including β-farnesene, that were not produced by females and could be as-yet unidentified pheromone components. Both sexes of *M. caryae* also produced trace quantities of 1-(methylthio)-2-methylbut-2-ene, which may be defensive in function. Adult *M. caryae* are now recognized as producing a total of 31 distinct isomers of

volatile chemicals, among the most complex suites of semiochemicals associated with any nonsocial insect species.

Introduction

Many species of diurnal beetles in the family Cerambycidae appear to be Batesian mimics of a wide variety of other insect species, including lycid and tenebrionid beetles (Raske et al. 1967, Slobodchikoff 1987, Eisner et al. 2008), ants (Berlocher et al. 1992), and wasps (Linsley 1959). Mimetic species can closely resemble their models in coloration, morphology, and even behavior (Raske 1967, Eisner et al. 2008, Linsley 1959). Among these presumed mimics are cerambycids in the genus *Megacyllene* (Cerambycinae, Clytini), a widespread New World group of 57 species (Monné and Bezark 2011) that exhibit striking black and yellow colorations (Lingafelter 2007). The two species *Megacyllene caryae* (Gahan), the painted hickory borer, and *M. robiniae* (Forster), the locust borer, are common in eastern North America (Lingafelter 2007) and appear to be mimics of sympatric species of aculeate Hymenoptera, such as *Vespula* and *Polistes* (Dusham 1921). In fact, *M. caryae* and *M. robiniae* are so similar in appearance that they originally were thought to represent two different broods of the same species (Dusham 1921). Adults of the two beetle species are active at opposite ends of the season, *Megacyllene caryae* in early spring, and adult *M. robiniae* in early autumn, consistent with the temporal disjunction of other types of mimetic insects that share similar models (Waldbauer 1988). The adult beetles feed on pollen and/or nectar, with *M. caryae* visiting flowers of *Crateagus* and *Quercus* species (Dusham 1921, L. M. Hanks, unpub. data) and *M. robiniae* feeding primarily on flowers of *Solidago* species (Wheeler et al., 1988). Larval hosts of

M. caryae include recently dead species of *Carya*, while larvae of *M. robiniae* develop in living *Robinia* (Lingafelter 2007).

Adult male *M. caryae* produce a volatile pheromone that is a blend of alkanediols, terpenoids, and an aromatic alcohol, and both sexes are attracted to synthetic reconstructions of the blend (Lacey et al. 2008). In fact, traps baited with only citral (a 1:1 mixture of neral and geranial) are highly attractive to adults (see Chapter 4). Adult male *M. robiniae* produce similar volatiles, including (*R*)-3-hydroxyhexan-2-one, (*R,S*)-2,3-hexanediol, and (*R*)-1-phenylethanol, but synthetic blends do not appear to be attractive to either sex in field bioassays (L. M. Hanks, pers. comm.). When handled, adult male *M. robiniae* produce secretions from metasternal glands that contain 1-phenylethanol, hexadecyl acetate, octadecyl acetate, and a unique compound, 2-(1,3-hexadien-1-yl)-5-methyltetrahydrofuran, that to date has been reported only from this species (Wheeler et al. 1988). The latter secretions were thought to serve a defensive function, and all but 1-phenylethanol also are produced by females (Wheeler et al. 1988).

When handling adult *M. caryae* that had been collected from the field, I noticed that both sexes produced a pungent, musty odor. A preliminary analysis of headspace volatiles indicated bicyclic spiroacetals, which bear some structural similarity to the monocyclic tetrahydrofuran reported by Wheeler et al. (1988). Spiroacetals are known to be produced by many species in the orders Hymenoptera, Diptera, Coleoptera, and Hemiptera (Francke et al. 2001), as well as several species of plants (Beck et al. 2008, Heiduk et al. 2010, Zhang et al. 2002). They apparently serve as defensive allomones in staphylinid beetles (Huth and Dettner 1990, Zhang et al. 1999), as sex pheromones in tephritid fruit flies (Booth et al. 2009), and as behavioral inhibitors in scolytine bark beetles (Zhang et al. 2002). Spiroacetals also are common constituents of venom gland secretions of social Hymenoptera, especially the Vespidae, and act

as alarm pheromones that induce aggressive and defensive behaviors (Bruschini et al. 2006, Dani et al. 2000, Fortunato et al. 2004, Francke and Kitching 2001). Spiroacetals alone may be sufficient to elicit defensive behavior in vespids (Dani et al. 2000) and may be used by other insects as cues to the presence of Hymenoptera (Heiduk et al. 2010).

This defensive function of spiroacetals among the stinging Hymenoptera suggests that their production by *M. caryae* adults adds an olfactory signal that complements their morphological and behavior mimicry of wasps. Here, I provide evidence supporting this hypothesis by characterizing the blend of volatiles produced by adult *M. caryae* and *M. robiniae* when they are agitated, and demonstrating that beetle-produced spiroactals are structurally similar to those produced by sympatric vespid wasps.

Methods

Source of insects

Adult *M. caryae* were captured in the field during May 2011 during field bioassays for another experiment that was conducted at Forest Glen Preserve, IL (Vermilion Co.; 730 ha; 40° 0'51.97"N, 87° 34'0.74"W; see Chapter 4). I collected beetles with black cross-vane flight intercept traps (Panel Trap model; AlphaScents, Portland, OR) that were baited with lures containing 50 mg citral in 1 ml isopropanol (for further details, see Chapters 2 and 3). Adult *M. robiniae* were collected by hand during September 2011 from inflorescences of *Solidago* species on roadsides near Mazonia State Fish and Wildlife Area (Grundy County, IL; 41° 10'47.57"N, 88° 14'35.28"W; 1,084 hectare), a 1,084 hectare reclaimed strip mine with several stands of *Robinia pseudoacacia* L. I also collected by hand three native species of vespid wasps that are common in central Illinois and may have served as Batesian models for *Megacyllene* species: the

eastern yellowjacket *Vespula maculifrons* (du Buysson), the paper wasp *Polistes fuscatus* (F.), and the potter wasp *Eumenes fraternus* Say (Buck et al., 2008). The latter two wasp species were collected in September 2011 as they foraged on inflorescences of *Solidago* species and other flowering forbs at Forest Glen. Specimens of *V. maculifrons* were collected by hand from garbage bins near campsites at the Preserve.

Conspecific beetles were housed together in the laboratory in 0.03 m^3 screen cages while vespid wasps were housed individually in cylinders of aluminum window screen (~10 cm tall, 9 cm diameter). Insects were held under ambient laboratory conditions (~12:12h L:D, ~20 °C) and provided 10% aqueous sucrose solution as food. Adult *M. robiniae* also were provided with freshly cut inflorescences of *Solidago* species.

Collection and identification of volatiles

In the laboratory, adult males and females of both *Megacyllene* species readily produced the pungent volatiles when handled. Volatiles were collected by placing individual beetles in a 500 ml glass bottle with a hose barb at the base, and the top covered with aluminum foil. The hose barb was connected with Teflon® tubing to a collector, a 10 cm glass tube containing ~150 mg of HayeSepQ (Sigma-Aldrich, St. Louis, MO) between plugs of silanized glass wool. The other end of the collector was connected to a vacuum source and air was drawn through the apparatus at 1 l/min for 2 min. The collector then was extracted into 1.5 ml of dichloromethane into a silanized glass vial.

Samples were concentrated to ~50 ul by leaving uncapped vials in a fume hood for ~3 hr at room temperature. Samples of 1 ul were injected into a Hewlett-Packard 6890 gas chromatograph (Hewlett Packard, Palo Alto, CA) with an AllTech AT-5ms column (30 m, 0.25

mm i.d., 0.25 µm film; Alltech Associates, Inc., Deerfield, IL) coupled to a 5973 mass selective detector (Hewlett-Packard). Injector temperature was 250 °C, and oven temperature was held at 40 °C for 1 min, increased at 10 °C/min to 160 °C, and held 1 min. Chemicals were identified by comparing spectra with those of standards in the software library (National Institute of Standards and Technology, Gaithersburg, MD) and with spectra of insect-produced spiroacetals reported by Francke and Kitching (2001). The small amount of chemical produced by individual beetles necessitated aerating beetles in groups of ten or more.

Solid phase microextraction (SPME) proved to be a more sensitive and efficient method for analyzing volatiles produced by individual insects. Beetles emitted the characteristic smell when handled and were immediately placed in 50 ml glass flasks that were covered with aluminum foil. I inserted a 1 cm SPME fiber coated with 100 µm polydimethylsiloxane (Supelco, Bellefonte, PA) through the foil and exposed it to headspace volatiles for 20 min. The fiber was then immediately desorbed in the GC injection port (settings as described above). SPME was used to analyze volatiles produced by ten individuals of each sex of *M. caryae*, and five female *M. robiniae*.

SPME fibers proved ineffective for collecting volatile chemicals from live vespids, even when they had been deliberately agitated. Instead, I extracted spiroacetals directly from insects by placing the entire insect in solvent (from Francke et al. 1979). One each of live female *V. maculifrons*, *P. fuscatus*, and *E. fraternus* was placed individually into a 4 ml silanized glass vial (Supelco), and 2 ml of pentane was added, which immediately killed the insect. The pentane was swirled vigorously for ~1 min, and insects were then removed from the vials and pinned as voucher specimens. Samples were concentrated to ~200 ul and analyzed as already described,

but with a final oven temperature of 300 °C held at 5 min to assure that cuticular waxes eluted from the column.

In samples that contained more than a single compound, peak areas were calculated as percentages relative to the dominant compound. Percentages of each compound were averaged across all samples within a species and gender.

Results

Headspace samples of *M. caryae* contained 28 different compounds, ten of which were specific to females, 12 specific to males, and six that were detected from both sexes (Table 5.1, Figs. 5.1, 5.2). Fifteen of the compounds from females, and five from males, presented mass spectra that were characteristic of spiroacetals of at least six motifs (Fig. 5.3; Francke and Kitching 2001), including dominant ion doublets of 84/87 (motif "A"), 98/101 ("B," "C," and "D"), or 112/115 ("E" and "F"). Mass spectra were identical or nearly identical within the motifs, and likely indicate multiple stereoisomers of the same chemical. To facilitate interpretation of the data, components have been labeled by the ion doublet motif followed by a number that referenced the order of elution (Table 5.1).

Headspace samples of female *M. caryae* contained spiroacetals representative of all six motifs, A through F. The A motif spectra were similar to that of 7-methyl-1,6-dioxaspiro[4.5]decane (Fig. 5.3A) and are probably members of the 1,6-dioxaspiro[4.5]decane family (Francke and Kitching 2001). The C motif (Fig. 5.3C) matched the spectrum of 2-methyl-1,6-dioxaspiro[4.5]decane (Francke and Kitching 2001). The D motif was very similar to A, but with an additional ion at 115, and could not be identified from published mass spectra (Fig. 5.3D). The E motif matched the spectrum of 2,8-dimethyl-1,7-dioxaspiro[5.5]undecane,

and the F motif matched the spectrum of 2-ethyl-7-methyl-1,6-dioxaspiro[4.5]decane (Francke and Kitching 2001; Figs. 5.3E,F).

The dominant compound in headspace samples of female *M. caryae* was identified by mass spectrum as 7-ethyl-2-methyl-1,6-dioxaspiro[4.5]decane (compound B1; Table 5.1, Fig. 5.4B) by W. Francke (pers. comm.). The next most abundant compound was B2 (~9% of B1), but all remaining compounds of the B motif were present in small quantities, usually <2% of B1 (Table 5.1), and as a result most yielded incomplete spectra. Nevertheless, these compounds probably represent some of the eight potential stereoisomers of 7-ethyl-2-methyl-1,6-dioxaspiro[4.5]decane. Headspace samples of male *M. caryae* contained only five distinct spiroacetals, all present in females (Table 5.1), with B1 again the dominant peak (Table 5.1, Figs. 5.2, 5.3).

Headspace samples of female *M. robiniae* contained five spiroacetals that matched retention time and mass spectra of those identified from female *M. caryae*, but in very different proportions (Table 5.1), E1 being dominant, and F2, B2, D1, C3 all in much higher relative quantities than were the minor peaks of female *M. caryae* (Table 5.1, Fig. 5.4A). A sixth spiroacetal, F1, was identified only from female *M. robiniae*. Peaks for spiroacetals in SPME samples of *M. robiniae* were consistently much closer to the baseline relative to *M. caryae*, suggesting that *M. robiniae* may release smaller quantities of spiroacetals.

Solvent extracts of the wasp *V. maculifrons* contained three distinct spiroacetals that were within the motifs of those produced by *M. caryae* females (Fig. 5.4B, Table 5.1). The dominant A2 was identical to the spectrum of 7-methyl-1,6-dioxaspiro[4.5]decane (Francke and Kitching 2001; Fig 5.5A), but differed in retention time from the A-motif compounds of *M. caryae*. The remaining two compounds were of the C motif, one matching the retention time of C3 produced

by female *M. caryae* (Fig. 5.5B). The wasps *P. fuscatus* and *E. fraternus* both produced a single spiroacetal that matched the retention time and mass spectrum of compound E1 (Figs. 5.4C, D and 4.5C, D).

Headspace samples of both sexes of *M. caryae* also contained a compound whose spectrum matched that of 1-(methylthio)-2-methylbut-2-ene. In addition, male *M. caryae* produced six compounds previously reported as pheromone components (Lacey et al. 2008; see Chapter 4), including (*S*)-(–)-limonene, 2-phenylethanol, (–)-α-terpineol, neral, geranial, and (*S*)-2-methylbutan-1-ol. During the present study, however, headspace samples of males consistently contained another six compounds (Fig. 5.6), five that were unidentified but suggested a terpenoid structure, and one that matched the spectrum of β-farnesene.

Discussion

The fact that adult *M. caryae* and *M. robiniae* produce spiroacetals when agitated suggests that they serve a defensive or alarm function, consistent with their role in other insects (Bruschini et al. 2006, Dani et al. 2000, Fortunato et al. 2004). The research summarized here supports the hypothesis that spiroacetals produced by the two *Megacyllene* species serve to enhance their mimicry of the vespid wasps with which they co-occur. Spiroacetal motifs produced by *M. caryae* are at least analogous in structure with those produced by all three vespid species. The complex blend of spiroacetals produced by the beetle may have evolved to include components that match spiroactals of other species of vespids that overlap in geographic distribution with *M. caryae* (Linsley 1964), and in fact spiroactals of motifs A, B, C, D, and F all are produced by Palearctic species of vespids (Francke and Kitching 2001, Bruschini et al. 2006). Alternatively, the presence of multiple motifs of spiroacetals in volatiles emitted by a

single beetle species could result from non-selective biosynthesis, which itself could be adaptive if complex blends confer a more general level of defense than would single compounds.

The hypothesis that spiracetals serve to enhance mimicry of *Megacyllene* species of its vespid models is further supported by the production of compounds matching motif E by the cerambycids *Molorchus minor* (L.) (Meyer 1993) and a *Callisphyris* species (J. Bergmann, pers. comm.), both of which are also believed to be mimics of wasps (Linsley 1959). Spiroacetals in general are used by many insects that appear to exploit aculeate Hymenoptera as Batesian models, including the staphylinid *Ontholestes murinus* (L.), tephritid flies of the genus *Bactrocera*, and several species of ichneumonid wasps (Francke and Kitching 2001).

Spiroacetals of *M. caryae* and *M. robiniae* may also serve as allomones that deter predation or parasitism, and in fact compounds of the A and E motifs are toxic to some types of insects (Dettner et al. 1992, Franke and Kitching 2001). Additionally, the spiroacetal conophthorin (motif A) is produced by some species of trees and inhibits aggregation in many species of scolytine bark beetles (Zhang et al. 2002 and Francke and Kitching 2001) and so could be used by *M. caryae* to avert competition with other wood-boring insects. The compounds also may be used by *Megacyllene* as alarm pheromones, or serve some other intraspecific function. For instance, the fact that spiroacetal B2 was present at relatively high levels in the blend produced by female *M. caryae*, but was absent in blends produced by males, suggests it carries sex-specific information. Alternatively, the more diverse blend produced by females may suggest a sexually dimorphic mimicry similar to that observed in the swallowtail butterflies (e.g., Kunte 2009).

Of course, the evidence I present for chemical mimicry is circumstantial, and it would be difficult to demonstrate its adaptive advantage. First, confirmation of chemical structures of

spiroacetals would require multiple isomeric standards that would be challenging to synthesize due to multiple chiral centers. Second, the appropriate bioassays would require gauging the response of potential predators to the chemical standards, which would in turn necessitate preliminary research to identify the key predators. To complicate matters, the key predators of any particular geographic region may not be the species that originally imposed the selective forces that drove evolution of mimicry. In fact, the complex of spiroacetals emitted by *M. caryae* and *M. robiniae* could include historical artifacts of extinct models.

In conclusion, my findings show that adult *M. caryae* and *M. robiniae* produce many more different types of volatile compounds than previously recognized. In addition to the 15 distinct spiroacetals produced by female and male *M. caryae*, males also are capable of producing several potential terpenoid components not previously described. Finally, the thioalkene 1-(methylthio)-2-methylbut-2-ene was produced by both sexes, and so probably serves a defensive function. Because the male-produced pheromone of *M. caryae* comprises a variety of terpenoids (Lacey et al. 2008), it seems likely that β-farnesene and the other unidentified terpenoids reported here also are minor pheromone components. In any case, the suite of volatile semiochemicals produced by this species now totals 28 distinct compounds, among the most complex reported for any nonsocial insect species to date (Keeling et al. 2003).

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Table 5.1. Spiroacetals produced by the cerambycid beetles *Megacyllene caryae* and *M. robiniae*, the yellowjacket *Vespula maculifrons*, the paper wasp *Polistes fuscatus*, and the potter wasp *Eumenes fraternus*. Compounds are labeled by a letter that indicates a motif based on mass spectrum (see text) and a number based on the retention time and order of elution on an AT-5ms column (1 min at 40 °C, 10 °C/min, 1 min at 60 °C). Motifs are tentatively identified based on known mass spectra of spiroacetals (Francke and Kitching 2001). Data are expressed as peak area relative to the dominant peak in the trace, and the ratio in parentheses indicates the number of headspace samples that contained the compound out of the total.

Motif	**Species**					
	M. caryae ♀	*M. caryae* ♂	*M. robiniae* ♀	*V. maculifrons* ♀	*P. fuscatus* ♀	*E. fraternus* ♀
"A": 7-methyl-1,6-dioxaspiro[4.5]decane (conophthorin)						
A1	0.03±0.02 (3/10)					
A2				100* (1/1)		
A3	0.62±0.61(2/10)					
"B": 7-ethyl-2-methyl-1,6-dioxaspiro[4.5]decane						
B1	100* (10/10)	100* (10/10)				
B2	9.19±2.4 (10/10)		12.4±6.4 (8/10)			
B3	0.15±0.06 (5/10)					
B4	1.51±0.32 (9/10)	N/A[1] (6/10)				
B5	1.10±0.44 (8/10)	3.49±2.3 (5/10)				
B6	0.06±0.04 (2/10)					
B7	0.02±0.02 (1/10)					
"C": 2-methyl-1,6-dioxaspiro[4.5]decane						

Table 5.1, continued

C1	0.24±0.11 (5/10)					
C2				23.7 (1/1)		
C3	0.10±0.05 (4/10)		6.82±4.3 (2/5)	16.8 (1/1)		
"D": Unknown spiroacetal						
D1	1.73±0.37 (9/10)	7.81±4.39 (7/10)	12.0±7.6 (2/5)			
"E": 2,8-dimethyl-1,7-dioxaspiro[5.5]undecane						
E1	0.76±0.2 (10/10)	0.89±0.89 (1/10)	100* (5/5)		100* (1/1)	100* (1/1)
"F": 2-ethyl-7-methyl-1,6-dioxaspiro[4.5]decane						
F1			69.9±9.1 (5/5)			
F2	0.20±0.08 (5/10)		31.8±14.6 (4/5)			
F3	<0.01 (1/10)					

*Dominant peak that was used as reference
[1]Peak area obscured by geranial

Fig. 5.1. Representative total ion chromatogram of headspace volatiles of an agitated adult female *Megacyllene caryae*. Labeled peaks are spiroacetals named by a letter that signifies a unique mass spectrum and a number designating the order of elution. Not pictured: spiroacetals B6 (retention time: 11.95), B7 (12.75), and F3 (10.25), and 1-(methylthio)-2-methylbut-2-ene (5.34). AT-5ms column, 40 °C/1 min, increasing at 10 °C/min, end at 160 °C/1 min.

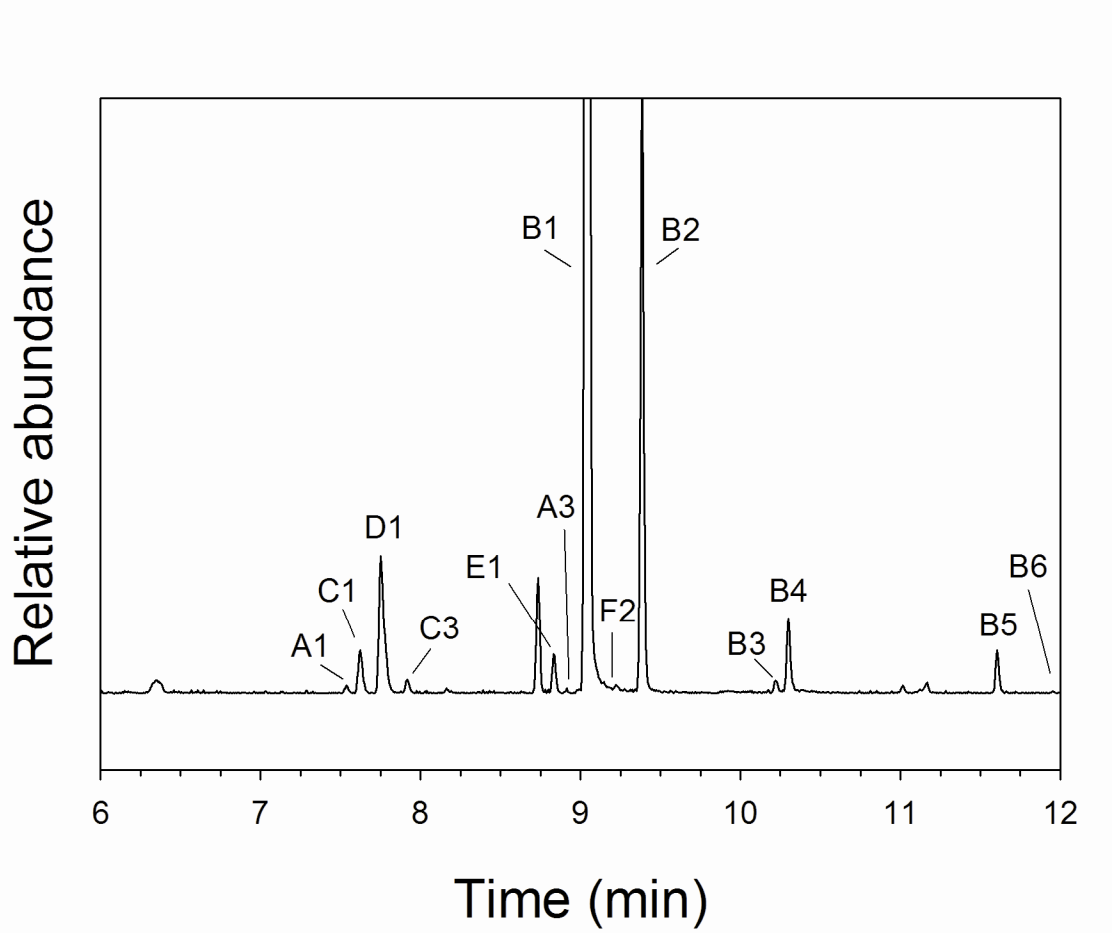


Fig. 5.2. Representative total ion chromatogram of headspace volatiles of an agitated adult male *Megacyllene caryae*. Labeled peaks are spiroacetals named by a letter that signifies a unique mass spectrum and a number designating the order of elution. Peak B4 is present, but obscured by geranial at 10.3. Unknown chemicals in the headspace are designated T1-T5. Peaks marked with asterisks are (left to right): 1-(methylthio)-2-methylbut-2-ene, (*S*)-(–)-limonene, (–)-α-terpineol, neral, geranial, and β-farnesene. AT-5ms column, 40 °C/1 min, increasing at 10 °C/min, end at 160 °C/1 min.

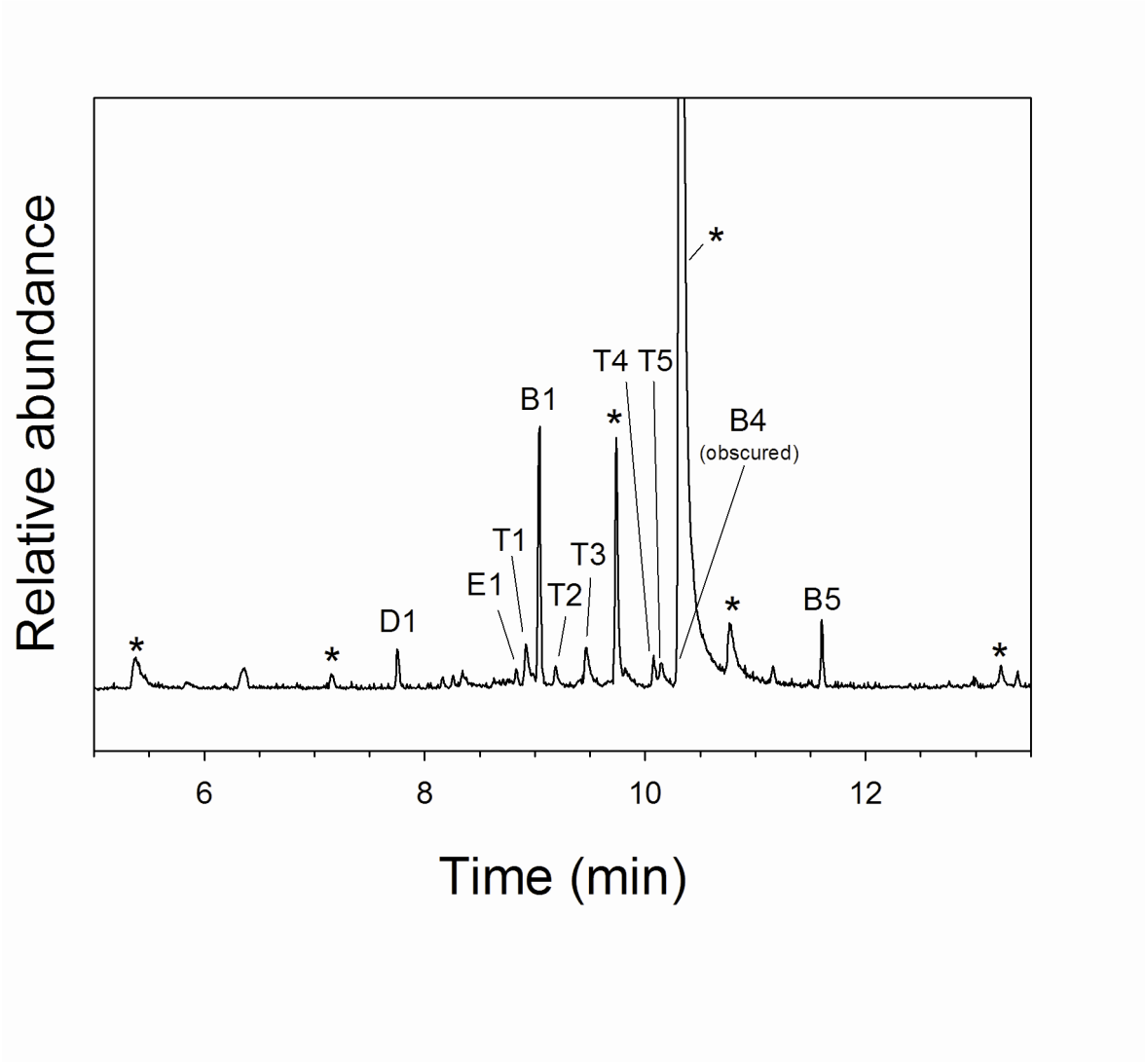


Fig. 5.3. Mass spectra of spiroacetal motifs recovered from headspace volatiles of agitated adult *Megacyllene caryae* and *M. robiniae*. Figure subheadings (**A**, **B**, **C**, etc.) indicate structural motif (Table 5.1).

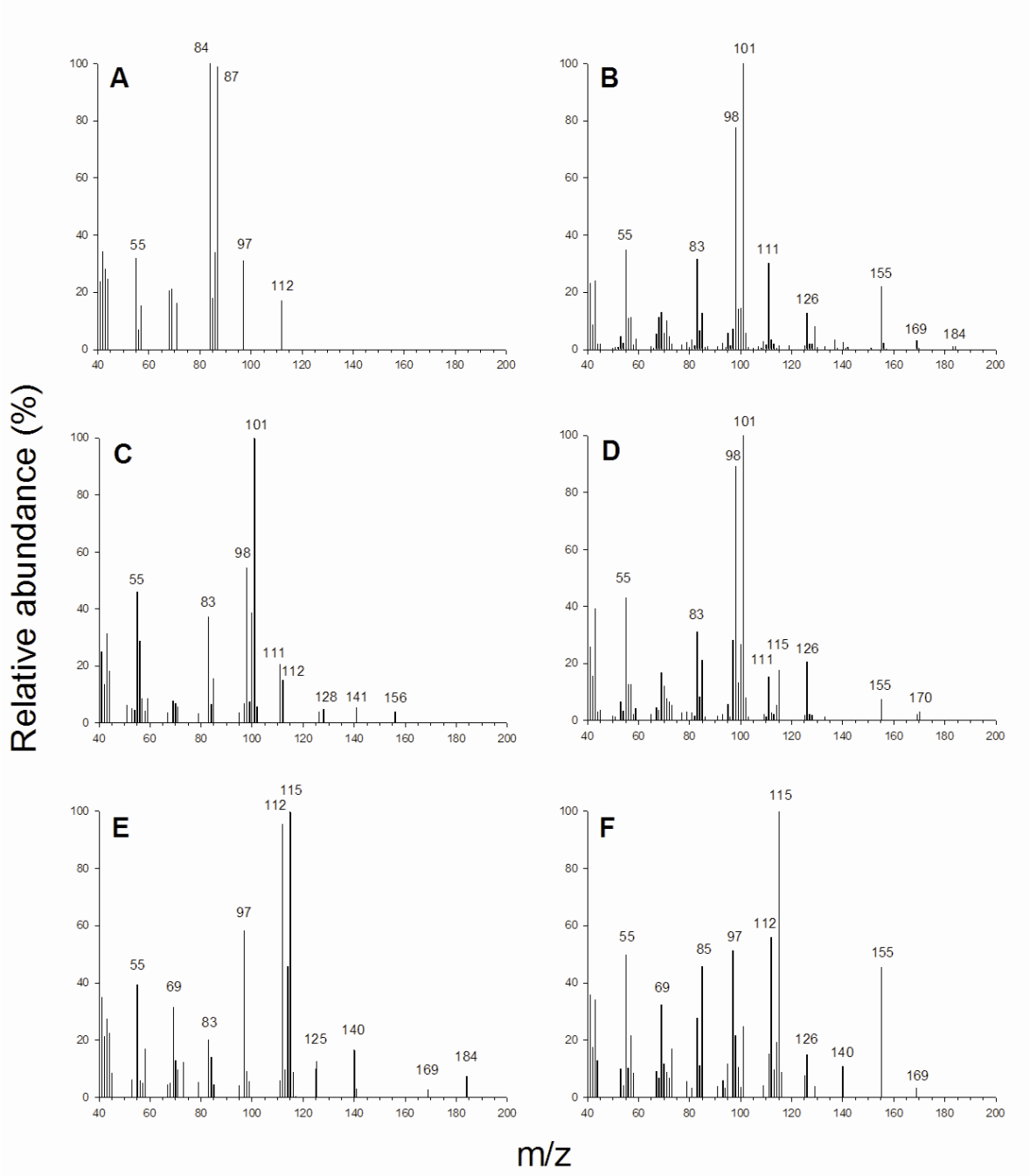


Fig. 5.4. Representative total ion chromatogram of pentane extracts of live adult female **A**) *Megacyllene robiniae*; **B**) *Vespula maculifrons*; **C**) *Polistes fuscatus*; and **D**) *Eumenes fraternus*. Chromatograms show only the period between 6-12 min to improve visibility of peaks. Labeled peaks are spiroacetals named by a letter that signifies a unique mass spectrum and a number designating the order of elution. The column was changed between aerations of A/B and C/D, changing apparent retention times of compound E1. AT-5ms column, 40 °C/1 min, increasing at 10 °C/min, end at 160 °C/1 min for **A** and 300 °C/5 min for **B-D**.

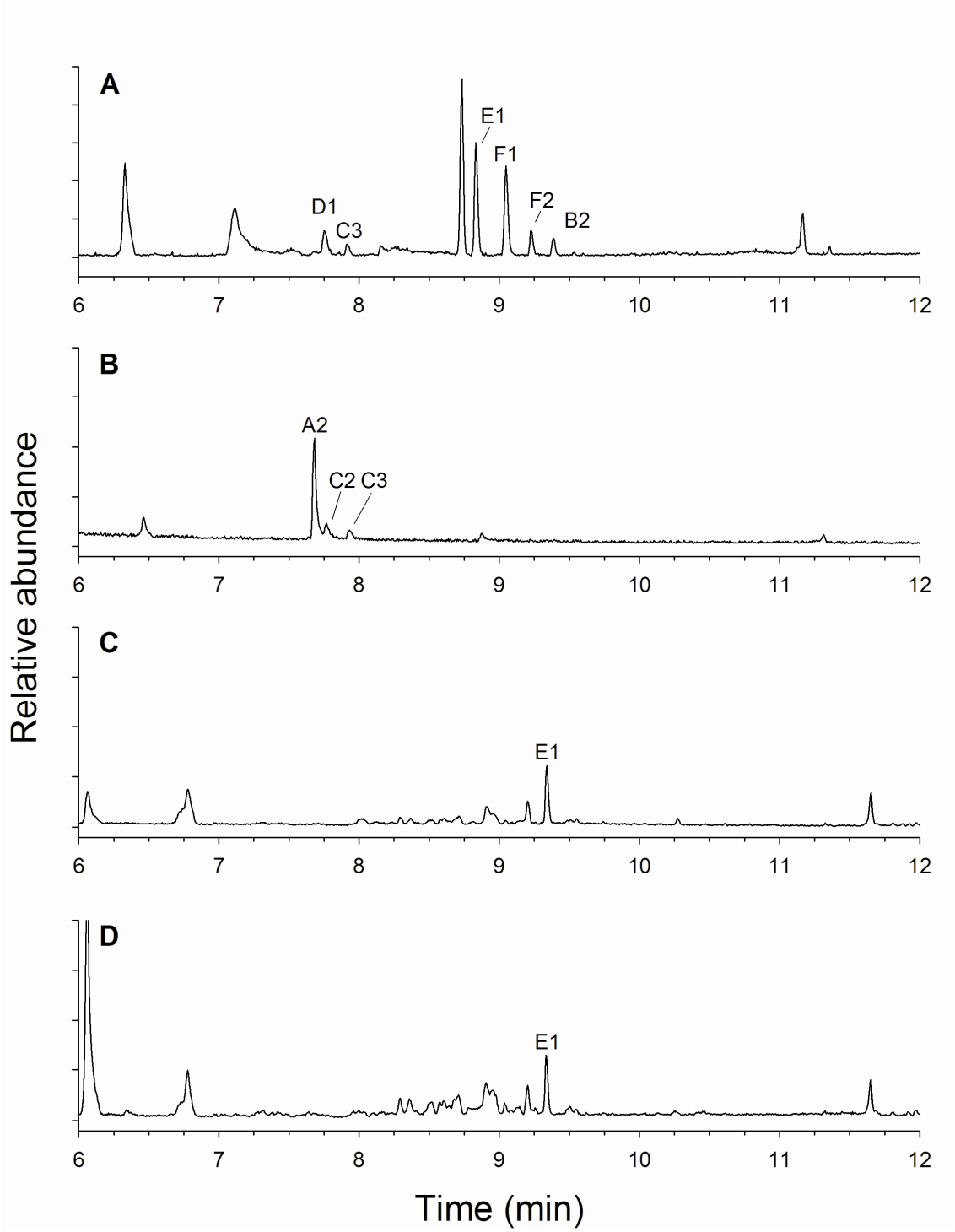


Fig. 5.5. Mass spectra of spiroacetal motifs recovered from pentane extracts of live adult female **A**) *Vespula maculifrons* (motif A; Table 5.1); **B**) *Vespula maculifrons* (motif B); **C**) *Polistes fuscatus* (motif E); and **D**) *Eumenes fraternus* (motif E).

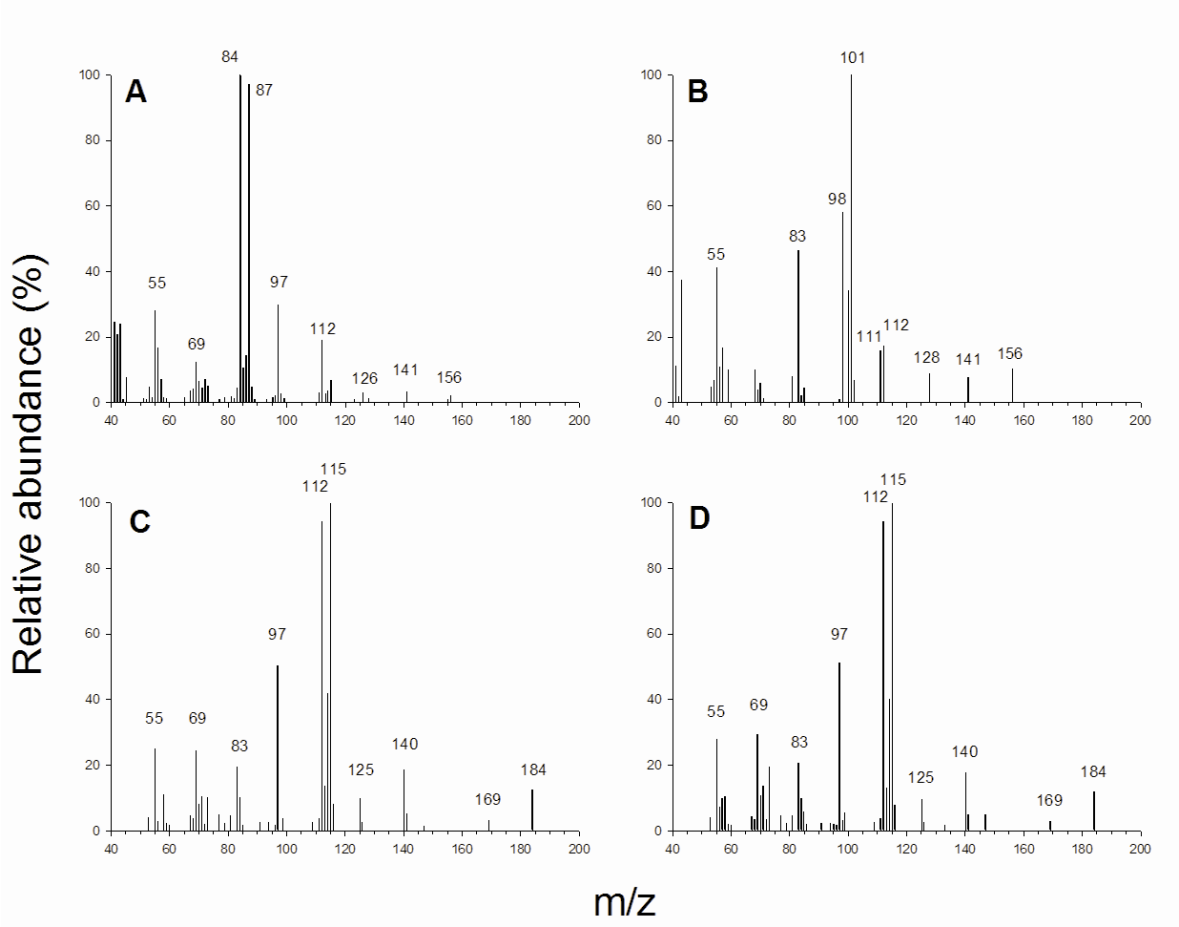
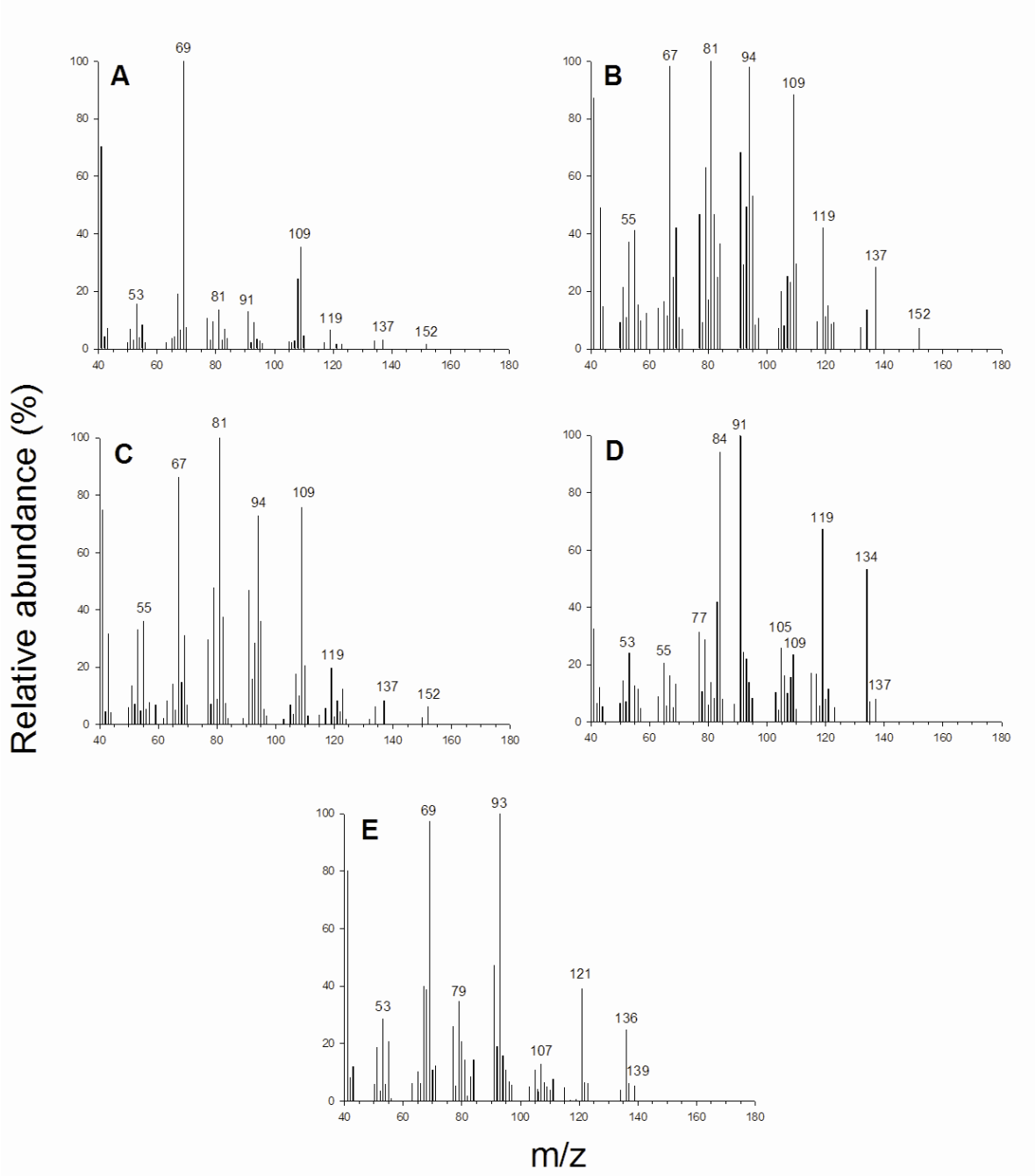


Fig. 5.6. Mass spectra of unidentified compounds recovered from headspace volatiles of agitated adult male *Megacyllene caryae*. **A**) compound T1; **B**) T2; **C**) T3; **D**) T4; **E**) T5.



CHAPTER 6: SEQUENCING AND CHARACTERIZING ODORANT RECEPTORS OF THE CERAMBYCID BEETLE *MEGACYLLENE CARYAE*[2]

Abstract

Odorant receptors (Ors) are a unique family of ligand-gated ion channels and the primary mechanism by which insects detect volatile chemicals. Here, I describe 57 putative Ors sequenced from an antennal transcriptome of the cerambycid beetle *Megacyllene caryae* (Gahan). The male beetles produce a pheromone blend of nine components, and I functionally characterized Ors tuned to three of these chemicals: receptor McOr3 is sensitive to (*S*)-2-methyl-1-butanol; McOr20 is sensitive to (2*S*,3*R*)-2,3-hexanediol; and McOr5 is sensitive to 2-phenylethanol. McOr3 and McOr20 are also sensitive to structurally-related chemicals that are pheromones of other cerambycid beetles, suggesting that orthologous receptors may be present across many cerambycid species. These Ors are the first to be functionally characterized from any species of beetle and lay the groundwork for understanding the evolution of pheromones within the Cerambycidae.

Introduction

Chemoreception by insects is mediated by a diverse array of receptor proteins that includes the gustatory, odorant, and ionotropic receptors (Benton et al. 2009, Hallem et al. 2006, Rützler and Zwiebel 2005). Among these groups, the family of odorant receptors (Ors) appears to be the primary mechanism by which insects detect volatile chemicals (Hallem and Carlson

[2] This chapter is in press and will appear in its entirety in the journal *Insect Biochemistry and Molecular Biology*: Mitchell, R. F., D. T. Hughes, C. W. Luetje, J. G. Millar, F. Soriano-Agatór, L. M. Hanks, and H. M. Robertson. 2012. Sequencing and characterizing odorant receptors of the cerambycid beetle *Megacyllene caryae*. Insect Biochem. Mol. Biol. This article is reprinted with the permission of the publisher. DTH and CWL aided in functionally characterizing receptors and designed Figures 6.2 and 6.3, JGM and FS synthesized chemical standards and authored sections on synthesis, LMH and HMR aided in experimental design and editing.

2006, Wang et al. 2010). This family is an expansion of seven-transmembrane domain proteins, unique to insects, which act as ligand-gated ion channels when bound to the conserved co-receptor *Orco* (Sato et al. 2008, Vosshall and Hansson 2011, Wicher et al. 2008).

Ors have been described from several insect species to date, but because of their rapid evolution, annotation has remained almost exclusive to genome projects (e.g., Clyne et al. 1999, Engsontia et al. 2008, Robertson et al. 2010, Vosshall et al. 1999). As such, understanding of Ors has been limited to the orders Diptera (*Drosophila* spp., species in the Culicidae), Lepidoptera (*Bombyx mori* [L.], *Helicoverpa zea* [Boddie]), and Hymenoptera (*Apis mellifera* L., *Nasonia* spp.; see Robertson et al. 2010 for a brief review). Moreover, the function of Ors remains poorly known for all but the model species *Drosophila melanogaster* Meigen and *Anopheles gambiae* Giles (Carey et al. 2010, Hallem and Carlson 2006, Wang et al. 2010). Beetles (Coleoptera), comprise a quarter of known insect species, and hundreds of important pest species, but are represented by only a single set of olfactory receptors described from the genome of *Tribolium castaneum* (Herbst) (Engsontia et al. 2008). *Tribolium castaneum* presented a surprisingly diverse array of 341 Ors, of which 111 were detected as transcripts in adult head tissues (Engsontia et al. 2008). However, only the conserved *Orco* receptor (TcOr1) has been functionally characterized, and no ligands are yet known for any of the TcOrs.

The research summarized in this article advances our understanding of insect olfactory receptors by describing Ors of a second beetle species, and more importantly, by initiating the first functional analysis of coleopteran chemoreceptors. Recent advances in sequencing have resulted in high-throughput techniques that permit extensive genetic surveys of non-model organisms (Toth et al. 2007, Vera et al. 2008), and transcripts of highly expressed receptors may be captured by sequencing RNA from antennal tissues (Ramsdell et al. 2010, Grosse-Wilde et al.

2011). This process is well suited to the extraction of receptors that detect chemicals critical to the life history of the insect, such as host odors or pheromones. Ors for pheromones should be highly sensitive to a small set of chemicals that already have been identified, and thus may offer convenient targets for sequencing and characterization.

The study species was the cerambycid beetle *Megacyllene caryae* (Gahan). The Cerambycidae constitute one of the largest insect families, with more than 35,000 described species (Grimaldi and Engel 2005), and include some of the most destructive pests of forests worldwide (Haack et al., 2010). Larvae of most species feed in the woody tissues of plants, and adults of many species depend on sex pheromones to locate and recognize mates (e.g., Millar et al. 2009, Ray et al. 2011, Rodstein et al. 2009, Silk et al. 2007). Pheromone structures frequently are shared among congeners, and even among more distantly related species (i.e., tribes, or even subfamilies). Known pheromone structures include 2,3-alkanediols and the related 3-hydroxy-2-alkanones (Hanks et al. 2007, Lacey et al. 2009), (*E*)-6,10-dimethyl-5,9-undecadien-2-ol and the corresponding acetate (Silk et al. 2007, Chapter 2), and 3,5-dimethyldodecanoic acid (Barbour et al. 2011). Male *M. caryae* produce an unusually complex blend for a species in its subfamily (Cerambycinae; see Millar et al. 2009), comprising several compounds that are better known as floral and wood volatiles (see Section 2.1), but also two compounds that are common pheromone components of cerambycids: 2,3-hexanediol and 2-methyl-1-butanol (Lacey et al. 2008, Chapter 4). This diversity of components improved chances of identifying pheromone receptors, and the commonplace nature of the ligands meant that receptor sequences might yield functionally similar orthologs in other cerambycid species. Here, I describe 57 likely odorant receptors identified from antennal tissue of *M. caryae*, and the functional characterization of three of those

odorant receptors by demonstrating their highly selective responses to several components of the insect's pheromone blend.

Materials and Methods

Sources of chemical standards and insects

The pheromone of male *M. caryae* consists of (2*S*,3*R*)- and (2*R*,3*S*)-2,3-hexanediol, (*S*)-(–) -limonene, 2-phenylethanol, (–)-α-terpineol, nerol, neral, geranial, and (*S*)-2-methylbutan-1-ol (Lacey et al., 2008; Chapter 4). Authentic standards of (*S*)-(–) and (*R*)-(+)-limonene, 2-phenylethanol, (–)-α-terpineol, and nerol were obtained from Sigma-Aldrich (St. Louis, MO), and (*S*)-2-methylbutan-1-ol from TCI America (Portland, OR). Because neral and geranial are not available as pure isomers, and readily isomerize, I instead used citral (a ~1:1 mixture of neral and geranial; Sigma-Aldrich). (*S*)- and (*R*)-3-hydroxyhexan-2-one were synthesized according to Lacey et al. (2007), in 93.4 and 97.8% ee respectively. Authentic standards of (2*R*,3*R*)-, (2*S*,3*S*)-, (2*S*,3*R*)- and (2*R*,3*S*)-2,3-hexanediol and (*R*)-2-methylbutan-1-ol were not commercially available and so were synthesized as described in Appendix A.

Adult *M. caryae* were captured alive with black flight-intercept traps (Panel Trap model, AlphaScents, Portland, OR) during May 2009 at Forest Glen Preserve, Vermilion Co., IL (40°0'51.97"N, 87°34'0.74"W). Traps were baited with plastic polyethylene sachets (Bagettes™ model 14770, 5.1 × 7.6 cm, Cousin Corp., Largo, FL; see Graham et al., 2010) loaded with 50 mg of citral in 1 ml of 95% ethanol. Citral is a blend of the primary components of the pheromone (Lacey et al. 2008) and attracts males and females in similar numbers (Chapter 4). Beetles were maintained on a diet of 10% sugar water for no more than 2 d before antennae were processed. I produced antennal transcriptomes from composite samples of 24 male and 21

female adult *M. caryae*, respectively. Subsequent assays used RNA extracted from antennae of an additional 10 males and 10 females captured during May 2009, and again in May of 2010 and 2011.

Isolation of total RNA and construction, sequencing, and assembly of library

Antennae of live adults were cut through the scape and immediately placed on dry ice, then homogenized in 1 ml TRIzol reagent with RNA extracted according to the manufacturer protocol (Invitrogen, Carlsbad, CA). Coupled gas chromatography-electroantennography revealed that antennae of both sexes respond in similar fashion to all components of the pheromone blend (see Chapter 4). I therefore combined RNA from males and females and the library was constructed from the pooled sample. Subsequent samples used for cloning were maintained as separate male and female stocks.

I provided 80 µg total antennal RNA to the Roy J. Carver Biotechnology Center High-Throughput Sequencing and Genotyping Unit of the W. M. Keck Center for Comparative and Functional Genomics (University of Illinois, Urbana, IL). Construction of a normalized cDNA library and 454 pyrosequencing were carried out at the Keck Center as described by Lambert et al. (2010). Assembly also was performed at the Keck Center using SeqMan Pro sequence assembly software (DNASTAR Inc., Madison, WI, USA).

Annotation of odorant receptor genes

I converted the assembled contigs into a searchable database using the formatdb tool of the BLAST+ toolkit (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/). Peptide sequences of Ors from *T. castaneum* (Engsontia et al.

2008) were used as BLAST queries (tBLASTn, National Center for Biotechnology Information; Altschul et al. 1997) to identify related genes in *M. caryae*. The encoded proteins of putative Ors were aligned with analogs from *T. castaneum* and raw reads from the library using CLUSTALX (Thompson et al. 1997) and, when necessary, manually extended or rebuilt from raw reads to create complete ORFs. Some ORFs could not be completed and were designated as "partial Ors". I did not attempt to extend these sequences through other methods because receptors with low copy numbers were unlikely to be involved in pheromone detection.

Each complete cerambycid Or was used as an additional BLAST query to identify other receptor genes. I used the complete set of Ors described from *D. melanogaster* (Clyne et al. 1999, Vosshall et al. 1999) and *Apis mellifera* L. (Robertson et al., 2006) as final queries to search for any subfamilies of Ors not present in *T. castaneum*. The phylogenetic relationships among the Ors of *M. caryae* (including partial Ors of at least 200 amino acids) and Ors of *T. castaneum* were assessed through a distance matrix corrected using the BLOSUM62 exchange matrix in TREEPUZZLE v5.0 (Schmidt et al. 2002). A tree based on these corrected distances was obtained using heuristic search and tree-bisection-and-reconstruction branch swapping in PAUP* (v4.0b10; Swofford 2001).

Identification and cloning of candidate pheromone receptors

I chose the initial set of candidate pheromone receptors as Ors that were represented by a high number of reads in the transcriptome, allowing me to build models with high confidence, and because genes with poor coverage were likely uncommon in the initial library and peripheral to the goal. Later candidates were chosen from clades that contained receptors that I had successfully characterized from the initial set.

I designed two pairs of primers to amplify each candidate pheromone receptor. The first pair amplified the exact ORF, whereas a second pair added overhangs with unique endonuclease sites (HindIII, EcoRI, BamH1, or XbaI) for unidirectional cloning. The PCR protocol for both stages was 2 min at 94 ^{o}C, 25 cycles of 94 ^{o}C for 1 min, 55-62 ^{o}C for 1 min (depending on primer T_M), and 72 ^{o}C for 1 min, and a final incubation for 5 min at 72 ^{o}C. Product was digested for 1 h with two enzymes in the appropriate buffer (New England Biolabs, Inc., Beverly, MA) and gel purified (Gel Extraction Kit; Qiagen Corp., Chatsworth, CA). I cloned sequences into the vector pGEMHE using the NEB Quick Ligation Kit (New England Biolabs), and confirmed all clones by sequencing. I also identified and cloned the cerambycid ortholog of the *Orco* receptor, because odorant receptors of insects will not function in the absence of this protein (Sato et al. 2008, Vosshall and Hansson 2011).

Expression of pheromone receptors in *Xenopus* oocytes

I characterized receptor function as described in earlier publications (Hughes et al., 2010; Wanner et al. 2007). Briefly, I synthesized capped cRNA in vitro from pGEMHE vectors encoding Ors with mMessage mMachine kits (Ambion, Austin, TX). The pGEMHE vector includes 3' and 5' UTR of a β-globin gene that facilitates high expression of receptors in oocytes of the frog *Xenopus laevis* (Daudin) (Liman et al. 1992). Oocytes were surgically removed from anesthetized frogs and follicle cells were removed by treatment with Collagenase B (Boehringer Mannhem) for 2 h at room temperature. Oocytes were co-injected with cRNA of a candidate pheromone receptor and *Mcar\Orco*, and incubated for 3-7 d before electrophysiological recording.

Initial screens for, and functional assay of pheromone receptors

Each receptor was tested against all components of the pheromone blend of *M. caryae*, as well as enantiomers and the following isomers of pheromone components: (2*S*,3*S*)- and (2*R*,3*R*)-2,3-hexanediol, (*S*)- and (*R*)-3-hydroxyhexan-2-one, (*R*)-(+)-limonene, and (*R*)-2-methylbutan-1-ol. I measured odorant-induced currents by two-electrode voltage clamp in an automated parallel electrophysiology system (OpusExpress 6000A; Molecular Devices, Union City, CA). Oocytes were bathed continuously with a buffered saline solution, ND96 (Hughes et al., 2010), and odorants were diluted in ND96 and applied to the oocyte for 20 s at 1.6 ml/min. Micropipettes were filled with 3 M KCl and had resistances of 0.2–2.0 MΩ. The holding potential was -70 mV. Current responses, filtered (4-pole, Bessel, low pass) at 20 Hz (-3 db) and sampled at 100 Hz, were captured and stored with OpusXpress 1.1 software (Molecular Devices, Sunnyvale, CA). I screened a range of concentrations (generally 1-3000 µM, half-log scale) for each odorant that activated a receptor. Data were fit to the following equation that models concentration-response (Prism 5, Graphpad Software, San Diego, CA): $I = I_{max}/(1+(EC_{50}/X)^n)$, where I = current response at a given concentration of odorant (X), I_{max} = maximal response; EC_{50} = concentration of odorant yielding a half-maximal response, and n is the apparent Hill coefficient.

Results

Identification of odorant receptors

The sequencing yielded 583 megabases in 1.56 million reads, with a mean fragment length of 404 bp. These data were assembled into 75,603 contigs with a mean length of 842 bp. I identified the *Orco*, 30 complete Ors, and 26 partial Ors by querying the database with Ors of

T. castaneum and through iterative searches with newly identified cerambycid Ors. No further Ors were identified by queries with receptors of *D. melanogaster* or *A. mellifera*. Contigs containing Ors were relatively rare in the database and built from an average of 22 reads and a median of 11 reads (exclusive of the *Orco*, which was extensively covered by 627 reads). Nucleotide and protein sequences of the receptors are included in Appendix B.

The majority of the expressed Ors of *M. caryae* were evenly placed among groups 1-3 of the six subfamilies described from *T. castaneum* (Engsontia et al. 2008, Fig. 6.1). A single cerambycid receptor, McOr44, was placed alongside TcOr275 as an outgroup to groups 4-6. Additionally, a novel expansion of receptors (noted as group 7 on the tree) was recovered from *M. caryae* that showed no close relationship to any TcOrs. This clade contained almost half (14) of the complete Or transcripts in the library, including the two most common Ors in the library (McOr30 and McOr28). The tree was rooted with *Orco* proteins from *M. caryae* and *T. castaneum* (McOr1, TcOr1), which retained the characteristically high degree of conservation found in the *Orco* lineage.

Characterization of pheromone receptors

I cloned nine putative odorant receptor genes of *M. caryae* into pGEMHE, as well as the *Orco* ortholog, and tested all clones for function in the *Xenopus* oocyte expression system. I first tested the five most common receptors in the library (McOr30, McOr28, McOr20, McOr3, and McOr19) and then included four additional candidates that were common in the library but also related to confirmed pheromone receptors from the initial group (McOr17, McOr18, McOr22, McOr5; Fig. 6.1). Unexpectedly, some receptors proved difficult to clone, and were recovered

with numerous deletions relative to the gene model. Complete ORFs were eventually cloned from all candidate pheromone receptors.

Two receptors from the initial group and one receptor from the subsequent group responded to components of the pheromone blend produced by *M. caryae* at a concentration of 30 µM (Figs. 6.2-6.3). In the initial group, McOr3 responded with high sensitivity to both enantiomers of 2-methyl-1-butanol (numbers **12** and **13**; Fig. 6.2A). McOr5, selected in the second group of receptors, was sensitive to 2-phenylethanol (**10**; Fig. 6.2B). McOr20 was sensitive to (*R*)-3-hydroxyhexan-2-one (**5**) and (2*S*,3*R*)-2,3-hexanediol (**4**; Fig. 6.2C). In a separate screen performed at 100 µM, this receptor also responded to (2*R*,3*S*)- and (2*S*,3*S*)-hexanediol (data not shown).

McOr3 was sensitive to (*S*)-2-methyl-1-butanol at a broad range of concentrations (Fig. 6.3A) with an EC_{50} of 25 µM (Fig. 6.3B, Table 6.1), and less sensitive to (*R*)-2-methyl-1-butanol at an EC_{50} of 100 µM (Fig. 6.3B, Table 6.1). McOr5 responded to 2-phenylethanol at an EC_{50} of 150 µM (Fig 6.3C, Table 6.1). Finally, McOr20 was sensitive to (2*S*,3*R*)-2,3-hexanediol at an EC_{50} of 120 µM, (*R*)-3-hydroxyhexan-2-one at an EC_{50} of 350 µM, and very slightly responsive to (2*R*,3*S*)- and (2*S*,3*S*)-hexanediol at EC_{50}s 1430 µM and 330 µM, respectively (Fig. 6.3D, Table 6.1). None of the remaining six receptors responded to the chemicals over the concentration range tested in the assay.

Discussion

The odorant receptors described here are only the second published set of receptors from the Coleoptera and include the first Ors functionally characterized from any species of beetle. It is highly unlikely that I have described the full complement of active receptors from adult *M.*

caryae, but the number of described receptors nevertheless compares favorably to those described from genomes of other insect species (Hill et al. 2002, Robertson et al. 2003) and permits a preliminary characterization of receptor evolution in beetles.

The minimal sequence similarity between receptors of *T. castaneum* and *M. caryae* is consistent with the overall family of olfactory receptors, which are remarkably divergent among insect groups (Robertson et al. 2010). In fact, the lineages of *T. castaneum* and *M. caryae* (Tenebrionoidea and Chrysomeloidea, respectively) are believed to have diverged between 220-236 Mya (Hunt et al. 2007), comparable to the timing of divergence between *Drosophila* and mosquitoes (i.e, the radiation of Nematocera; Wiegmann et al. 2011). It is thus unsurprising that the relationship between cerambycid and tenebrionid receptors follows a similar pattern of substantial gene loss and radiation (Hill et al. 2002). I did identify a small number of potentially orthologous pairs within the coleopteran receptors (McOr2/TcOr47, McOr8/TcOr64, McOr44/TcOr275) that may bind odorants important to both species. In fact, McOr3 is placed alongside the receptor pair TcOr65/66, suggesting these *Tribolium* receptors may bind 2-methyl-1-butanol or related chemicals. Such speculation is admittedly risky, given the rapid evolution of chemoreceptors, and receptors must be tested experimentally to verify ligands.

The apparent lack of cerambycid receptors among *T. castaneum* groups 4-6 supports the hypothesis that these large expansions are recent and specific to *Tribolium*, or at least to a tenebrionoid lineage (Engsontia et al. 2008). Conversely, the group 7 receptors are not present in *Tribolium*, and have not been identified from any other insect genome to date. Receptors tested from group 7 did not respond to any components of the pheromone blend, but the large number of Ors in this group suggests an important function in *M. caryae*, especially because most are

strongly represented in the library. This family could instead be sensitive to host plant odors rather than pheromone components (Hanks 1999, Ginzel and Hanks 2005).

I were surprised by the high frequency of flawed transcripts characterized by apparent deletions, which were present in the library and recovered in cloned receptors from all pools of RNA used in the experiments. Deletions ranged from a dozen to hundreds of base pairs, and probably stemmed from changed or incorrect splicing of exons, but this mechanism can only be confirmed with a genome sequence. Some genes such as McOr11 were entirely crippled, with complete ORFs unrecoverable, whereas other McOrs were present at considerably reduced frequencies. It is possible that cerambycid Ors are regulated in some manner by alternative splicing, either changing or eliminating their functionality. I characterized receptors for three pheromone components of *M. caryae* that were spread across two lineages of Ors (groups 1 and 2). This suggests that pheromone receptors of the Cerambycidae do not share a recent evolutionary origin, in contrast with the pheromone receptors of moths that apparently arose from a single lineage (e.g., Wanner et al. 2010). The most sensitive of the cerambycid receptors, McOr3, detected ligands at an EC_{50} of 25 µM, a high value relative to other reports (Wanner et al. 2007; Nakagawa et al. 2005), but not unusual for Ors tested in the *Xenopus* assay. For example, the odorant receptor AmOr11 of *A. mellifera* was sensitive to the queen substance 9-oxo-2-decenoic acid at an EC_{50} of 280 nM (Wanner et al. 2007), BmOr 1 of *Bombyx mori* L. was sensitive to the female-produced pheromone bombykol at 1.5 µM, and BmOr3 was sensitive to bombykal at 260 nM (Nakagawa et al. 2005). Receptors described from the moths *Ostrinia nubilalis* (Hübner) and *Heliothis virescens* (F.) responded to pheromone components at EC_{50} values ranging from 260 nM – 24 µM (Wanner et al. 2010, Wang et al., 2011). However, many of the recently characterized odorant receptors of *A. gambiae* responded to ligands at EC_{50} values

in the tens or hundreds of μM (Wang et al. 2010). Regardless, the sensitivity of odorant receptors may be highly modulated by odorant binding proteins (Große-Wilde et al. 2007), which were not tested in the present study.

Two of the cerambycid pheromone receptors were sensitive to components that are produced by numerous cerambycid species (2-methyl-1-butanol and 2,3-hexanediol; Hanks et al. 2007, Lacey et al. 2009, Chapter 4). Male *M. caryae* produce specific stereoisomers of these chemicals, which could impart species specificity to the signal, but the chiral specificity of the receptors was not as exact: McOr3 was responsive to (*S*)-2-methyl-1-butanol, but also to the unnatural (*R*)-enantiomer. Moreover, McOr20 was more narrowly tuned to a specific isomer, (2*S*,3*R*)-2,3-hexanediol, but also responded to two of the three other stereoisomers, as well as the structurally related (*R*)-3-hydroxyhexan-2-one. Synthesized standards of (2*R*,3*S*)- and (2*S*,3*S*)-2,3-hexanediol contained trace amounts of (2*S*,3*R*)-2,3-hexanediol, but standards of 3-hydroxyhexan-2-one were free of 2,3-hexanediol (see Appendix A), and (*R*)-2-methyl-1-butanol was chirally pure, suggesting that the apparent flexibility of these receptors is not due to contamination.

Electroantennographic assays support the dual sensitivity of antennae to the enantiomers of 2-methyl-1-butanol, but antennae of males and females appear highly sensitive to all four isomers of 2,3-hexanediol (RFM, unpub. data). This finding suggests that McOr20 is not the primary receptor for other isomers of hexanediol, but rather that the low affinity for these isomers could be a vestige of an ancestral receptor for the hydroxyalkanones and diols that are common pheromone components in the family. In fact, McOr20 pairs with a similar receptor, McOr17 (45.3% identical peptide sequence; Fig. 6.1), although McOr17 appeared unresponsive to any isomer of 2,3-hexanediol or 3-hydroxy-2-hexanone.

Functional flexibility of receptors that detect pheromones is consistent with previous findings for the moth *Ostrinia nubilalis* (Hübner) (Wanner et al, 2010), and suggests that other cerambycids that produce isomers of 2-methyl-1-butanol and 2,3-hexanediol will detect these chemicals with receptors closely related, or orthologous to McOr3 and McOr20. If multiple species rely on a single lineage of similar pheromone receptors, it may be possible to create generic primers that can serve as markers for pheromone sensitivity, and these genes also may inform phylogenetic study of the family. Additionally, many insects produce or respond to 2-methyl-1-butanol (e.g., Matsuura et al. 2010, Pontes et al. 2008) and the sequence of McOr3 may inform study of these species as well. I am currently exploring these potential applications by searching for orthologs of McOr3 and McOr20 in other species of *Megacyllene* and closely related genera.

In summary, this research has proven that olfactory receptors can be identified through high-throughput sequencing of an antennal transcriptome, and verifies this method as a powerful and efficient means of describing Ors in lieu of a genome sequence. Key receptors for important odorants, such as pheromones, should be represented strongly in the resulting transcriptome and their function can be quickly characterized. It is my hope that this study will encourage research on other non-model species that will improve understanding of the evolution of chemoreception within the Insecta.

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Table 6.1. Cerambycid pheromone components that were detected by odorant receptors of the cerambycid *Megacyllene caryae*. Receptors were expressed in oocytes of *Xenopus laevus* and tested against a panel of chemicals in a voltage clamp. Sensitivity is expressed as the concentration of odorant yielding a half-maximal response (EC_{50} ± SEM).

Odorant Receptor	Ligand	EC_{50} (μM)
McOr3	(*S*)-methylbutan-1-ol	25±7.36
	(*R*)-methylbutan-1-ol	100±36.3
McOr5	2-phenylethanol	150±32.5
McOr20	(2*S*,3*R*)-2,3-hexanediol	120±57.4
	(2*R*,3*S*)-2,3-hexanediol	1430±1331
	(2*S*,3*S*)-2,3-hexanediol	330±77.2
	(*R*)-3-hydroxyhexan-2-one	350±154.6

Fig. 6.1. Phylogram showing peptide sequences of odorant receptors from *Tribolium castaneum* (TcOrs) and *Megacyllene caryae* (McOrs). Partial sequences of at least 200 amino acids are included in the tree and denoted by the suffix "PAR". Receptors are organized into groups 1-6 as defined by Engsontia et al. (2008), but including a group described only from *M. caryae* (7). Receptors from Groups 4-6, not present in the antennal transcriptome of *M. caryae*, are represented as black triangles to indicate the number of omitted receptors. Receptors of *M. caryae* that are sensitive to pheromone components (and the *Orco*) are indicated by "+", those insensitive to components are indicated by "–", and those that were not tested (ligands unknown) are indicated by "•". The tree is rooted with *Orco* proteins from both species (McOr1, TcOr1).

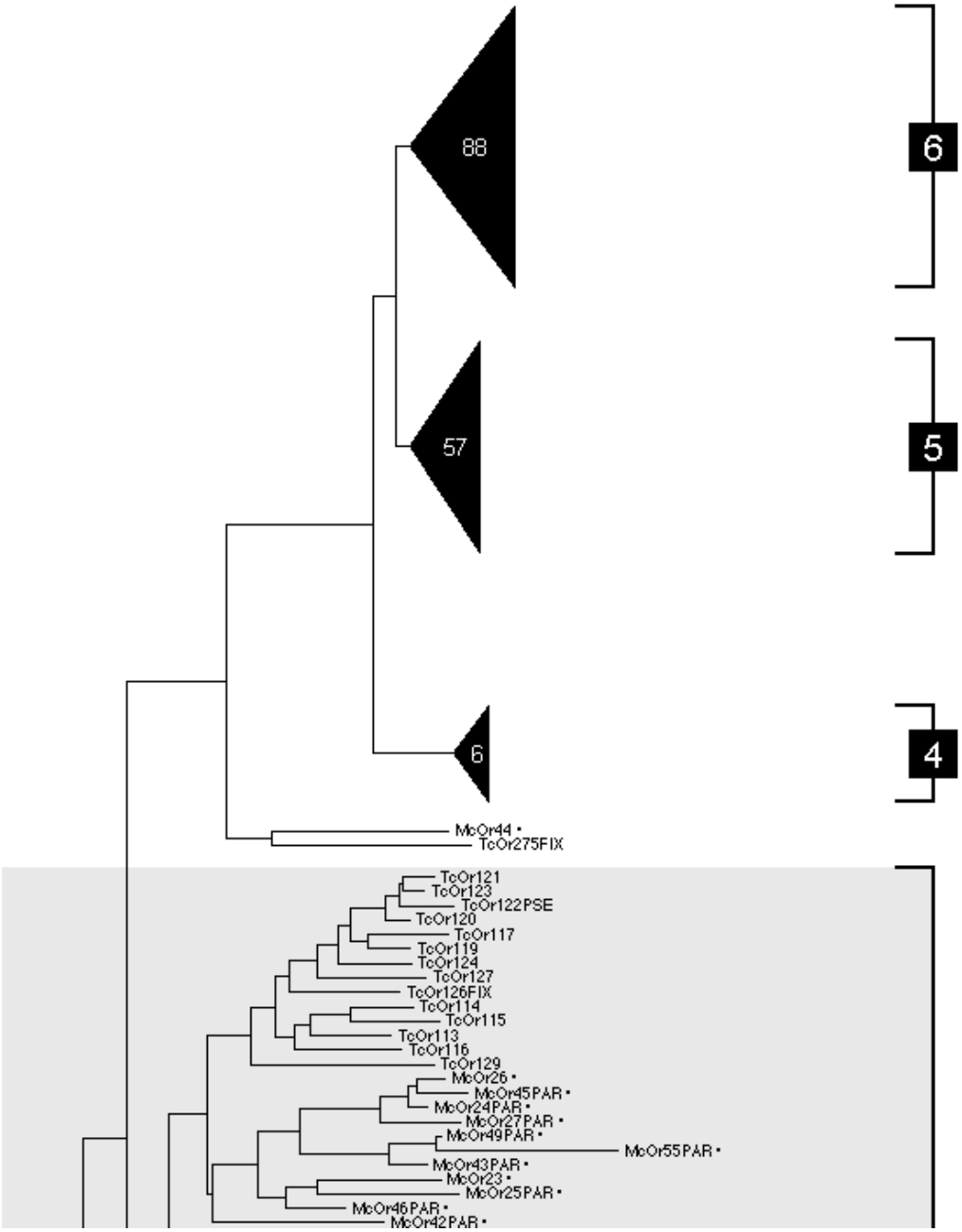


Fig. 6.1, continued.



Fig. 6.1, continued.

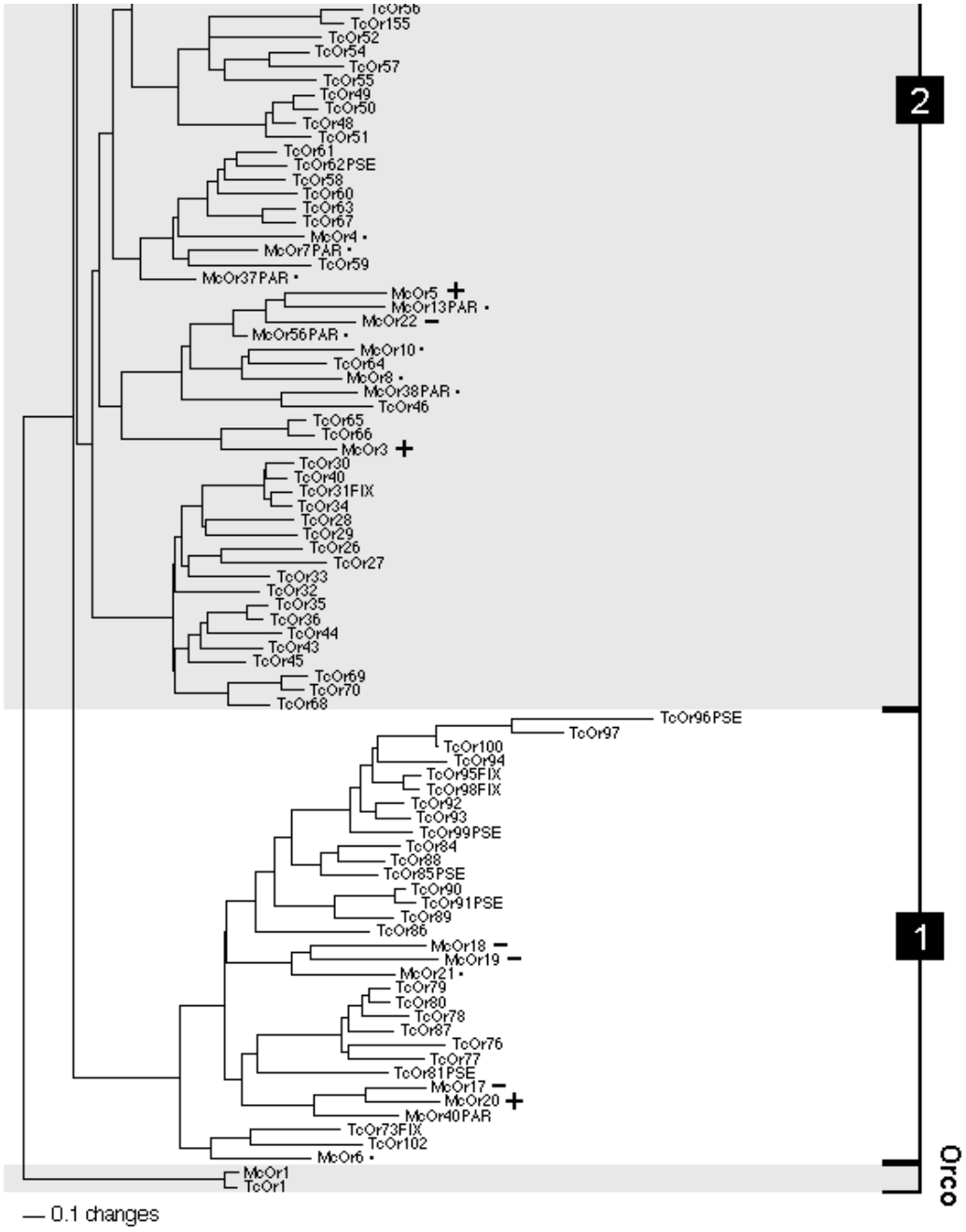


Fig. 6.2. Change in electric current induced by candidate odorant receptors, expressed in *Xenopus* oocytes, in response to pheromone components of *Megacyllene caryae*. Frog oocytes that expressed receptors **A)** McOr3, **B)** McOr5, and **C)** McOr20 were sequentially challenged with 30 µM of each of the following pheromone components (applied for 20 s): 1. (2*R*,3*R*)-2,3-hexanediol, 2. (2*S*,3*S*)-2,3-hexanediol, 3. (2*R*,3*S*)-2,3-hexanediol, 4. (2*S*,3*R*)-2,3-hexanediol, 5. (*R*)-3-hydroxyhexan-2-one, 6. (*S*)-3-hydroxyhexan-2-one, 7. (*S*)-(–)-limonene, 8. (*R*)-(+)-limonene, 9. (–)-α-terpineol, 10. 2-phenylethanol, 11. Citral, 12. (*R*)-2-methylbutan-1-ol, and 13. (*S*)-2-methylbutan-1-ol. Diagonal hatching indicates short breaks in recording.

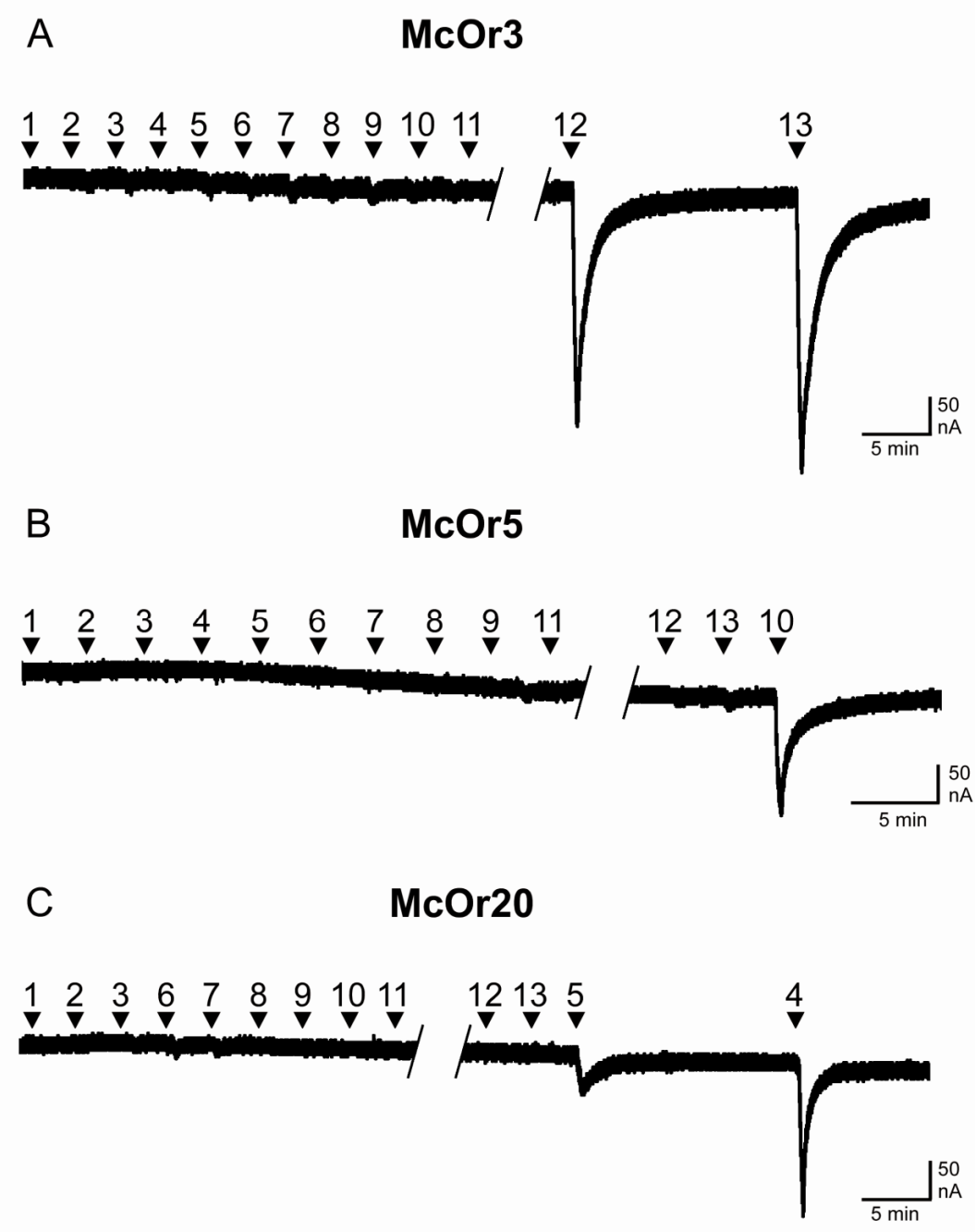
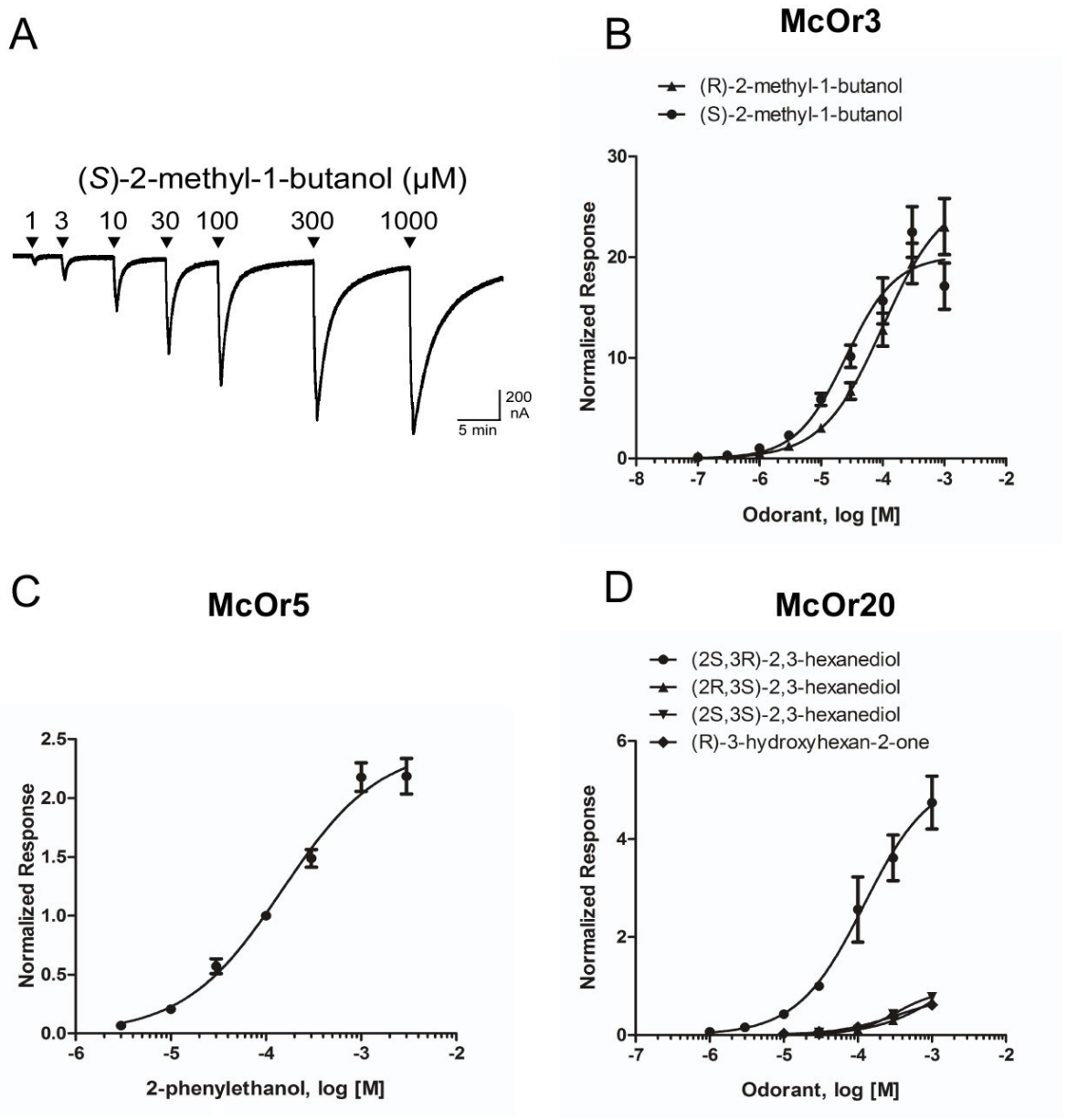


Fig. 6.3. Relationships between concentrations of pheromone components and the responses of *Xenopus* oocytes that are expressing pheromone receptors of *Megacyllene caryae*. **A)** An example of a trace used to generate concentration-response curves (described in section 2.6): an oocyte expressing McOr3 is challenged with 20-s applications of (*S*)-2-methylbutan-1-ol at a range of concentrations. Concentration-response curves of **B**) McOr3 challenged with (*R*)- and (*S*)-2-methyl-1-butanol (normalized to the response of each oocyte to 1 µM [*S*]-2-methylbutan-1-ol; n = 5–15); **C**) McOr5 challenged with 2-phenylethanol (normalized to 100 µM 2-phenylethanol; n = 6); **D**) McOr20 challenged with three isomers of 2,3-hexanediol and (*R*)-hydroxyhexan-2-one (normalized to 30 µM [2*S*,3*R*]-2,3-hexanediol; n = 5-7).



APPENDIX A: SYNTHESIS OF HOMOCHIRAL 2,3-HEXANEDIOL AND 2-METHYLBUTAN-1-OL[3]

Stereospecific syntheses of (2*R*,3*R*)- and (2*S*,3*S*)-hexanediols from (*L*)- and (*D*)-threonine (Figure A.1)

Unless otherwise specified, worked up reaction mixtures were dried over anhydrous Na_2SO_4 and concentrated by rotary evaporation with a vacuum of 40-200 Torr, depending on the solvent being removed. THF was distilled from sodium-benzophenone ketyl, and reactions with air- or water-sensitive reagents were carried out under argon in oven-dried glassware.

Synthesis of (4*S*,5*R*)-2,2,5-trimethyl-4-(methoxycarbonyl)-1,3-dioxolane (4)

(*L*)-Threonine **1** (59.5 g, 500 mmol; Alfa Aesar, Ward Hill MA) was dissolved in 595 ml water in a 3-neck flask fitted with a bubbler to allow release of gas, and the solution was cooled in an ice-bath. Five ml aliquots of $NaNO_2$ solution (38 g, 550 mmol in 80 ml water) and 3.5 ml aliquots of 5M H_2SO_4 solution (280 mmol, 56 ml) were added sequentially to the cooled mixture at ~20 min intervals. After the additions were complete, the solution was allowed to warm to room temperature overnight, by which time gas evolution had ceased. The mixture was concentrated by rotary evaporation. The residue was taken up in 500 ml EtOH, and the mixture was filtered through a pad of Celite® (Fisher Scientific, Fairlawn, New Jersey), rinsing twice with 100 ml EtOH. The filtrate was concentrated by rotary evaporation, then pumped under vacuum to yield 69 g of the diol acid **2** as a viscous yellow oil.

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[3] This appendix is in press and will appear as supplementary material in the journal *Insect Biochemistry and Molecular Biology*: Mitchell, R. F., D. T. Hughes, C. W. Luetje, J. G. Millar, F. Soriano-Agatór, L. M. Hanks, and H. M. Robertson. 2012. Sequencing and characterizing odorant receptors of the cerambycid beetle *Megacyllene caryae*. Insect Biochem. Mol. Biol. This material is reprinted with the permission of the publisher. JGM and FS authored this section in its entirety.

Crude diol acid **2** was dissolved in a mixture of THF (150 ml) and MeOH (150 ml), and chlorotrimethylsilane (32 ml) was added dropwise over ~30 min. The slightly cloudy solution was stirred overnight, then filtered through a pad of Celite, and the filtrate was concentrated at 40 Torr to yield the diol methyl ester **3** as a viscous yellow oil, which was used without further purification. ^{1}H NMR ($CDCl_3$): δ 4.11 (qd, 1H, J = 6.4, 2.8 Hz), 4.04 (d, 1H, J = 2.8 Hz), 3.84 (s, 3H), 3.63 (br s, 2 x OH), 1.32 (d, 3H, J = 6.4 Hz).

Crude diol methyl ester **3** was dissolved in a mixture of acetone (300 ml) and 2,2,-dimethoxypropane (67 ml), and 1 g of p-toluenesulphonic acid was added. The mixture was stirred overnight at room temperature, then concentrated by rotary evaporation. The residue was taken up in ether (200 ml), washed twice with 75 ml saturated aqueous $NaHCO_3$ (foams!) and once with brine, then concentrated to a yellow oil. Kugelrohr distillation (oven temp to 60ºC, 0.5 Torr) gave 34.8 g of the protected diol ester **4** (40% from threonine). ^{1}H NMR ($CDCl_3$): δ 4.21 (qd, 1H, J = 8.0, 6.0 Hz), 4.07 (d, 1H, J = 8.0 Hz), 3.79 (s, 3H), 1.48 (s, 3H), 1.45 (s, 3H), 1.44 (d, 3H, J = 6.0 Hz). ^{13}C NMR ($CDCl_3$): δ 18.7, 25.9, 27.3, 52.5, 75.3, 80.6, 110.8, 171.1 ppm. The proton NMR was in accord with that previously reported (Servi 1985).

Synthesis of (4*R*,5*R*)-5-Hydroxymethyl-2,2,4-trimethyl-1,3-dioxolane (5)

Protected diol ester **4** (34.45 g, 198 mmol) was added dropwise to a slurry of $LiAlH_4$ in ether cooled in an ice-bath and under Ar atmosphere. When the addition was complete, the mixture was allowed to warm to room temperature and stirred overnight. The mixture was again cooled in an ice-bath, and water (5 ml; vigorous H_2 evolution!), 20% aq. NaOH (3.75 ml), and water (17.5 ml) were sequentially added dropwise. The resulting mixture was stirred vigorously for 30 min to allow the white precipitate to granulate, and was then filtered through a pad of

Celite, rinsing with ether. The filtrate was dried, concentrated, and Kugelrohr distilled (over temp to 70ºC, 8 Torr), yielding alcohol **5** (25.7 g, 89%) as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 4.02 (dq, 1H, J = 8.4, 6 Hz), 3.81 (ddd, 1H, J = 12, 5.2, 4,8 Hz), 3.66 (m, 1H), 3.62 (ddd, 1H, J = 12, 7.2, 4.4 Hz), 2.05 (dd,1H, J = 7.2, 5.2 Hz, OH), 1.43 (s, 3H), 1.41 (s, 3H), 1.30 (d, 3H, J = 6.0 Hz) . ^{13}C NMR ($CDCl_3$): δ 17.8, 27.1, 27.6, 61.6, 72.9, 82.9, 108.7 Hz. The proton NMR was in accord with that previously published (Nagashima and Ohno 1991).

Synthesis of (2*R*,3*R*)-hexanediol (8)

Trifluoromethanesulphonic anhydride (33.6 ml, 200 mmol) was added dropwise over ~30 min to a solution of alcohol **5** (25.5 g, 175 mmol), CH_2Cl_2 (300 ml), and pyridine (14.2 ml, 200 mmol) cooled in an ice-acetone bath to ~-10ºC. When the addition was complete, the mixture was warmed to 0ºC, and was stirred until all the starting alcohol had been consumed. The mixture was washed successively with ice-water, saturated aqueous $NaHCO_3$, and brine, and the organic phase was dried and filtered through a 2 cm plug of silica gel with suction, rinsing well with CH_2Cl_2. The filtrate was concentrated without heating, and pumped under vacuum for 5 min. The resulting brown oil was immediately taken up in 100 ml dry ether and kept at 0º until used in the coupling step below.

A dried flask under Ar was charged with CuI (3.8 g, 20 mmol) and dry ether (500 ml), and after cooling to ~-10ºC in an ice-acetone bath, EtMgBr (2M in THF, 110 ml, 220 mmol) was added slowly, keeping the temperature <0ºC. The mixture was stirred for 15 min at <0º, then the ether solution of triflate was added dropwise over ~90 min, keeping the temperature <0ºC. When the addition was complete, the mixture was allowed to slowly warm to room temperature overnight. The mixture was then poured into 500 ml saturated aqueous NH_4Cl. The organic

layer was separated and washed with saturated aqueous NH_4Cl and brine, dried, and concentrated. The residue was taken up in a mixture of THF (150 ml) and 3M HCl (150 ml) and stirred at room temperature for 16 hr. The mixture was cooled to 0ºC and quenched by addition of cold 5M NaOH in portions. The mixture was then saturated with salt and extracted with EtOAc (2 x 100 ml). The combined extracts were washed with brine, dried, and concentrated by rotary evaporation followed by partial Kugelrohr distillation (room temperature, 6.5 Torr). The residue was taken up in 10% ether in pentane (50 ml) and purified by vacuum flash chromatography on silica gel in a 150 ml Buchner funnel, eluting with 10% ether in pentane (3 x 100 ml), then ether (5 x 100 ml). The first two fractions contained a small amount of the ketal starting material, and the diol eluted in fractions 5-8, which were combined, concentrated, and Kugelrohr distilled, giving 9.43 g of the diol (46% from alcohol 5; 53% based on recovered ketal; 97.5% chemically pure by GC). The diol was 97% enantiomerically pure, and contaminated with 0.9% of the (*R*,*S*)-diastereomer, as determined by GC on a chiral stationary phase Cyclodex B column (30 m × 0.25 mm × 0.25 micron film thickness, J&W Scientific, Folsom CA), programmed from 50 ºC/1 min, then 3ºC/min to 200ºC. Under these conditions, the four stereoisomers elute in the following order: (2*S*,3*S*) 21.89 min, (2*R*,3*R*) 22.25 min, (2*R*,3*S*) 23.06 min, (2*S*,3*R*) 23.25 min. ^{1}H NMR ($CDCl_3$): δ 3.58 (quint, 1H, J = 6.4 Hz), 3.36-3.31 (m, 1H), 2.44 (br s, 2 × OH), 1.56-1.32 (m, 4H), 1.18 (d, 3H, J = 6.4 Hz), 0.94 (t, 3H, J = 6.8 Hz). ^{13}C NMR ($CDCl_3$): δ 14.3, 19.0, 19.7, 35.7, 71.1, 76.1 ppm. The spectra matched those of the racemic compound prepared as described in Lacey et al. (2004).

(2*S*,3*S*)-hexanediol was synthesized in identical fashion from (*D*)-threonine (Chem-Impex International, Wooddale IL), ee 94.2 %, and 0.6% (2*S*,3*R*)-diastereomer.

Kinetic resolution of (2*R*,3*S*)- and (2*S*,3*R*)-hexanediols from racemic (2*R,3*S**)-hexanediol**

Racemic (2*R**,3*S**)-hexanediol (10 g, 85 mmol, prepared by OsO_4-catalyzed dihydroxylation of (*Z*)-2-hexene as described in Lacey et al. (2004) was added to a slurry of vinyl acetate (24 ml, 260 mmol), t-butyl methyl ether (350 ml), and Amano lipase PS (5 g), and the mixture was stirred at room temperature for 10 d. The mixture was then filtered, concentrated, and fractionated by vacuum flash chromatography on 300 g silica gel, eluting with 40% EtOAc (5 x 200 ml), then 100% EtOAc (3 x 300 ml). The combined mono- and diacetates eluted in the first two fractions, whereas the unreacted diols eluted in fractions 5-7. The unreacted diols (3.65 g) were an 86:14 mixture of (2*S*,3*R*): (2*R*,3*S*)-diols.

After concentration, the combined acetates were hydrolyzed in 1.25 M NaOH overnight, and the diols were recovered by saturating the solution with salt and extracting twice with EtOAc. The resulting crude diols consisted of a 14:86 mixture of (2*S*,3*R*): (2*R*,3*S*) diols.

The two scalemic mixtures, each enriched in a different enantiomer of hexanediol (3.5 g of each), were again stirred in two separate reactions in mixtures of vinyl acetate (8 ml) and Amano lipase PS (3.5 g) in t-butyl methyl ether (100 ml) for 3 d, then worked up as described above. After vacuum flash chromatography, the hexanediols were Kugelrohr distilled (oven temperature to 85ºC at 5 Torr), yielding 2.4 g of the less reactive (2*S*,3*R*)-hexanediol (91.4% e.e., 2.8% (2*R*,3*R*)-diastereomer) and 2.68 g of the more readily acetylated (2*R*,3*S*)-diol (93.4 % ee, 1.1% (2*R*,3*R*)-diastereomer).

Synthesis of (*R*)-2-methyl-1-butanol

(*S*)-(+)-3-bromo-2-methyl-1-propanol (7 g, 46 mmol; Aldrich Chemical Co.), 3,4-dihydro-2*H*-pyran (4.2 g, 50 mmol), and 100 mg of *p*-toluenesulfonic acid were stirred 2 h at

room temperature in diethyl ether (100 mL). The mixture was washed with saturated aqueous $NaHCO_3$ and brine, and the organic layer was dried and concentrated. Kugelrohr distillation (oven temp to 90ºC, 9 mm Hg) yielded 9.2 g (85%) of the protected alcohol [(*S*)-3-bromo-2-methylpropoxy]-2-tetrahydro-2*H*-pyran. MS (*m/z* rel. intensity): 235, 237 (M^+-1, 4), 135, 137 (6), 115 (6), 101 (6), 85 (100), 67 (9), 55 (54), 41 (45).

Methyl magnesium chloride (3 M in THF, 36 ml, 108 mmol) was added dropwise to a mixture of the protected alcohol (8.5 g, 36 mmol) and Li_2CuCl_4 (0.1 M in THF, 18 ml, 1.8 mmol) in THF (20 mL) at –30°C. The mixture was warmed to room temperature and stirred overnight. After quenching the reaction mixture with dilute HCl, added slowly at 0°C with vigorous stirring, the organic layer was separated. The aqueous layer was extracted with diethyl ether (3 x 100 ml) and the combined organic extracts were washed with saturated aqueous $NaHCO_3$, water, and brine, yielding 5.0 g (80 %) of [(*R*)-2-methylbutoxy]-2-tetrahydro-2*H*-pyran as a yellow liquid. MS (*m/z* rel. intensity): 171 (M^+, 4), 142 (4), 115 (14), 101 (10), 85 (100), 71 (30), 56 (40), 43 (64).

The crude THP-protected alcohol (4 g, ~23 mmol), 1-tetradecanol (10 g, 46 mmol), and 100 mg of *p*-toluenesulfonic acid were stirred for 5 h at room temperature under vacuum (~5 Torr), collecting the liberated (*R*)-2-methyl-1-butanol in a trap cooled to –78°C. (*R*)-2-methyl-1-butanol was recovered as a colorless liquid as a single enantiomer, as determined by analysis on the Cyclodex B chiral GC column (oven temperature 35º isothermal; [*R*]-enantiomer 12.00 min; [*S*]-enantiomer 12.30 min). The retention times and spectra matched those of a racemic standard (Aldrich Chem. Co.).

Results of chemical syntheses

Synthesis of (2*R*,3*R*)- and (2*S*,3*S*)-hexanediols in high enantiomeric purity and in multigram scale was made possible by the ready availability of the two enantiomers of the starting material, threonine, with some modification of the route described by Bianchi et al. (2001). Thus, the amino group of (*L*)-threonine (**1**, Supplementary Fig. 1) was converted to a hydroxyl group with retention of configuration, followed by conversion of the carboxylic acid function to the corresponding methyl ester by treatment with methanol and chlorotrimethylsilane (Nakao et al. 1981) and then protection of the vicinal diol as a ketal, giving ester **4**. It proved advantageous to carry out these three successive steps before purification of ester **4** because the intermediates **2** and **3** were viscous, highly polar, and rather intractable oils. Purified ester **4** was reduced with $LiAlH_4$ to give alcohol **5**, which was then converted to the unstable triflate **6**. After rapid purification by passage through a pad of silica gel, the triflate **6** was immediately alkylated with ethylmagnesium bromide in ether/THF, with CuI catalysis. Removal of the ketal protecting group with aqueous acid then gave (2*R*,3*R*)-hexanediol **8** in high chemical and enantiomeric purity. The analogous series of reactions beginning with (*D*)-threonine provided ready access to the (2*S*,3*S*)-enantiomer.

Although it would have been possible to prepare the (2*R*,3*S*)- and (2*S*,3*R*)-stereoisomers from the threonine enantiomers by a somewhat longer and less efficient route than that described above (Ibuka et al. 1988), I opted instead to prepare the enantiomers by kinetic resolution of the racemate with vinyl acetate and Amano PS lipase in t-butyl methyl ether. After two reaction cycles, the less reactive (2*S*,3*R*)-hexanediol was obtained in 86% enantiomeric excess, whereas the more reactive (2*R*,3*S*)-enantiomer was obtained in ~96% ee. Thus, kinetic resolution did not result in enantiomeric purities as high as those of the (2*R*,3*R*)- and (2*S*,3*S*)-stereoisomers obtained from the threonine chiral synthons, but it did involve fewer steps overall.

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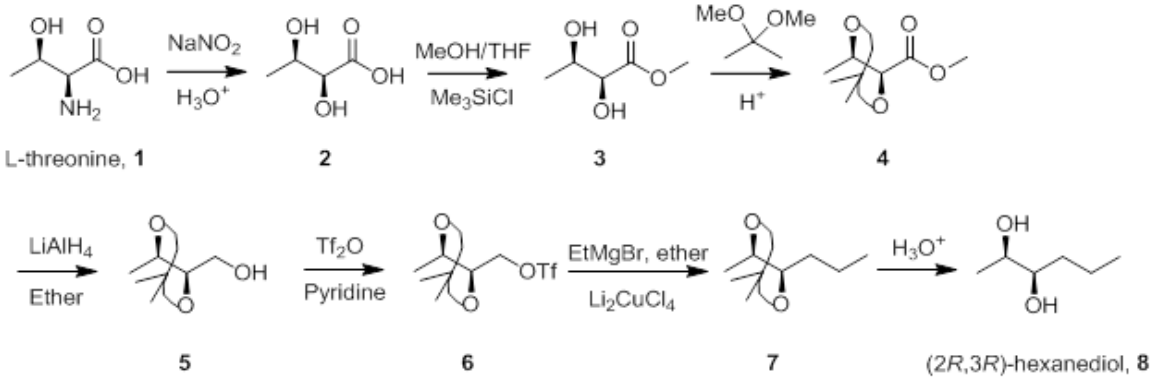
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Fig. A.1. Schematic for synthesis of (2*R*,3*R*)-hexanediol from (*L*)-threonine.



APPENDIX B: NUCLEOTIDE AND PROTEIN SEQUENCES OF ODORANT RECEPTORS OF *MEGACYLLENE CARYAE*

The nucleotide and peptide sequences of odorant receptors referenced in Chapter 6 may be found in supplemental files named “MCOR_nucleotide.txt” and “MCOR_protein.txt”. Sequence data in these files are presented in FASTA format.