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Biosynthesis and Site of Production of Sex Pheromone Components of the Cerambycid Beetle, *Hedypathes betulinus*

Paulo H. G. Zarbin · Marcy G. Fonseca · Daiane Szczerbowski · Alfredo R. M. Oliveira

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Abstract We determined the site of pheromone production tissues and a partial route for the biosynthesis of the sex pheromone in *Hedypathes betulinus* (Coleoptera: Cerambycidae: Lamiinae), Brazil's main green maté pest. Pheromone was found predominantly in the prothorax of males, suggesting that this is the region of production of pheromones in this insect. Scanning electron microscopy revealed small pores that may be associated with pheromone release in males; these pores also were observed in females. A deuterium-labeled putative precursor (geranyl acetone-D_5) of the sex pheromone of *H. betulinus* was synthesized. When applied to the prothorax of males, label from the precursor was incorporated into the pheromone components, confirming that pheromone production occurs in the prothorax and that the pheromone components are biosynthesized from geranyl acetone.

Keywords SEM · Pheromone biosynthesis · Deuterated precursor · Fuscumol acetate · Coleoptera · Cerambycdae · Insect pest

Introduction

Hedypathes betulinus (Coleoptera: Cerambycidae: Lamiinae) is the most important pest of green maté (*Ilex paracguariensis*), a popular kind of tea in the southern region of Brazil. Larvae of this insect burrow into, and feed on, the branches and trunks of green maté, disrupting translocation and killing the plant. As larvae bore through the plant they produce characteristic sawdust, which accumulates at the base of the stalk (Brandão Filho, 1945; Mazuchowski, 1991; Cassanello, 1993).

In olfactometer biossays, female *H. betulinus* responded to volatiles released by male conspecifics, suggesting the presence of a male-produced sex pheromone (Fonseca and Zarbin, 2009). Three male-specific compounds that were bioactive in olfactometer bioassays were identified as (*E*)-6,10-dimethyl-5,9-undecadien-2-yl acetate (major component; fuscumol acetate), (*E*)-6,10-dimethyl-5,9-undecadien-2-ol (minor component; fuscumol), and (*E*)-6,10-dimethyl-5,9-undecadien-2-one (geranyl acetone) (Fonseca et al., 2010). Enantioselective synthesis and comparative gas chromatography using a chiral column revealed that the natural constituents are enantiopure (*R*)-fuscumol acetate and a mixture of (*R*)- and (*S*)-fuscumol in a ratio of 82.4 % and 17.6 %, respectively (Vidal et al., 2010).

Fuscumol also has been identified as a male-produced aggregation pheromone in the cerambycid species *Tetropium fuscum*, *T. cinnamopterum* (Silk et al., 2007), and *Steirastoma breve* (Liendo et al., 2005), while fuscumol acetate has been identified only in *H. betulinus*. However, Mitchell et al. (2011) recently demonstrated that fuscumol and fuscumol acetate attract numerous cerambycid species from different tribes and subfamilies.

In males of several species in the subfamily Cerambycinae, pheromones are produced in sex-specific glands in the prothorax, and discharged through pores situated within depressions in the cuticle (Ray et al., 2006; Lacey et al., 2007a). A morphological survey of 65 Cerambycinae species revealed that 49 species in 14 tribes had analogous male-specific prothoracic pores (Ray et al., 2006). The sex-specific nature of pheromone glands in these Cerambycinae species suggests

P. H. G. Zarbin (✉) · M. G. Fonseca · D. Szczerbowski · A. R. M. Oliveira
Laboratório de Semioquímicos, Departamento de Química, Universidade Federal do Paraná, CP 19081, 81531-990, Curitiba, PR, Brazil
e-mail: pzarbin@ufpr.br

that gland pores could serve as a convenient morphological indicator of pheromone production by males (Ray et al., 2006). In contrast to this survey of Cerambycinae species, the site of a sex pheromone gland has not been determined for any member of the subfamily Lamiinae.

Among families of Coleoptera, pheromone biosynthesis has been studied most extensively in the Scolytidae, although other species in other families, including Scarabaeidae, Tenebrionidae, Curculionidae, and Silvanidae, also have been studied (Seybold and Vanderwel, 2003). Because advances in the chemical ecology and pheromone chemistry of Cerambycidae have occurred mainly over the last decade, little is known about the biosynthetic pathways and regulatory factors that control pheromone production in this family. This paper reports the site of the sex pheromone-producing tissue of *H. betulinus* and describes our initial studies on the biosynthesis of the pheromone components.

Methods and Materials

Source of Beetles Adults insects were collected from green maté in São Mateus do Sul, Parana, Brazil and transferred to the laboratory. Insects were sexed according to Cassanello (1993), on the basis that males have thicker antennae scapes and femur forelegs than do females. Males and females were held separately in plastic boxes (7.5 cm diam.×8.5 cm high), fed with green maté branches, and maintained at 25±2 °C, 60±5 % RH and a photoperiod of 12:12 L:D.

Site of Pheromone Storage During the fourth to ninth hour of the photophase, when adult beetles were active (Fonseca et al., 2009), we dissected five body regions of four males. Body regions were sampled sequentially in the order: head, prothorax, elytra, meso plus metathorax, and abdomen, as shown in Fig. 1. After sampling, each body part of a specimen was immersed, separately, in 1 ml of hexane for 2 h. Tridecane (50 ng) was added as an internal standard to each extract, and samples were concentrated to 100 µl under an argon stream (Zarbin et al., 1999). The presence of the major sex pheromone component, fuscumol acetate (Fonseca et al., 2009), in each sample was determined by gas chromatography/mass spectrometry (GC/MS).

Statistical Analysis Differences in mean quantities of the major pheromone component in extracts of the various body parts were tested by ANOVA, followed by Tukey's test. The analyses were performed using the program BioEstat 3.0 (Ayres et al., 2003).

Analytical Procedures The analyses were carried out by electron ionization GC/MS using a Shimadzu 17A gas chromatograph coupled to a Shimadzu QP5050A. The instrument was operated in the splitless mode with the injector set at 250 °C. A DB-5 (Agilent Technologies; 30 m×0.25 m×0.25 µm) capillary column was used, with the column temperature maintained at 50 °C for 3 min, before being increased to 250 °C at a rate of 7 °C.min^{-1}; the final temperature was maintained for 10 min.

Scanning Electron Micrographs Scanning electron microscopy (SEM) was performed on the prothorax of one adult of each sex. The prothorax was prepared for SEM, following the procedure described by Ray et al. (2006), by soaking overnight in 4 % Triton X100 (Amresco) to remove dirt and lipids. The prothorax then was dehydrated by successive 5 min. submersions in 70 % EtOH and 95 % EtOH. Lipids

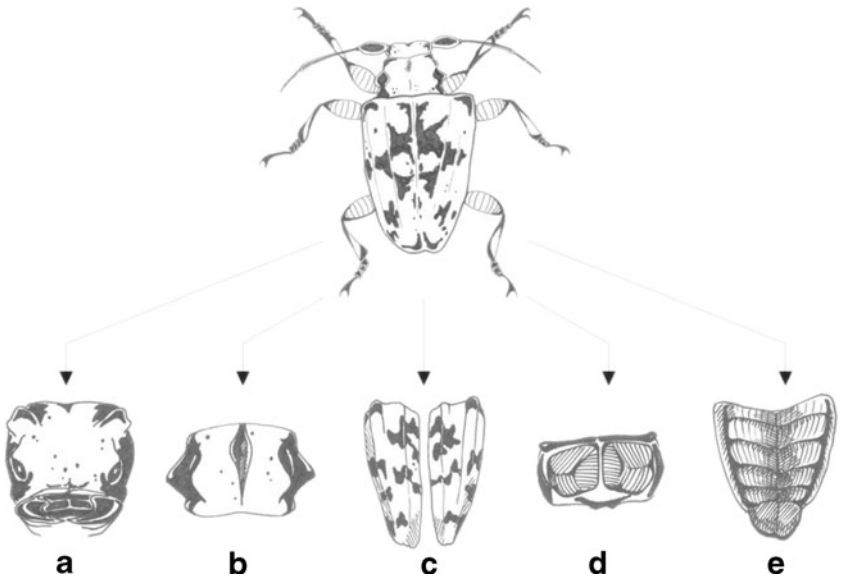


Fig. 1 The dissection of *Hedypathes betulinus* into five parts for detection of sex pheromone components: head (**a**), prothorax (**b**), elytra (**c**), meso plus metathorax (**d**), and abdomen (**e**)

were stripped by soaking specimens in hexanes (Vetec, Rio de Janeiro, Brazil) for at least 2 h, and then sonication in hexanes for 30 sec. Specimens were dried in air before being coated with 0.6 nm of gold (Balzers Union, Germany). A Jeol JSM 6360 LV, operated at 15 KV, was used to obtain the micrographs.

Synthesis of Labeled Compound Deuterium-labeled geranyl acetone-D_5 was obtained by reacting commercially available geranyl acetone (pure isomer; Fluka) with an excess of sodium methoxide in deuterated methanol, according to the following procedure (Gäbler and Boland, 1991). A mixture of geranyl acetone (0.54 g, 2.78 mmol) was dissolved in a mixture of MeOD/MeO^- (1.41 mmol MeO^- in 8.46 ml of MeOD) and stirred for 12 h at room temperature. After this, H_2O was added (20 ml), and the product was extracted with ethyl ether (3× 50 ml). The formation of the deuterium-labeled compound was confirmed by mass spectral analysis, and the deuterated product used immediately after preparation to avoid any possible degradation.

Application of Deuterated Precursor and Extraction Procedures At hours 4–9 h of the photophase (the time that pheromone is produced; Fonseca et al., 2010), an acetone solution (2 µl) of the labeled compound (50 µg/µl) was topically applied to the prothorax of an insect (N=6). Two hours later, the beetle was immobilized by placing it in the freezer for 15 min, before its prothorax was dissected and extracted with hexane for 15 min. Crude extracts were concentrated to 20 µl, and 1 µl of each extract was analyzed by GC/MS.

Results and Discussion

Greater amounts of the major pheromone component were found in extracts of the prothorax of male *H. betulinus* (F= 18.67, df=4, P =0.001) than in extracts of body parts, suggesting that the source of the pheromone is in the prothorax (Fig. 2). Small amounts of pheromone also were detected in other areas, but likely resulted from contamination during dissection. Similar results were obtained in dissections of male *Neoclytus acuminatus acuminatus*, in which pheromone was detected in high abundance in the prothorax, but small amounts of pheromone were present in other parts, due to contamination (Lacey et al., 2007b).

SEMs of the prothorax of a male revealed pores distributed over the entire cuticle (Fig. 3a). Similar pores also were present in females (Fig. 3b). This contrasts with studies on species of Cerambycinae, including *Neoclytus mucronatus mucronatus* (Lacey et al., 2007a), *Xylotrechus nauticus, Phymatodes lecontei, Neoclytus modestus modestus* (Hanks et al., 2007), *N. acuminatus* (Lacey et al., 2007b), and other species (Ray et al., 2006), showing male-specific pores and glands situated within depressions in the cuticle of the prothorax. These male-specific pores are associated with the production of pheromone (Ray et al., 2006). It is possible that the pores found in the prothorax of male *H. betulinus* also may be associated with the production of pheromone. However, it is

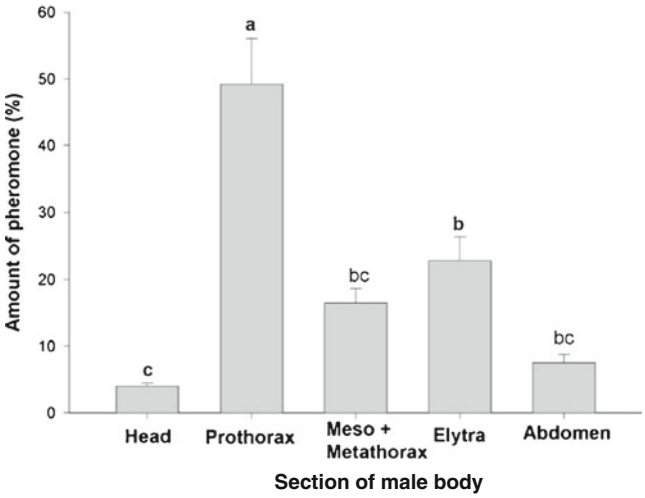


Fig. 2 The amount of fuscumol acetate in extracts of body parts of male *Hedypathes betulinus*. Means followed by the same letter are not different, as determined by ANOVA followed by a Tukey's test; N=4

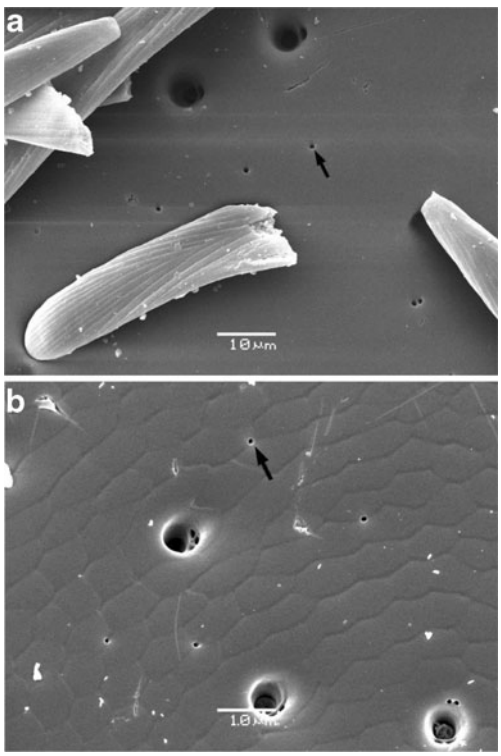
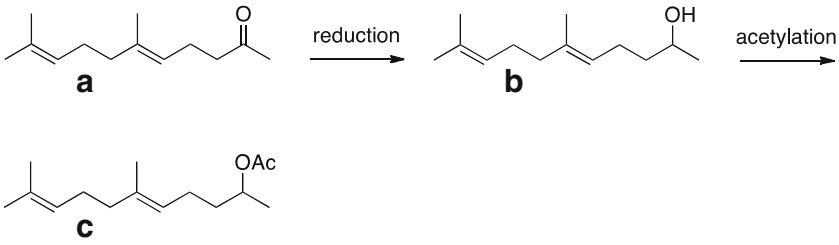


Fig. 3 Scanning electron micrographs of the prothorax of an adult *Hedypathes betulinus* male (**a**) and female (**b**); pores are indicated by *arrows*

Scheme 1 Proposed biosynthetic pathway for fuscumol (**b**) and fuscumol acetate (**c**) via geranyl acetone (**a**)



unclear what the function is of the pores in the prothorax of female *H. betulinus*.

To confirm that pheromone is produced in the prothorax of males, we topically applied a deuterium-labeled putative precursor to the insect's prothorax, with the aim of observing incorporation of the labeled compound into the final products (Leal et al., 1999), according to a biosynthetic pathway in which fuscumol acetate **(C)** is obtained by carbonyl reduction of geranyl acetone **(A)** to fuscumol **(B)**, with subsequent acetylation (Scheme 1).

The mass spectrum of unlabeled fuscumol acetate has a base peak at *m/z* 109, indicating five α-acetyl hydrogen atoms. Incorporation of deuterium into these positions would shift the base peak to *m/z* 114; this ion has a low intensity in the unlabeled compound. A sample of deuterium-labeled geranyl acetone-D_5 was synthesized (Gäbler and Boland, 1991), with the exchange of the five α-carbonyl hydrogens for deuteriums confirmed by mass spectral analysis (Fig. 4).

Topical application of geranyl acetone-D_5 to the prothorax showed clear incorporation of this compound into fuscumol acetate, through a strong increase in the intensity of *m/z* 114, relative to that in unlabeled fuscumol acetate (Fig. 5). The deuterated product eluted almost 0.10 min earlier than unlabeled fuscumol acetate. Despite the deuteriums, the mass spectra of the labeled and unlabeled compounds were largely similar; for example, the fragment at *m/z* 69 is common to both molecules because it does not contain atoms of deuterium (Fig. 5).

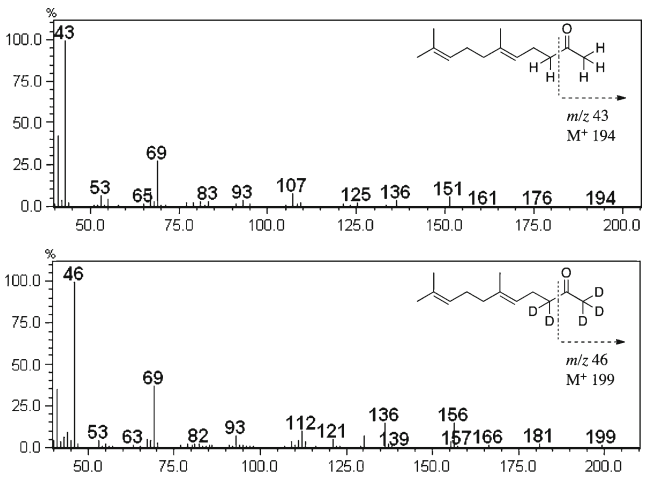


Fig. 4 Mass spectra of geranyl acetone and geranyl acetone-D_5

The production of labeled geranyl acetone-D_5 ranged from 6 % to 11 % that of unlabeled compound. Accordingly, we could not detect the expected molecular ion at *m/z* 243. Instead, the fragment of greatest molecular weight that we detected was *m/z* 200, resulting from the loss of the acetyl group through a McLafferty rearrangement (Silverstein et al., 2005). This fragment is equivalent to *m/z* 195 in unlabeled pheromone (Fig. 5). We also detected incorporation of label from geranyl acetone-D_5 into fuscumol (data not shown), by a change in relative intensities of *m/z* 109: 114 and *m/z* 153: 158, confirming the reduction step of the proposed biosynthetic pathway. It was difficult to obtain a clean mass spectrum of the deuterated alcohol as it eluted immediately after deuterated geranyl acetone and was present in a very low amount relative to that of the major component (Fonseca et al., 2010).

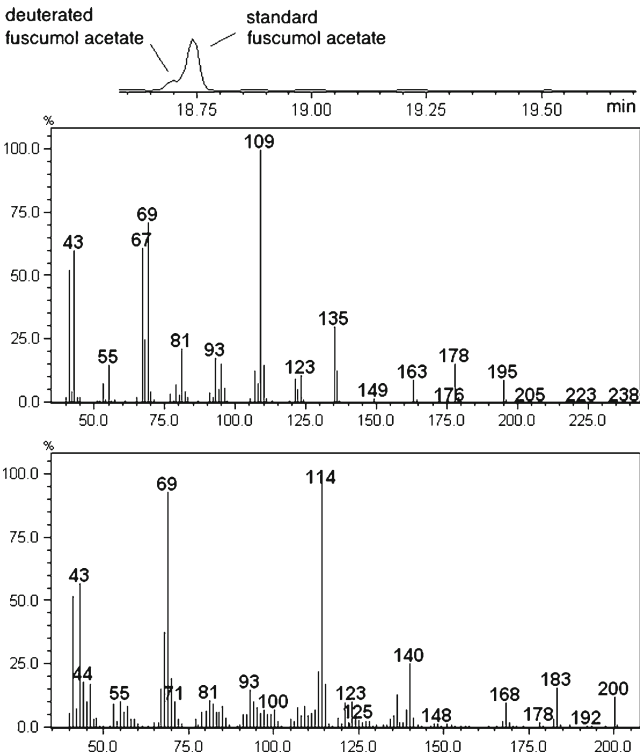


Fig. 5 Gas chromatography/mass spectrometry data for standard fuscumol acetate (*top spectrum*) and for fuscumol acetate obtained after topical application of geranyl acetone-D_5 to the prothorax of male *Hedypathes betulinus* (*bottom spectrum*)

In contrast to the result with males, we did not detect any natural or deuterated pheromone when we applied geranyl acetone-D_5 to the prothorax of females, confirming male specificity of pheromone production.

We previously described the presence of both enantiomeric forms of fuscumol in extracts from *H. betulinus* males at a ratio of 82.4 % (*R*)- to 17.6 % (*S*)-fuscumol, while the major component was enantiopure (*R*)-fuscumol acetate (Vidal et al., 2010). Our results demonstrate that both fuscumol and fuscomol acetate are biosynthesized from geranyl acetone, which also is found in male extracts. Therefore, it seems that the biosynthetic pathway involves a stereoselective reduction of geranyl acetone, followed by a stereospecific acetylation of the resulting alcohol (Scheme 1).

Definitive proof that pheromone production and release occurs in certain tissues comes from studies in which isolated tissue incorporates labeled precursors into pheromone components (Tillman et al., 1999). The sites of sex or aggregation pheromone synthesis, accumulation and/or release have been examined in many species of Coleoptera, with the preponderance of studies demonstrating the presence of abdominal glands, as in the beetles *Lasioderma serricorne* (Anobiidae) (Levinson et al., 1983), *Bruchidius atrolineatus* (Bruchidae) (Biemont et al., 1992), *Diabrotica virgifera* (Chrysomelidae) (Lew and Ball, 1978), *Selatosomus latus* (Elateridae) (Ivastschenko and Adamenko, 1980), *Carpophilus freemani* (Nitidulidae) (Dowd and Bartelt, 1993), and in scarab beetles of the sub families Melolonthine and Ruteline (Tada and Leal, 1997).

Although several studies indicate that pheromones are produced by glands located on the male prothorax in many species of Cerambycinae, there has been only one report, thus far, describing pheromone biosynthesis. Males of *Xylotrechus pyrrhoderus* produce (*S*)-2-hydroxy-3-octanone and (2*S*,3*S*)-2,3-octanediol as sex pheromone components. These compounds were synthesized with four deuterium atoms as labels, and the labeled compounds were applied topically to the prothorax of *X. pyrrhoderus* males. While no conversion of the diol into ketol could be detected, the ketol was converted into diol, indicating that the biosynthesis of (2*S*,3*S*)-2,3-octanediol occurs via (*S*)-2-hydroxy-3-octanone (Kiyota et al., 2009).

Geranyl acetone is the third most abundant component in the essential oil of green maté leaves (Bastos et al., 2006) and the second most abundant pheromone component in extracts of *H. betulinus* males (Fonseca et al., 2010). We reported previously that production of pheromone components of *H. betulinus* is dependent on the presence of the host plant; males in the presence of green maté branches released larger amounts of volatiles than did starved males (Fonseca et al., 2009). This supports the hypothesis that males sequester geranyl acetone from the host plant during feeding and use it as a precursor for pheromone production.

Studies have shown that some insects obtain compounds from host plants and use them as sex pheromones or pheromone precursors (Reddy and Guerrero, 2004). One example of the use of host plant precursors in pheromone synthesis is the boll weevil, *Anthonomus grandis* (Coleoptera: Curculionidae). Pheromone biosynthesis in this species is associated with the precursors geraniol and nerol (Thompson and Mitlin, 1979). Further studies are necessary to confirm whether *H. betulinus* sequesters host plant volatiles or produces its sex pheromone components via a *de novo* biosynthetic pathway.

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