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Enantiomers of fuscumol acetate comprise the aggregation-sex pheromone of the South American cerambycid beetle *Psapharochrus maculatissimus*, and likely pheromones of the cerambycids *Eupromerella plaumanni* and *Hylettus seniculus*

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Abstract

There is increasing evidence that pheromone chemistry within the large coleopteran family Cerambycidae is often highly conserved, with numerous related species sharing the same pheromone components. As a result, traps containing these components can attract multiple cerambycid species simultaneously. In the present study, we exploited this concept in the identification of the male-produced aggregation-sex pheromone of the South American species *Psapharochrus maculatissimus* (Bates) (Coleoptera: Cerambycidae, subfamily Lamiinae, tribe Acanthoderini). Initially, live adults of both sexes were caught using a trap baited with a lure containing a blend of known cerambycid pheromone components. Headspace volatiles were collected from live beetles and analyzed by coupled gas chromatography-mass spectrometry. Males of *P. maculatissimus* sex-specifically produced a 1:38 blend of (*R*)-fuscumol acetate ([2*R*,5*E*]-6,10-dimethylundeca-5,9-dien-2-yl acetate) and (*S*)-fuscumol acetate, which were both components of the pheromone lures to which they had been attracted. In more focused field trials, traps baited with the (*S*)-enantiomer, or a blend approximating the natural 1:38 ratio of (*R*)- to (*S*)-enantiomers, attracted adults of both sexes in approximately equal numbers. During bioassays, adults of the lamiine species *Eupromerella plaumanni* (Fuchs) (tribe Acanthoderini) and *Hylettus seniculus* (Germar) (Acanthocinini) also were attracted, but to different lures, with *E. plaumanni* being attracted to the racemic mixture of the two enantiomers of fuscumol acetate, whereas *H. seniculus* was attracted specifically to (*R*)-fuscumol acetate. Our results suggest that differences between these sympatric species in the stereochemistry of fuscumol acetate impart species-specificity to pheromone communication channels, similar to what has been found recently with lamiine species from other continents.

Introduction

Over the past 15 years, substantial progress has been made in the identification of attractant pheromones for the large beetle family Cerambycidae (Coleoptera) (Hanks & Millar, 2016). One noteworthy point from this cumulative body of research is that there often appears to be a high degree of parsimony in the use of pheromone components by closely related species, to the extent that congeners native to different continents, which have been geographically isolated for millions of years, still frequently share the same pheromones (Hanks & Millar, 2016). For example, 3-hydroxyalkan-2-ones and the analogous 2,3-alkanediols have been identified as male-produced aggregation-sex pheromones (sensu Cardé, 2014), or pheromone candidates for

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numerous species in the subfamily Cerambycinae (reviewed by Millar & Hanks, 2017). In analogous fashion, male-produced hydroxyethers such as monochamol (2-[undecyloxy]ethanol) and terpenoid derivatives such as fuscumol ([*E*]-6,10-dimethyl-5,9-undecadien-2-ol) and fuscumol acetate ([*E*]-6,10-dimethyl-5,9-undecadien-2-yl acetate) represent common pheromone motifs identified from cerambycid species in the subfamilies Lamiinae and Spondylidinae (Millar & Hanks, 2017).

As a consequence of these shared pheromones, traps baited with single compounds or blends of similar components may attract multiple cerambycid species simultaneously. This pattern has now been observed in field bioassays carried out on different continents (e.g., Asia: Sweeney et al., 2014; Wickham et al., 2014; Africa: Bobadoye et al., 2018; Australia: Hayes et al., 2016; Europe: Flaherty et al., 2019; Rassati et al., 2019; North America: Millar et al., 2018), and it can be exploited in surveillance programs for detecting quarantine pests (Fan et al., 2019). This parsimony also implies that identification of pheromone components for one cerambycid species may subsequently expedite the identification of pheromones or likely pheromones for related target species (Millar et al., 2018, 2019).

Despite rapid advances in our understanding of the pheromone chemistry of cerambycid beetles, there are still vast gaps in our knowledge. For example, only a few pheromone compounds have been identified from species in the largest subfamily, the Lamiinae, especially those native to South America (Millar & Hanks, 2017). To our knowledge, possible pheromone components have been identified from only two South American lamiine species, both in the tribe Acanthoderini, specifically, *Hedypathes betulinus* (Klug), whose adult males produce a blend of (*R*)-fuscumol, (*R*)- and (*S*)-fuscumol acetate, and geranylacetone (Fonseca et al., 2010), and *Steirastoma breve* (Sulzer), whose males produce fuscumol of unknown chirality (Liendo-Barandiaran et al., 2010). Even for these two species, the results of field bioassays of the putative pheromones have not yet been reported.

As part of an ongoing project to assess possible attractant pheromones for cerambycid species native to Brazil, we describe the identification and field testing of a male-produced aggregation-sex pheromone for the lamiine *Psapharochrus maculatissimus* (Bates) (also tribe Acanthoderini). To our knowledge, there is no published information on the biology of this species. Its distribution appears to be restricted to South America, specifically Brazil (states of Pará, Goiás, Mato Grosso, and Amazonas), Bolivia, and Peru (Monné, 2018; Tavakilian & Chevillotte, 2018). We serendipitously captured this species in 2016 during field screening trials conducted in the Brazilian state of São Paulo (which incidentally represents a new state record for this species) while screening multicomponent cerambycid pheromone lures. Based on the history of conservation of pheromone structures among related cerambycid species, we hypothesized that these lures likely contained one or more components of the pheromone of *P. maculatissimus*. We report here the identification and field testing of two volatile compounds produced by males of this species. We further report the attraction of two additional lamiine species, *Eupromerella plaumanni* (Fuchs) (Acanthoderini) and *Hylettus seniculus* (Germar) (Acanthocinini), during field trials of the *P. maculatissimus* compounds. As with *P. maculatissimus*, to our knowledge there is no published information on the biology of *E. plaumanni*, other than the fact that it has been reported from several states in Brazil and from Bolivia (Monné, 2018). For *H. seniculus*, its reported range embraces much of Central and South America, ranging from Costa Rica in the north to Paraguay in the south. It apparently has a broad host range which comprises plant species in the families Anacardiaceae, Burseraceae, Flacourtiaceae, Malvaceae, Pinaceae, and Rutaceae (including, for instance, mango, citrus, boxwood, and pines) (Monné, 2018).

Materials and methods

Source of chemicals

Racemic 3-hydroxyhexan-2-one, monochamol, racemic fuscumol, and racemic fuscumol acetate were purchased from Bedoukian Research (Danbury, CT, USA), and racemic 2-methylbutan-1-ol from Sigma-Aldrich (St. Louis, MO, USA). *Syn*- and *anti*-2,3-hexanediol were synthesized as described in Lacey et al. (2004). The (*R*)- and (*S*)-enantiomers of fuscumol acetate were prepared in 96.6 and 98.0% enantiomeric excess, respectively, by enzyme-based kinetic resolution of racemic fuscumol, as described in Hughes et al. (2013).

Source of beetles

Adult males and females of *P. maculatissimus* initially were collected alive with a cross-vane intercept panel trap (black corrugated plastic) hung from an inverted L-shaped frame made from PVC pipe (for details see Silva et al., 2018). The trap basin was replaced with a 5-l jar with 2-mm holes drilled in its base for rainwater drainage. Internal surfaces of the trap and collection jar were coated with a 50% aqueous dispersion of Fluon (Insect-a-Slip; Bioquip, Rancho Dominguez, CA, USA). The lure consisted of a clear plastic press-seal sachet (polyethylene, 5 × 7.5 cm, 0.05 mm wall thickness; Bagettes model 14770; Cousin, Largo, FL, USA), containing a cotton dental roll loaded with 275 mg of a multicomponent pheromone blend in 725 µl of

isopropanol. This blend contained known attractant pheromones previously identified from several cerambycid species in the subfamilies Cerambycinae [racemic 3-hydroxyhexan-2-one (50 mg), racemic *syn*- and *anti*-2,3-hexanediol (50 mg), and racemic 2-methylbutan-1-ol (50 mg)], and Lamiinae [racemic fuscumol and fuscumol acetate (50 mg each), and monochamol (25 mg)]. The lure was hung from the central slot in the trap and replaced every 2 weeks.

The trap was deployed in a remnant forest of cerrado (Brazilian savanna) located in Valentim Gentil, state of São Paulo, Brazil (−20.372 latitude, −50.080 longitude). The trap was serviced daily throughout September 2017. Male and female beetles were sexed by the length of the fifth abdominal sternite (longer in females) and placed individually in plastic containers with sucrose solution (10%) dispensed from glass vials plugged with cotton rolls for moisture and nutrition. Beetles were shipped via overnight courier to University of São Paulo, Piracicaba (ca. 400 km from Valentim Gentil) where they were allowed to acclimate under laboratory conditions (23 ± 2 °C, 60 ± 10% r.h., and 12 h photophase) for 24 h prior to collection of headspace volatiles.

Collection of beetle-produced volatiles

Adult beetles of the same sex were aerated in groups of five, in custom-made cylindrical glass chambers (25 cm long × 6 cm inner diameter) containing two glass vials with sugar solution. Volatiles emitted by beetles were trapped on 150 mg of 80/100 mesh HayeSep Q adsorbent (Supelco, Bellefonte, PA, USA) in a glass pipette (8.5 cm long × 0.5 cm i.d.) with the adsorbent held in place with glass wool plugs. Collectors were connected to the outlets of the chambers with a screw cap fitted with a teflon ferrule. Charcoal-filtered air was pushed through the chambers at 200 ml per min. Volatiles were collected continuously from groups of beetles for 48 h under the environmental conditions described above, and collections were made from each group no more than twice. Volatiles also were collected from chambers containing feeder vials but no beetles, as controls to monitor for system contaminants.

Trapped volatiles were eluted from collectors with three 500-µl aliquots of methylene chloride into silanized amber glass vials. Each extract was then concentrated to 500 µl under a gentle flow of N_2 and stored at −30 °C until analysis. We obtained eight aeration extracts from males and five from females.

Identification of pheromone candidates

Extracts of volatiles were initially analyzed in Brazil by gas chromatography (GC) with flame ionization detection to confirm the presence of sex-specific compounds. Two microliters of extract was injected into a GC-2010 gas chromatograph (Shimadzu, Kyoto, Japan) fitted with an HP5-MS capillary column (30 m × 0.25 mm i.d. × 0.25 µm film; Agilent Technologies, Santa Clara, CA, USA). Injections were made in splitless mode (purge valve off for 1 min) with an injector temperature of 250 °C and helium carrier gas at a linear velocity of 25 cm s^{-1}. The GC oven was programmed from 35 °C for 1 min, increased to 40 °C at 2 °C per min, held 1 min, and increased to 250 °C at 10 °C per min, held 10 min. Representative extracts (four from males and two from females) were sent to the University of California, Riverside, where they were reanalyzed by coupled GC-mass spectrometry with an Agilent 7820A GC fitted with a DB-5 column (30 m × 0.25 mm diameter, 0.25 µm film thickness; J&W Scientific, Folsom, CA, USA) interfaced to an Agilent 5977E mass selective detector. The GC oven was programmed from 40 °C for 1 min, 10 °C per min to 280 °C, held 20 min. One microliter injections were made in splitless mode (injector 250 °C, transfer line 280 °C, split vent opened at 0.5 min), with helium carrier gas (inlet pressure 89.6 kPa). Mass spectra were recorded at 70 eV in EI mode, from 40 to 450 m/*z*, with a 3-min solvent delay.

To determine which enantiomer(s) of fuscumol acetate the insects produce, extracts of volatiles from the male beetles were further analyzed on a chiral stationary phase Cyclodex B GC column (30 m × 0.25 mm i.d., 0.25 µm film thickness; J&W Scientific). Injections were made in split mode (injector temperature 200 °C), and the GC was programmed from 50 °C for 1 min, 3 °C per min to 220 °C, held 20 min. The GC was equipped with a flame ionization detector (240 °C). Authentic standards of (*R*)- and (*S*)-fuscumol acetate were analyzed under the same conditions, and the identifications of the insect-produced enantiomers were verified by coinjection of a blend of an insect extract with the racemic standard.

Field bioassays of pheromone candidates

Field bioassays of synthesized compounds were conducted in the remnant of cerrado forest in Valentim Gentil as described above, from 4 September to 10 October 2018. The same type of traps was used, except that the supplied collection jars were filled with 300 ml of a saturated aqueous solution of NaCl with a few drops of dish detergent, to kill and preserve the captured beetles. Lures consisted of the polyethylene sachets described above, which were loaded with 1 ml solutions of synthetic pheromones diluted in isopropanol. Treatments were: (1) (*S*)-fuscumol acetate (25 mg); (2) (*R*)-fuscumol acetate (25 mg); (3) natural blend of (*R*) = 0.65 mg + (*S*) = 25 mg; (4) 1:1 blend of (*R*)- + (*S*)-fuscumol acetate (25 mg

each = racemic); and (5) control (1 ml neat isopropanol). Five traps were positioned 15 m apart in each of four blocks (30 m apart), and treatments were assigned randomly to traps within blocks on the day of setup. Traps were serviced every 2 days, at which time the treatments were rotated within blocks to minimize positional effects. Lures were replaced every 2 weeks. Voucher specimens of captured beetles have been retained at the Laboratory of Chemical Ecology and Insect Behavior, Department of Entomology and Acarology, USP, Piracicaba.

Statistical analysis

Differences between treatment means were tested separately for each species that was represented by at least 10 specimens, using the non-parametric Friedman's test (Proc FREQ, option CMH; SAS SAS Institute, 2011) because field data violated assumptions of ANOVA (Sokal & Rohlf, 1995). Replicates were defined by block and collection date. We considered in each analysis only replicates that had a minimum number of specimens (ranging from 1 to 11 depending on species), which was determined to guarantee a sufficient number of replicates for a robust analysis ($n \geq 12$ replicates). In recognition of the multiple statistical tests of treatment effects, significance levels were adjusted to $\alpha = 0.017$ according to the Bonferroni procedure (Quinn & Keough, 2002), assuming five treatments and three independent analyses. Pairs of means were compared using the REGWQ multiple range test, which controls the type I experiment-wise error rate (SAS Institute, 2011). The sex ratio of adults of *P. maculatissimus* captured by traps baited with the optimal attractant ([*S*]-fuscumol acetate; see Results) was compared to a nominal proportion of 0.5 with 95% Clopper–Pearson exact confidence intervals (Newcombe, 1998). Sexes of captured adults of *E. plaumanni* and *H. seniculus* could not be separated based on morphology.

Results

Identification of pheromone candidates

Analyses of headspace volatiles emitted by adults of *P. maculatissimus* revealed the presence of a single prominent peak in extracts of males that was absent in equivalent extracts from females (Figure 1), as well as from system controls. The retention time and EI mass spectrum of the compound matched those of an authentic standard of fuscumol acetate. Further analyses of the extracts on a Cyclodex B chiral stationary phase GC column showed that the beetles produced a non-racemic mixture of the two enantiomers of fuscumol acetate, with the average ratio of (*R*)- and (*S*)-fuscumol acetate in three representative samples being ca. 1:38.

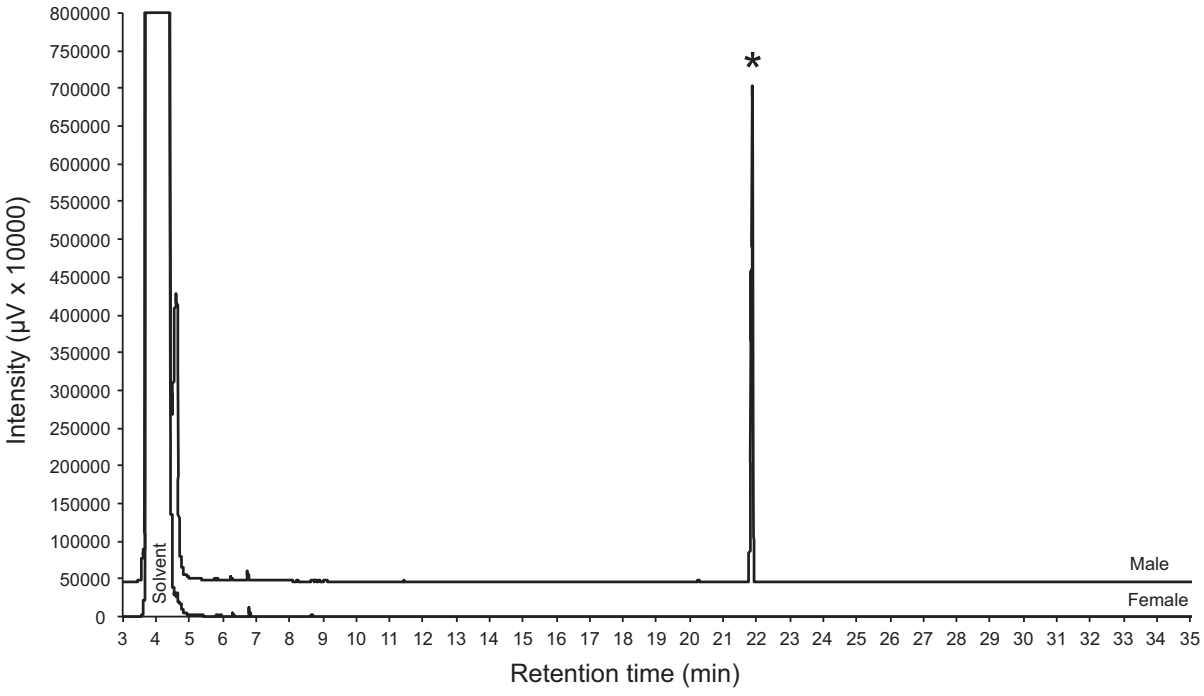

Figure 1 Representative gas chromatograms of extracts of headspace odors from adult males (upper trace) and females (lower trace) of *Psapharochrus maculatissimus*. The asterisk indicates the single, large sex-specific peak in extracts from males.

Field bioassays of pheromone candidates

In total, 518 cerambycid beetles representing 13 species were caught during field bioassays, with most of the species being caught in small numbers with no pattern to the catches, which probably represent random encounters with traps. The most numerous species was the targeted *P. maculatissimus* (n = 417), but two other lamiine species were caught in numbers sufficient for statistical analysis, *E. plaumanni* (n = 35) and *H. seniculus* (n = 19).

Adults of *P. maculatissimus* were attracted in significant numbers only to traps baited with (*S*)-fuscumol acetate alone, or with the 38:1 blend with (*R*)-fuscumol acetate that approximated the natural ratio. The racemic blend and (*R*)-fuscumol acetate were not more attractive than the unbaited control (Figure 2). The sex ratio of captured beetles attracted by (*S*)-fuscumol acetate was not significantly different from 1:1 (46% females; 95% Clopper–Pearson exact confidence interval of 0.39–0.53, P = 0.22).

In contrast, adults of *H. seniculus* were only significantly attracted to (*R*)-fuscumol acetate, and were apparently antagonized by the (*S*)-enantiomer because the racemic fuscumol treatment did not attract this species (Figure 3A). Furthermore, adults of *E. plaumanni* also showed a unique response to the test treatments by responding only to the racemic blend of fuscumol acetate isomers (Figure 3B), and not to the 38:1 blend, nor to the individual enantiomers.

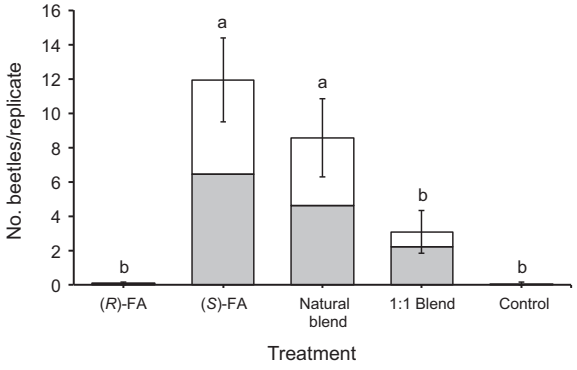

Figure 2 Mean (± SE) number of adults of *Psapharochrus maculatissimus* (sexes combined; males, gray; females, white) caught in traps baited with synthetic enantiomers of fuscumol acetate (individually or as blends). Treatments: (*R*)-FA = (*R*)-fuscumol acetate; (*S*)-FA = (*S*)-fuscumol acetate; Natural blend = 1:38 blend of (*R*)- and (*S*)-fuscumol acetate (same ratio produced by adult males); 1:1 Blend = racemic blend of (*R*)- and (*S*)-fuscumol acetate; and Control = neat isopropanol. Bait type had a significant effect on trap catch (Friedman's Q = 46.9, d.f. = 4,70, P<0.0001). Means capped by the same letter are not significantly different (REGWQ multiple range test: P>0.05).

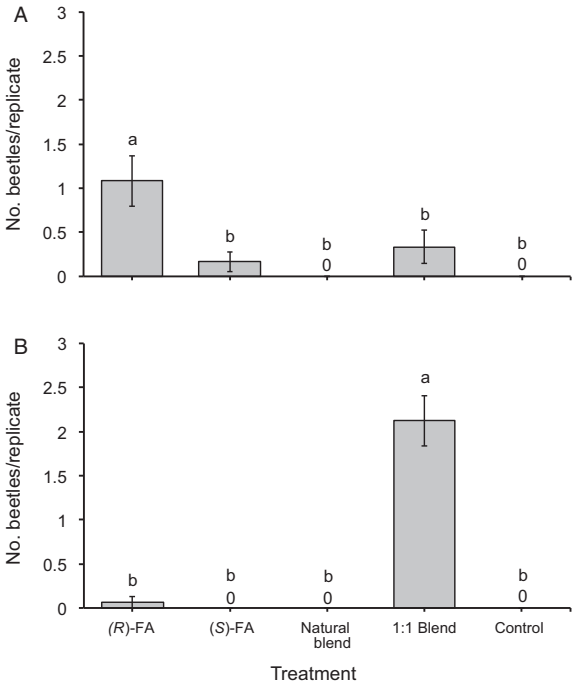

Figure 3 Mean (± SE) number of adults of (A) *Hylettus seniculus* and (B) *Eupromerella plaumanni* captured in traps baited with synthetic enantiomers of fuscumol acetate (individually or as blends). Treatments: (*R*)-FA = (*R*)-fuscumol acetate; (*S*)-FA = (*S*)-fuscumol acetate; Natural blend = 1:38 blend of (*R*)- and (*S*)-fuscumol acetate (same ratio produced by adult males of *Psapharochrus maculatissimus*); 1:1 Blend = racemic blend of (*R*)- and (*S*)-fuscumol acetate; and Control = neat isopropanol. Bait type had a significant effect on trap catch (Friedman's Q = 73.6, d.f. = 4,80, P<0.0001 and Q = 21.8, d.f. = 4,60, P = 0.0002, respectively). Means within a panel (species) capped by the same letter are not significantly different (REGWQ multiple range test: P>0.05).

Discussion

Fuscumol acetate was first identified as a pheromone component of cerambycid beetles in the subfamily Lamiinae from the South American species *H. betulinus*, males of which produce both enantiomers of (*R*)- and (*S*)-fuscumol acetate, (*R*)-fuscumol, and geranylacetone (Fonseca et al., 2010). In laboratory bioassays, only the complete blend was attractive to females (Vidal et al., 2010), but to our knowledge, the results of field tests with the putative pheromone components have not yet been reported. Thus, the study described here represents the first reported

confirmation of the biological activity of an attractant pheromone from field trials for a South American lamiine. We determined that the sex-specific volatiles emitted by adult males of *P. maculatissimus* were composed primarily of (*S*)-fuscumol acetate, along with trace amounts of (*R*)-fuscumol acetate. However, in the field bioassay, the synthetic recreation of the naturally produced 1:38 blend of (*R*)- to (*S*)-fuscumol acetate was not more attractive to beetles than (*S*)-fuscumol acetate as a single component, indicating that the (*S*)-enantiomer is both necessary and sufficient for full attraction. Increasing the ratio of enantiomers to 1:1 resulted in decreased attraction, demonstrating that adults of *P. maculatissimus* do indeed detect the (*R*)-enantiomer, and that it antagonizes attraction when present in higher proportions relative to the (*S*)-enantiomer. Overall, males and females were attracted in approximately equal numbers, confirming that the pheromone is an aggregation-sex pheromone attractive to both sexes, as is the case with all other lamiine species for which volatile pheromones have been identified (Millar & Hanks, 2017).

In contrast, adults of *E. plaumanni* were significantly attracted only by lures containing the 1:1 racemic blend of (*R*)- and (*S*)-fuscumol acetate, indicating that the two enantiomers were both necessary and sufficient for attraction. On the other hand, adults of *H. seniculus* were significantly attracted only to the pure (*R*)-enantiomer. The actual pheromone chemistry of these species remains to be determined, but these results strongly suggest that both enantiomers of fuscumol acetate will be found in headspace extracts from males of *E. plaumanni*, and at least the (*R*)-enantiomer will be found in extracts from males of *H. seniculus*.

These results also suggest that partitioning of the pheromone channel minimizes cross-attraction among these sympatric species that overlap in seasonal flight period. That is, *P. maculatissimus* and *H. seniculus* would not cross-attract because they appear to use different enantiomers of fuscumol acetate, whereas *P. maculatissimus* and *E. plaumanni* also would not cross-attract, because the former uses primarily the (*S*)-enantiomer in its pheromone whereas the other appears to require an approximately equal blend of both enantiomers. Similarly, *H. seniculus* was only significantly attracted to the (*R*)-enantiomer and not to the racemate, whereas *E. plaumanni* was only attracted to the racemate, so these two species also would not cross-attract one another. Several other lamiine species native to North America and Europe recently have been found to use similar strategies to minimize cross-attraction of sympatric heterospecifics (Meier et al., 2016; Millar & Hanks, 2017). Thus, this manipulation of the enantiomers of fuscumol acetate and/or fuscumol to create species-specific pheromone channels appears to be an emerging theme among the pheromones of this subfamily.

It is also noteworthy that the captures of *P. maculatissimus* and *E. plaumanni* represent new state records because to our knowledge, neither species had been recorded previously in the state of São Paulo (Monné, 2018; Tavakilian & Chevillotte, 2018). From a practical viewpoint, our findings reinforce the concept that pheromone-baited traps can be efficient and effective tools for detecting specific target species, and for delineating their geographical distributions. Efficient detection is important in the multiple contexts of detection of potentially dangerous invasive species, monitoring populations of rare or endangered native species, and monitoring populations of native species which are emerging as new pests due to changes in agricultural or forestry practices. For example, *H. seniculus* has been reported to cause damage in citrus orchards in northern Brazil (Moreira et al., 2003) and has been intercepted in timber imported to Europe from South America (Duffy, 1953). Therefore, the pheromone-based methods described here could be applied for monitoring the range and spread of this species within South America, and for its early detection in shipments at ports of entry on other continents.

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