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EVIDENCE THAT CERAMBYCID BEETLES MIMIC VESPID WASPS IN ODOR AS WELL AS APPEARANCE

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Abstract

We present evidence that cerambycid species that are supposed mimics of vespid wasps also mimic their models in odor by producing spiroacetals, common constituents of vespid alarm pheromones. Adults of the North American cerambycids *Megacyllene caryae* (Gahan) and *Megacyllene robiniae* (Forster) are conspicuously patterned in yellow and black, and believed to be mimics of aculeate hymenoptera such as species of *Vespula* and *Polistes*. Adult males of *M. caryae* produce an aggregation-sex pheromone, but both sexes produce a pungent odor when handled, which was assumed to be a defensive response. Headspace aerations of agitated females of *M. caryae* contained 16 compounds that presented mass spectra characteristic of spiroacetals of eight distinct chemical structures, the dominant compound being (7*E*,2*E*)-7-ethyl-2-methyl-1,6-dioxaspiro[4.5]decane. Headspace samples of agitated males of *M. caryae* contained five of the same compounds, and with the same dominant compound. Females of *M. robiniae* produced six different spiroacetals, one of which was not produced by *M. caryae*, (2*E*,7*E*)-2-ethyl-7-methyl-1,6-dioxaspiro[4.5]decane, and five that were shared with *M. caryae*, including the dominant (2*E*,8*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane. The latter compound was the sole spiroacetal produced by both males and females of a South American cerambycid species that also is thought to be a wasp mimic, *Callisphyris apicicornis* (Fairmaire & Germain). Preliminary work also has identified spiroacetals of similar or identical structure released by vespid wasps that co-occur with the *Megacyllene* species.

Keywords

Spiroacetal; chemical defense; chemical mimicry; Cerambycidae; *Megacyllene caryae*; *Megacyllene robiniae*; *Callisphyris apicicornis*

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Introduction

Batesian mimics are species that have gained protection from natural enemies by resembling other species that are unpalatable or otherwise defended (Ruxton et al. 2004). Many species of beetles in the Cerambycidae appear to be Batesian mimics of other insects, including tenebrionid beetles (Raske 1967; Slobodchikoff 1987), ants (Berlocher et al. 1992), and even spiders and true bugs (Linsley 1959). Mimicry of lycid beetles is well-documented, and predatory cerambycids may mimic their lycid prey (Eisner et al. 2008) or emit similar warning odors (Dettner 1987). Mimicry of wasps is also particularly common among diurnal cerambycids that feed on pollen, and such vespiform mimics have been described from several genera in the cerambycid subfamilies Cerambycinae, Lepturinae, and Necydalinae (Švácha and Lawrence 2014). In fact, some of these species so closely resemble wasps in color pattern, morphology, and walking and flight behavior that human observers may be deceived (Linsley 1959).

Closer scrutiny of mimicry systems recently has revealed that mimicry extends beyond visual cues, with mimics also resembling their models in production of sound (Barbero et al. 2009) and odor (Vereecken and McNeil 2010). Here, we present evidence that cerambycid species that mimic hymenopterans also resemble their models in odor by producing spiroacetals, common constituents of alarm pheromones released from venom glands of the models (Francke and Kitching 2001). Our study species are the congeners *Megacyllene caryae* (Gahan) and *M. robiniae* (Forster) (Cerambycinae), native to eastern North America, and *Callisphyrus apicicornis* (Fairmaire & Germain) (Necydalinae), native to Chile and Argentina (Bezark and Monné 2013).

Adult *M. caryae* and *M. robiniae* are similar in appearance, conspicuously patterned in yellow and black, and are believed to be mimics of aculeate hymenoptera, as are many other members of the cerambycine tribe Clytini (Linsley 1959). Adults of the two species are active at opposite ends of the season, with *M. caryae* flying in early spring and *M. robiniae* flying in early autumn (Dusham 1921). Similar temporal allopatry has been reported for syrphid fly mimics of Hymenoptera and is thought to minimize contact with insectivorous birds during the summer nesting season, when fledglings have not yet learned to avoid the vespid models (Waldbauer 1988). Adults of both *Megacyllene* species are diurnal and feed on pollen: adults of *M. caryae* visit flowers of *Crataegus* and *Quercus* species (Dusham 1921; LMH unpub. data) whereas adults of *M. robiniae* visit flowers of *Solidago* species (Wheeler et al. 1988). The diurnal adults of *C. apicicornis* also are frequently found on flowers (Curkovic and Ferrera 2012), and, like adults of closely related species, are thought to mimic hymenopterans such as species of *Hypodynerus* (Vespidae) and *Pepsis* (Pompilidae), resembling them in color, form, and flight behavior (Barriga and Peña 1994).

Males of *M. caryae* emit an aggregation-sex pheromone (sensu Cardé 2014) composed of nine components, including isomers of 2,3-hexanediol and 2-methylbutan-1-ol (common pheromone components among cerambycid species in the subfamily Cerambycinae; Millar and Hanks 2016), but also including compounds that are better known as floral and wood volatiles: (*S*)-(-)-limonene, 2-phenylethanol, (-)- α -terpineol, nerol, neral, and geranial (Lacey et al. 2008; Mitchell et al. 2012). Adult *M. robiniae* of both sexes produce a secretion

from metasternal glands when they are handled, which is composed primarily of 2-(1,3-hexadien-1-yl)-5-methyltetrahydrofuran (Wheeler et al. 1988).

During research on *M. caryae* (Mitchell 2012), it was discovered that adult beetles of both sexes emit a pungent odor when handled, which was assumed to be a defensive response. The research reported here initially was intended to characterize the chemical nature of this defensive response, as well as that of a similar response by adults of *M. robiniae*. Research on *C. apicicornis* was conducted independently, and was included here because this species is similar to the *Megacyllene* species in the chemistry of its defensive response, and of course in its mimicry of aculeate Hymenoptera.

Methods and Materials

Sources of Insects

Adults of *M. caryae* were captured alive with cross-vane panel traps (black corrugated plastic; AlphaScents, Portland, OR, USA; see Graham et al. 2010) during May 2011 at Forest Glen Preserve (Vermilion County, IL, USA; 40.015°, -87.568°). Trap lures were polyethylene resealable sachets containing 50 mg of citral (Sigma-Aldrich, St. Louis, MO, USA) in 1 ml isopropanol. Citral is an isomeric blend of neral and geranial, both pheromone components of *M. caryae* (Lacey et al. 2008), and attracts adults of both sexes (e.g., Handley et al. 2015). Adults of *M. robiniae* were collected by hand from inflorescences of *Solidago* species on roadsides near Mazonia State Fish and Wildlife Area (Grundy County, IL, USA; 41.197°, -88.269°) during September 2011. Conspecific adults of the *Megacyllene* species were housed together in the laboratory in aluminum screen cages under ambient conditions (~12:12h L:D, ~20 °C), and provided 10% aqueous sucrose solution as nourishment (glass vial with cotton wick). Adults of *M. robiniae* also were provided freshly cut inflorescences of *Solidago* species.

Adults of *C. apicicornis* were reared from branches of larval host plants (primarily quince, *Cydonia oblonga* Miller, Rosaceae) which had been collected at several locations in the vicinity of Santiago, Chile (“Lo Cañas”, -33.533°, -70.557°; “El Olivar”, -34.232°, -70.874°) during August-September of 2007 and 2008. Branches were placed in Flanders cages (Curkovic and Ferrera 2012) in the laboratory (~20 ± 5 °C, natural photoregime). Cages were checked every morning to collect freshly emerged adults, which were sexed (males are smaller than females, and have longer antennae). Adults emerged during September – December and were used in experiments 1–13 d after emergence. Beetles were held in individual 500 ml plastic vials, and provided 5% aqueous sugar solution as nourishment.

Collection and Analysis of Volatiles

Solid phase microextraction (SPME) was used to collect volatiles produced by individual beetles of the two *Megacyllene* species. Beetles were placed in 50 ml glass flasks that were capped with aluminum foil, and agitated by vigorously shaking the flasks for a few seconds. A SPME fiber (100 µm polydimethylsiloxane, Supelco Inc., Bellefonte, PA, USA) was inserted through the foil and exposed to headspace volatiles for 20 min. Headspace volatiles

were analyzed from ten beetles of each sex of *M. caryae*, and five females of *M. robiniae*. Trace compounds identified in these initial samples were later isolated by collecting volatiles in this same manner, but from groups of 4–5 agitated beetles.

SPME fibers were desorbed into the injection port of a Hewlett-Packard 6890 gas chromatograph coupled to a 5973 mass selective detector (Hewlett Packard, Palo Alto, CA, USA). The GC was fitted with an AT-5ms column (30 m, 0.25 mm i.d., 0.25 μ m film; Alltech Associates, Inc., Deerfield, IL, USA), the injector temperature was set to 250 °C, and the oven programmed from 40 °C (1 min hold) to 160 °C at 10 °C/min. Helium was used as carrier gas, and the mass spectra were acquired at 70 eV. Chemical structures of compounds were tentatively assigned according to their mass spectra using the software library (National Institute of Standards and Technology, Gaithersburg, MD, USA) or by comparison with published spectra (Francke and Kitching 2001). Structure assignments were unambiguously confirmed by comparison of retention times and mass spectra with those of authentic reference samples. Peak areas were calculated as percentages relative to the dominant spiroacetal structure, and percentages of each compound then were averaged across samples within a species and gender.

Volatiles from *C. apicicornis* were collected by placing individual males and females in cylindrical glass chambers (4 cm diameter $\times$ 30 cm long) connected by Teflon[®] tubing to an adsorbent collector (~150 mg activated charcoal; Fisher Scientific, Waltham, MA, USA). Beetles were allowed 5 min to acclimate, then charcoal-purified air was pulled through the apparatus at 1 l/min for 2–4 h. Beetles were aerated between 1000 and 1400 h (when adults were most active in the field), under the same environmental conditions as used in rearing adult beetles (see above), but under fluorescent lighting. Collectors were eluted with ~1.5 ml hexane (SupraSolv, Merck, Darmstadt, Germany), and extracts were concentrated to 5 μ l under a gentle nitrogen stream. Samples of 1 μ l were injected into a GC17A gas chromatograph coupled to a GCMSQP5050A mass spectrometer (Shimadzu, Kyoto, Japan). The GC was equipped with a 30 m $\times$ 0.25 mm VF5-ms fused silica capillary column (Agilent, Santa Clara, CA, USA), the injector temperature was set to 250 °C, and the oven programmed from 50 °C to 270 °C at 8 °C/min. Helium was used as the carrier gas, and the mass spectra were acquired at 70 eV. Structures of compounds were confirmed by co-injection of the natural extract with authentic reference samples.

Source of Reference Compounds

Standards of spiroacetals were not commercially available, so racemates of diastereomeric mixtures of the known structures **A–G** (Francke and Kitching 2001, Booth et al. 2006) were prepared according to established methods (Doubek et al. 2004; Jacobson et al. 1982; Phillips et al. 1980). Following the approach of Jacobson et al. (1982), the syntheses of the new 2-methyl-7-propyl-1,6-dioxaspiro[4.6]undecane (structure **H**) and 8-propyl-1,7-dioxaspiro[5.6]dodecane (structure **I**) are described in Online Resource 1.

Structural Features and Identification of Spiroacetals

Intramolecular ring closure of a straight chain ketone, with stereochemically non-defined secondary hydroxy groups at either side of the carbonyl group, yields a mixture of bicyclic

acetals called spiroacetals (synon. spiroketals). The two rings of the system share a new stereogenic carbon, referred to as the spirocenter. In the most widespread spiroacetal system, the 1,6-dioxaspiro[4.5]decane, a six membered ring (tetrahydropyran) and a five membered ring (tetrahydrofuran) share the spirocenter (Fig. 1A). In the other frequently found system, the 1,7-dioxaspiro[5.5]undecane, two tetrahydropyran substructures are linked (Fig. 1B). When the substituent adjacent to the oxygen of the first ring is positioned at the opposite side as the oxygen of the second ring, it is assigned to keep the more stable "*E*-configuration", while the alternative represents the less stable "*Z*-configuration". An alkyl substituent adjacent to the oxygen in the tetrahydropyran substructure of a spiroacetal keeps the equatorial position (Deslongchamps et al. 1981, Francke et al. 1980), whereas the stabilizing anomeric effect (Perron and Albizzati 1989) directs the oxygen of the alternate ring to the axial position causing (*E*)-conformation. The thermodynamical stability and polarity of *E/Z*-isomers are different (Booth et al. 2009, Pothier et al. 1992), and they separate readily in gas chromatography. The (*E,E*)-isomers elute first (Francke et al. 1981, Heiduk et al. 2015, Kitching et al. 1989), because the oxygens are more shielded by the substituents, which renders these stereoisomers less polar. The stereoisomers showing (*Z,Z*)-conformation (Fig. 1) elute last. The two oxygens, keeping equatorial positions, are even less shielded. These more polar compounds are unstable and almost absent in synthetic mixtures (Heiduk et al. 2015, Kitching et al. 1989).

Spiroacetals constituting 1,6-dioxaspiro[4.5]decane or 1,6-dioxaspiro[5.5]undecane produce highly significant mass spectra with a diagnostic doublet forming the most abundant signals. Due to retrocleavage at the six-membered ring, these fragments represent the corresponding 2-methylene-5-alkyltetrahydrofuran or 2-methylene-6-alkyltetrahydropyran sub-structures and the corresponding protonated lactone(s). A second set of key-fragments are produced by α -cleavage of the alkyl substituents adjacent to the ring oxygen (Francke and Kitching 2001). In the present study, those diagnostic signals, the molecular masses of the target compounds, and gas chromatographic retention times served to unambiguously classify spiroacetals into different chemical structures, each of which represented a set of stereoisomers that shared unequivocal analytical data. Chemical structures were termed by letters **A–H**, and individual isomers within each structure were further designated by a number that indicated the order of elution (e.g., **A1** and **A2** had similar mass spectra, and **A1** eluted before **A2**).

Results

As expected, headspace samples from males of *M. caryae* contained various components of the aggregation-sex pheromone, absent in samples from females, including 2-phenylethanol, (*S*)-(-)-limonene, (-)- α -terpineol, neral, and geranial (Online Resource 2). Headspace samples of both sexes contained a compound that was tentatively identified as 1-(methylthio)-2-methylbut-2-ene by a high confidence match of its mass spectrum with that reported in the NIST database, and which may contribute to the characteristic pungent odor of the agitated beetles. Unexpected, however, was the discovery of a suite of spiroacetals in headspace aerations of females and males of *M. caryae*. Samples from females consistently contained 16 compounds that presented mass spectra characteristic of spiroacetals of at least eight distinct chemical structures (Figs. 2, 3, Table 1).

According to its mass spectrum (Francke and Kitching 2001) showing a dominant ion doublet at m/z 84, m/z 87, and $M^+=156$, structure **A** (Fig. 3) was assigned to be 7-methyl-1,6-dioxaspiro[4.5]decane, represented by its (7*E*)-isomer, **A1**, and by its less stable (7*Z*)-isomer, **A2** (Table 1). Because of its low concentration, the identification of the latter was hampered by an incomplete spectrum. Structure **B** with m/z 98, m/z 101, and $M^+=156$ proved to be 2-methyl-1,6-dioxaspiro[4.5]decane, and **B1** was its (2*E*)-isomer (Fig. 3B). Structure **C** with m/z 98, m/z 101, m/z 155, and $M^+=170$ was shown to be 2,7-dimethyl-1,6-dioxaspiro[4.5]decane, with **C1** as the (2*E*,7*E*)-isomer and **C2** as the (2*Z*,7*E*)-isomer (Fig. 3C). Structure **D** with m/z 112, m/z 115, m/z 169, and $M^+=184$ was 2,8-dimethyl-1,7-dioxaspiro[5.5]undecane with the (2*E*,8*E*)-isomer **D1** and the (2*E*,8*Z*)-isomer **D2** (Fig. 3D). **E**, with m/z 98, m/z 101, m/z 155, m/z 169, and $M^+=184$ was 7-ethyl-2-methyl-1,6-dioxaspiro[4.5]decane. Compound **E1**, the dominant component in headspace samples of females of *M. caryae* (Figs. 1 and 3E), proved to be the (7*E*,2*E*)-isomer (Table 1), whereas the second most abundant compound, **E2**, was the (7*E*,2*Z*)-isomer (~9% of **E1**; Table 1). The remaining two compounds, assigned to structure **E** (**E3** and **E4**, Fig 1) were present in small amounts (usually <2% of **E1**; Table 1), and their (incomplete) spectra suggest the thermodynamically less favored (7*Z*,2*E*)- and (7*Z*,2*Z*)-stereoisomers, respectively. This is corroborated by the fact that the same compounds were present as trace amounts in the corresponding synthetic samples of **E1** and **E2**. Structure **F** with m/z 112, m/z 115, a prominent fragment at m/z 155, and $M^+=184$ was confirmed as 2-ethyl-7-methyl-1,6-dioxaspiro[4.5]decane (Fig. 3F), and **F1** and **F2** were the (2*E*,7*E*)- and (2*Z*,7*E*)-stereoisomers. Structure **G** with m/z 98, m/z 101, a diagnostic fragment at m/z 155, and $M^+=212$, was confirmed as 7-butyl-2-methyl-1,6-dioxaspiro[4.5]decane. The structure of **G1**, the (7*E*,2*E*)-stereoisomer, could be easily confirmed, however, the mass spectrum of the (7*E*,2*Z*)-isomer **G2** was overlaid by that of another spiroacetal (Fig 2., Online Resource 3A).

Due to the low intensity of the target GC-peak, the mass spectrum obtained from the mixture was incomplete but presented m/z 169 as the highest detectable signal. The structure of this spiroacetal was tentatively assigned after monitoring the diagnostic signals at m/z 98, m/z 101, m/z 111, m/z 155, and m/z 169 (SIM; Online Resource 3A). The ion pair at m/z 98 and m/z 101 indicated a 2-methyltetrahydrofuryl or an unsubstituted tetrahydropyranyl substructure, whereas the intensity of the fragment at m/z 111 suggested the second ring to be an oxepane (Francke and Kitching 2001). As (7*E*,2*Z*)-7-butyl-2-methyl-1,6-dioxaspiro[4.5]decane (**G2**) presents a molecular mass of $M^+=212$ and a significant signal at m/z 155 (M^+ -butyl), the most likely structure of the unknown compound (postulated mass of $M^+=212$, but producing a key-fragment at m/z 169 [M^+ -propyl] instead of m/z 155), was either 2-methyl-7-propyl-1,6-dioxaspiro[4.6]undecane (**H**), or 8-propyl-1,7-dioxaspiro[5.6]dodecane (**I**). The scope of m/z 169 and m/z 155 (Online Resource 3A) suggested that the unknown spiroacetal should elute as a shoulder in front of **G2**.

Stereoisomeric mixtures of **H** and **I** were synthesized as references for unambiguous identification (Online Resource 1), and (2*E*,7*E*)-2-methyl-7-propyl-1,6-dioxaspiro[4.6]undecane, (**H1**) (Fig. 3H) showed the expected mass spectrum and almost the same gas chromatographic retention time as **G2** (Online Resource 3B). The isomeric non-

natural **I**, though showing a similar fragmentation pattern than **H**, eluted significantly later; for its mass spectrum see Online Resource 1.

Headspace samples from males of *M. caryae* contained only five of the spiroacetals that were emitted by conspecific females, but **E1** again was dominant (Table 1). During the present study, however, headspace samples from males of *M. caryae* consistently contained another seven compounds that were not spiroacetals. Two of these compounds were identified by their mass spectra and authentic standards as (*E*)- β -farnesene and β -bisabolene. The remaining five compounds were not fully identified, but showed mass spectra indicating oxygenated monoterpenes (U1-U5; Online Resource 4).

Headspace samples from females of *M. robiniae* contained five spiroacetals that matched retention time and mass spectra of those identified from female *M. caryae*, but in very different proportions: **D1** was dominant, and **F2**, **E2**, **C1**, and **C2** were in much higher relative amounts than in samples from females of *M. caryae* (Table 1, Fig. 4). A sixth spiroacetal, identified only from females of *M. robiniae*, was (2*E*,7*E*)-2-ethyl-7-methyl-1,6-dioxaspiro[4.5]decane, **F1**.

Aeration extracts from both sexes of *C. apicicornis* (Fig. 4) contained one spiroacetal: (2*E*,8*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, **D1** (Fig. 3D, Online Resource 5).

Discussion

Adults of *M. caryae* and *M. robiniae* apparently produced spiroacetals as a form of chemical defense, because the compounds were released only when the beetles were agitated. This finding explains why spiroacetals were not detected in our earlier work on pheromones of the two species (Lacey et al. 2008; unpub. data). The glandular source of the chemicals has yet to be determined. Males of both species have pores on the prothorax that in other species of cerambycines are the source of pheromones (Ray et al. 2006), but their absence in females suggests that they are not the source of spiroacetals. Although Wheeler et al. (1988) did not find spiroacetals in exudate from metasternal glands of agitated adult *M. robiniae* of both sexes, the 11-carbon dominant component, *trans*-2-(*trans*-1-*cis*-3-hexadien-1-yl)-5-methyltetrahydrofuran, is structurally related to spiroacetals, suggesting these glands as a possible source. Metasternal glands of other species of cerambycids produce supposed defensive exudates of different chemical natures, primarily unsaturated cyclic aldehydes and phenols (reviewed by Millar and Hanks 2016).

Production of spiroacetals by adults of *M. caryae*, *M. robiniae*, and *C. apicicornis* suggests that they mimic their Hymenopteran models in odor, as well as in appearance. Social Hymenoptera recruit nestmates for defense by producing alarm pheromones, which commonly contain spiroacetals as major components (Bruschini and Cervo 2011; Bruschini et al. 2006; Fortunato et al. 2004; Francke and Kitching 2001). In fact, spiroacetals alone may be sufficient to induce an alarm response by the colony (Dani et al. 2000). Vertebrate predators may learn to associate the odor with the sting, and come to be repelled by the odor alone. In that case, spiroacetals produced by mimics should be the same as those produced by their sympatric models.

Consistent with this hypothesis, adult *C. apicicornis* produce the same spiroacetal as the Neotropical vespid *Polybia occidentalis* (Olivier): (2*E*,8*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (Dani et al. 2000), designated **D1** in our study. Moreover, the same compound is produced by the vespids *Polistes fuscatus* (F.) and *Eumenes fraternus* Say (RFM, unpub. data), and is the primary spiroacetal produced by adults of *M. robiniae*, which often forage side-by-side with those wasps on inflorescences of goldenrod (RFM, unpub. data). **D1** is also among the compounds produced by both sexes of *M. caryae*, which emerge in early spring when overwintering female vespids are active. Another potential model of the *Megacyllene* species is the vespid *Vespula maculifrons* (du Buysson), adults of which produce spiroacetals of structures **A** and **B** (RFM, unpub. data). The role of spiroacetals in mimicry is further supported by their production by the Palearctic cerambycids *Molorchus minor* (L.) and *Agapanthia villosoviridescens* (Meyer 1993), both of which may be visual mimics of Hymenoptera (Linsley 1959; RFM, pers. obs.). In fact, this hypothesis could explain production of spiroacetals by many other insect species that also appear to be Batesian mimics of aculeate Hymenoptera, including staphylinid beetles, tephritid flies, and ichneumonid wasps (Francke and Kitching 2001).

The variety of spiroacetals produced by adult *Megacyllene* may be the product of selection for chemical mimicry across a broad range of hymenopteran models that differ in the chemistry of their alarm pheromones, and in their geographic distribution. In fact, spiroacetals matching the **A**, **B**, **D**, **E**, and **F** structures all are produced by various vespid species native to the Palearctic (Francke and Kitching 2001). Alternatively, the multiple structures of spiroacetals produced by *M. caryae* could be the product of non-selective biosynthesis, which nevertheless could be adaptive if potential predators are repelled by the appearance and general spiroacetal odor of vespid wasps, rather than by particular isomers of spiroacetals. Therefore, we did not specifically look for the enantiomeric composition of spiroacetals in the investigated cerambycid species. However, detailed analyses using enantioselective gas chromatography may be necessary to examine special relationships.

Spiroacetals are also known to be produced by many insect species that do not appear to be Hymenopteran mimics (Francke and Kitching 2001), as well as several species of plants (Beck et al. 2008; Heiduk et al. 2015; Zhang et al. 2002). They are believed to serve as defensive allomones in staphylinid beetles (Huth and Dettner 1990; Zhang et al. 1999), behavioral inhibitors in scolytine bark beetles (Zhang et al. 2002), and sex pheromones for some species of flies in the genus *Bactrocera* (Booth et al. 2009). Thus, spiroacetals of cerambycids may serve purposes other than odor mimicry. For example, the spiroacetal conophthorin (structure **A**) is produced by some species of trees and apparently serves to inhibit aggregation in scolytine bark beetles (Francke and Kitching 2001; Zhang et al. 2002), and disrupts the kairomonal attraction of pine sawyers (*Monochamus* species, Cerambycidae) to pheromones of bark beetles (Morewood et al., 2003). The compounds also may be used by *Megacyllene* species as defensive allomones or alarm pheromones (e.g., Greeney and DeVries 2004), or serve some other intraspecific function. For instance, the fact that spiroacetal **E2** was present at relatively high levels in the blend produced by female *M. caryae*, but was absent in blends produced by males, suggests that it could serve as a sex-specific signal.

It should also be noted that the quantitative composition of a mixture of synthetic stereoisomeric spiroacetals (e.g. **E1–E4**, Fig. 1) will differ from that of the natural bouquet: the lab synthesis is thermodynamically controlled, whereas the biosynthesis of the natural products is enzymatically (kinetically) controlled. As a result, natural blends contain much higher amounts of the less stable isomers (e.g., **E3** and **E4** in Fig. 1) (Francke and Kitching 2001). Consequently, relative proportions will change with time. It is yet unknown whether such an “inbuilt clock” of the bouquet is of any biological significance.

In conclusion, spiroacetals are produced by cerambycid species of diverse taxonomic lineages, and it seems likely that they play an important defensive role in mimicry systems. Moreover, the association of spiroacetals with aculeate Hymenoptera extends beyond the social wasps (e.g., the potter wasp *E. fraternus*) and may itself be a Muellierian mimicry system. Our study suggests a rich ecological context for the production and use of spiroacetals, and we hope it will serve as a foundation for future research on their role in interactions between insects and their vertebrate predators.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

A: 2,9-Dihydroxyundecan-5-one forms 4 pairs of enantiomers of 7-ethyl-2-methyl-1,6-dioxaspiro[4.5]decane (Structure **E**). Only one of each is shown. B: 2,10-Dihydroxyundecan-6-one forms 2,8-dimethyl-1,7-dioxaspiro[5.5]undecanes (Structure **D**).

Fig. 2.

Representative total ion chromatogram of headspace volatiles produced by an agitated adult female of *Megacyllene caryae*. Peak labels indicate general chemical structures and elution order, as in Table 1. Spiroacetal **F1** may be obscured by **E1**. Spiroacetals **G3** (12.75) and **D2** (10.25), and 1-(methylthio)-2-methylbut-2-ene (5.34) are not present in the headspace. AT-5ms column, programmed from 40 °C (1 min hold) to 160 °C at 10 °C/min).

Fig. 3. Representative mass spectra of spiroacetal structures recovered from headspace volatiles of agitated adults of *Megacyllene caryae* and *M. robiniae*. Figure labels (A, B, C, etc.) indicate general chemical structures, as in Table 1 (spectra obtained with an HP5973 instrument).

Fig. 4.

Representative total ion chromatogram of headspace volatiles from agitated adults of *Megacyllene robiniae* (AT-5ms column, 40 °C/1 min, 10 °C/min, end at 160 °C/1 min) and *Callisphyrus apicicornis* (VF5-ms column, 50 °C/2 min, 8 °C/min, end at 270 °C). Chromatograms trimmed to 6–12 min (above), or paired with a negative control (below), to improve visibility of peaks. Peak labels indicate general chemical structures and elution order, as in Table 1.

Table 1

Spiroacetals produced by adults of the cerambycid beetles *Megacyllene caryae*, *M. robiniae*, and *Callisphyrus apicicornis*. Compounds are labeled by a letter that indicates the general chemical structure (based on mass spectrum; see text) and a number indicating elution order of stereoisomers from an AT-5ms column, programmed from 40 °C (1 min hold) to 160 °C at 10 °C/min. The relative configuration is indicated for compounds whose structure could be confirmed with authentic standards. Data are expressed as averaged peak areas (± 1 SE) relative to the dominant peak in the traces, per sex and species, and in parentheses are the numbers of headspace samples that contained the compound in detectable quantities.

Structure (isomer)	Species and sex (sample size)			
	<i>M. caryae</i> ♀ (N=10)	<i>M. caryae</i> ♂ (N = 10)	<i>M. robiniae</i> ♀ (N = 5)	<i>C. apicicornis</i> ♀/♂ (N = 13)
“A”: 7-methyl-1,6-dioxaspiro[4.5]decane				
A1 (7 <i>E</i>)	0.03 $\pm$ 0.02 (3)			
A2 (7 <i>Z</i>)	0.62 $\pm$ 0.61 (2)			
“B”: 2-methyl-1,6-dioxaspiro[4.5]decane				
B1 (2 <i>E</i>)	0.24 $\pm$ 0.11 (5)			
“C”: 2,7-dimethyl-1,6-dioxaspiro[4.5]decane				
C1 (2 <i>E</i> ,7 <i>E</i>)	1.73 $\pm$ 0.37 (9)	7.81 $\pm$ 4.39 (7)	12.0 $\pm$ 7.6 (2)	
C2 (2 <i>Z</i> ,7 <i>E</i>)	0.10 $\pm$ 0.05 (4)		6.82 $\pm$ 4.3 (2)	
“D”: 2,8-dimethyl-1,7-dioxaspiro[5.5]undecane				
D1 (2 <i>E</i> ,8 <i>E</i>)	0.76 $\pm$ 0.2 (10)	0.89 $\pm$ 0.89 (1)	100 (5)	100 (11)
D2 (2 <i>E</i> ,8 <i>Z</i>)	<0.01 (1)			
“E”: 7-ethyl-2-methyl-1,6-dioxaspiro[4.5]decane				
E1 (7 <i>E</i> ,2 <i>E</i>)	100 (10)	100 (10)		
E2 (7 <i>E</i> ,2 <i>Z</i>)	9.19 $\pm$ 2.4 (10)		12.4 $\pm$ 6.4 (4)	
E3 ¹	0.15 $\pm$ 0.06 (5)			
E4 ¹	1.51 $\pm$ 0.32 (9)	N/A ² (6)		
“F”: 2-ethyl-7-methyl-1,6-dioxaspiro[4.5]decane				
F1 (2 <i>E</i> ,7 <i>E</i>)			69.9 $\pm$ 9.1 (5)	
F2 (2 <i>Z</i> ,7 <i>E</i>)	0.20 $\pm$ 0.08 (5)		31.8 $\pm$ 14.6 (4)	
“G”: 7-butyl-2-methyl-1,6-dioxaspiro[4.5]decane				
G1 (7 <i>E</i> ,2 <i>E</i>)	1.10 $\pm$ 0.44 (8)	3.49 $\pm$ 2.3 (5)		
G2 ³ (7 <i>E</i> ,2 <i>Z</i>)	0.06 $\pm$ 0.04 (2)			
G3 ¹	0.02 $\pm$ 0.02 (1)			
“H”: 2-methyl-7-propyl-1,6-dioxaspiro[4.6]undecane				
H1 ³ (2 <i>E</i> ,7 <i>E</i>)	0.06 $\pm$ 0.04 (2)			

¹ Relative configuration unknown;

² Peak area obscured by geranial;

³ Peak area comprises sum of **G2** and **H1**