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Defensive Compounds and Male-Produced Sex Pheromone of the Stink Bug, *Agroecus griseus*

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Abstract *Agroecus griseus* is a serious corn pest in Brazil. Contents of the dorsal abdominal glands (DAGs) in nymphs, and the metathoracic gland (MTG) in adults of this species were characterized and quantified. Compounds found were similar to those of other Pentatomidae species and included aliphatic hydrocarbons, aldehydes, oxoalkenals, and esters. However, two compounds were found in the MTG that have not been described previously for this family. Mass spectrometry, infrared spectroscopy, and gas chromatographic analysis using coinjection with authentic standards confirmed the identities of the compounds as enantiopure (*S*)-2-methylbutyl acetate and 3-methyl-2-butenyl acetate. The five nymphal instars showed significantly different ratios of components, mainly between those of the first and later instars. No significant differences were detected in MTG contents between sexes. Gas chromatography (GC) analysis of aeration extracts of males and females showed the presence of a compound released exclusively by males. Gas chromatography – electroantennographic detection (GC-EAD) assays indicated that the male-specific compound is bioactive in females, suggesting the presence of an attractant pheromone. The mass spectrum and infrared data for this compound matched with methyl 2,6,10-trimethyltridecanoate, a sex pheromone component previously detected in the stink bugs, *Euschistus heros* and *E. obscurus*. The synthetic standard coeluted with the natural pheromone on two different GC stationary phases, confirming the proposed structure. Y-tube olfactometer assays showed that the synthetic standard was strongly attractive to females, and GC-EAD tests produced responses with antennae from females similar to those of the natural pheromone.

Keywords GC-FTIR · Heteroptera Pentatomidae · Dorsal abdominal gland · Metathoracic gland · (*S*)-2-methylbutyl acetate · Methyl 2,6,10-trimethyltridecanoate · Agricultural pest

Introduction

Stink bugs (Heteroptera: Pentatomidae) are one of the main agricultural pests in the world, and they have become increasingly problematic with the advent of genetically modified crops (Bundy and McPherson, 2000). These pests produce large quantities of strong-smelling and irritating defense chemicals (allomones), which are released when the bugs are disturbed, serving as an alarm pheromone as well as a defense against predators (Aldrich, 1988).

Heteropteran nymphs produce exocrine secretions from dorsal abdominal glands (DAGs), the contents of which are shed along with the exuviae each time the nymph molts. Extraction of exuviae is a convenient method to obtain compounds made in DAGs for their identification (Borges and Aldrich, 1992). In adults, allomones are produced in the metathoracic scent gland (MTG). The identification of the contents of MTG secretions has received considerable attention, partly because these secretions constitute such an obvious defense, and because the large quantities of simple compounds produced are easy to analyze and identify (Aldrich, 1988; Ho and Millar, 2001; Fávaro et al., 2011).

The composition of stink bug allomones is similar for most species and includes hydrocarbons, as well as saturated and unsaturated aldehydes and esters (Aldrich, 1988). It is well-known that the proportions of compounds present in DAG secretions differ among the five nymphal instars

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(Borges and Aldrich, 1992; Fávaro et al., 2011; Fávaro and Zarbin, 2012). However, it has only recently been reported that the composition of the pentatomid MTG secretions may also differ according to the insect adult age (Fávaro et al., 2011; Fávaro and Zarbin, 2012).

Unlike defensive compounds, attractant pheromones of many stink bugs are produced in clusters of unicellular glands that are difficult to isolate by dissection and extraction (Millar, 2005). Thus, the most appropriate method for extraction is by aeration of live insects (Zarbin et al., 1999). In contrast to lepidopterans, pheromones identified to date for Pentatomidae exhibit a wide variety of chemical structures (Moraes et al., 2008). Pentatomid pheromones that have been identified include long-chain saturated hydrocarbons [*Phthia picta,* (Soldi et al., 2012)], terpenes [*Piezodorus hybneri,* (Leal et al., 1998) and *Nezara viridula,* (Aldrich et al., 1989)], and methyl esters [*Euschistus heros,* (Borges et al., 1998, 1999) and *Edessa meditabunda*, (Zarbin et al., 2012)].

Agroecus griseus Dallas (1851) is a major corn pests found in Brazil. These pests feed at the base of the plants, affecting tissue formation at the growth stage (Gassen, 1996). Besides *A. griseus*, the genus *Agroecus* contains another five described species (*A. brevicornis*, *A. lizerianus*, *A. scabricornis*, *A. ecuadoriensis,* and *A. reticultus*) that have been reported in several South America countries (Rider and Rolston, 1987). Although these insects are well-known, this work constitutes the first study of semiochemicals of *Agroecus* species.

The present work sought to: (a) identify and quantify nymphal exocrine compounds from the exuviae of the five nymphal instars; (b) identify and quantify the chemical components of the MTG in adults; and (c) identify the sex pheromone(s) of *A. griseus* by artificially testing the attractiveness of synthetic pheromone.

Methods and Materials

Insects Nymphs and adults of *A. griseus* were collected at EMBRAPA soybean fields in Londrina, Paraná State, Brazil (23º11' S, 51º11' W). Adults were sexed and separated from nymphs, and insects were maintained in plastic cages (35 x 20 x 20 cm) at 26±2 °C with 70 % relative humidity, and a 14/10 hL/D photoperiod. The colony was reared on soybean seeds (*Glycine max*), green beans (*Phaseolus vulgaris*), peanuts (*Arachis hypogaea*), and glossy privet fruits (*Ligustrum lucidum*). The food was replaced every 3 d.

Extraction of Dorsal Abdominal Glands Contents Hexane extracts of DAGs of the five instars were prepared by suspending exuviae in analytical-grade hexane after being collected≤24 h after ecdysis. Concentrations of the extracts were three exuviae per 100 µl for the first instar, one exuviae per 100 µl for the second and third instars, one exuviae per 200 µl for the fourth instar, and one exuviae per 400 µl for the fifth instar. Exuviae were extracted for 24 h at room temperature, after which the hexane extracts were transferred to another clean vial and stored at -20 °C until analysis. Three extracts were prepared and analyzed for each nymphal instar.

Extraction of Metathoracic Gland Contents A 30-d-old *A. griseus* adult was pinned dorsal side up through the prothorax in a Petri dish and submerged in tap water. The dissection process (using small surgical scissors and sharpened forceps) consisted of removing the wings, cutting the lateral margins of the abdomen anteriorly up to the metathorax, and transversely cutting the anterior margin of the scutellum. The tergal cuticle was pulled back, and the viscera were removed. The scent gland complex, located in the ventral metathoracic region, could subsequently be reached and removed by cutting laterally through the meso- and metathorax, turning the preparation over, and cutting transversely between the meta- and prothorax. The gland reservoir, including the lateral accessory glands (Aldrich et al., 1978), was removed, dried with tissue paper, immersed in 1000 µl of analytical grade hexane, and stored at -20 °C until being analyzed.

Collection of Volatile Compounds Volatile compounds were collected by the aeration method (Zarbin et al., 1999). Groups of 5 males and females were separately placed in glass chambers (25 x 9 cm ID) containing privet fruits. The collecting apparatus was maintained at the same temperature and photoperiod as the colony. A continuous 1 L/min flow of humidified and charcoal-filtered air was pulled through each chamber, drawing the volatile compounds through glass traps (11 x 1 cm ID) containing 60 mg of Super-Q adsorbent polymer (Alltech Associates Inc., Deerfield, IL, USA). Adsorbed volatile compounds were eluted from the polymer once a day with doubly distilled hexane (2 ml), concentrated with argon to 50 µl (10 µL per insect), and stored at -20 °C until analysis.

Chemical Analysis Volatile extracts were analyzed with a Shimadzu GC2010 gas chromatograph (GC) equipped with a FID detector, RTX-5 (Restek Bellefonte, PA, USA; 30 m x 0.25 mm x 0.25 µm film thickness) capillary column and helium as carrier gas. The GC was operated in splitless mode (250 °C). The temperature profile for gland extract analyses began at 50 °C for 1 min, increasing at 7 °C/min until reaching 250 °C, and maintaining this temperature for 10 min. Aeration extract analyses were conducted by starting the temperature profile at 100 °C for 1 min, increasing at 7 °C/min until reaching 250 °C, and holding at this temperature for 10 min. To determine the Kovats indices (Lubeck

and Sutton, 1983) and to examine the coinjection of the natural products with synthetic standards, RTX-WAX (Restek, 30 m x 0.25 mm x 0.25 μm film thickness) and EC™-1 (Alltech Associates, Inc., Deerfield, IL, USA; 30 m x 0.25 mm x 0.25 μm film thickness) capillary columns were employed. Chiral separations were obtained on a β-DEX™ 325 capillary column [25 % 2,3-di-O-methyl-6-O-TBDMS-β-cyclodextrin embedded in SPB-20 poly (20 % phenyl/80 % dimethylsiloxane), 30 m x 0.25 mm x 0.25 μm ID; Supelco, Bellefonte, PA, USA] using helium as carrier gas (1 ml/min) at 40 °C.

Gas chromatography-mass spectrometry (GC-MS) data were acquired using a Shimadzu QP2010-Plus electron ionization mass detector operating in the electron impact mode (70 eV) with an RTX-5 (Restek, 30 m x 0.25 mm x 0.25 μm) capillary column. The injector mode and program temperature were the same as described above. The National Institute of Standards and Technology (NIST) mass spectra chemical database was used.

Extracts also were analyzed by GC-Fourier transform infrared spectroscopy (GC-FTIR) with a Shimadzu GC2010 GC coupled to a DiscovIR-GC infrared detector (4000-750 cm^{-1}, resolution of 8 cm^{-1}; Spectra Analysis, Marlborough, MS, USA). The capillary column, injector mode and program temperature were the same as those previously described.

Gas chromatography-electroantennographic detection (GC-EAD) analysis data were acquired with a Shimadzu GC2010 coupled to a Syntech® electroantennographic detector (Hilversum, Netherlands). The GC was equipped with an RTX-5 capillary column (Restek, 30 m x 0.25 mm x 0.25 μm), operated in splitless mode (250 °C), and programmed to run at 100 °C for 1 min and subsequently increase by 7 °C/min to 250 °C. The antennae were fixed between the stainless steel electrodes using conductive gel (Signa gel, Parker Laboratories, Inc., Fairfield, NJ, USA). The electroantennogram were recorded on the Syntech GC-EAD32 program (version 4.6).

Micro-Derivatization

Hydrogenation with Palladium on Charcoal (Pd/C) To a hexane MTG extract in a glass vial~0.5 mg of Pd/C (5 % Pd) were added. A balloon filled with hydrogen was attached to the vial, and the reaction was stirred for approximately 3 h. The product solution was filtered and analyzed by GC (Attygalle, 1998).

Chemical Standards (*E*)-2-Hexen-1-ol, (*E*)-2-octenal, and (*E*)-2-decen-1-ol were purchased from Acros Organics (Geel, Turnhout, Belgium). Undecane, dodecane, tridecane, 1-tridecene, and 1-tetradecanol were purchased from Aldrich Chemical Company (Milwaukee, WI, USA). (*E*)-4-Oxo-2-hexenal and (*E*)-4-oxo-2-decenal were gifts from Dr. K. Chauhan of the USDA-ARS (Beltsville, MD, USA) (Feldlaufer et al., 2010), and Dr. J. G. Millar of the University of California Riverside (CA, USA) (Moreira and Millar, 2005), respectively.

(*S*)-2-Methylbutyl, 3-methyl-2-butenyl, (*E*)-2-hexenyl, and (*E*)-2-octenyl acetates were prepared from their respective precursor alcohols (Ho and Millar, 2001; Fucarino et al., 2004). Additionally, 3-methylbutyl acetate was made via the hydrogenation of 3-methyl-2-butenyl acetate over Pd/C (5 % Pd) at room temperature and 20 psi H_2 in a Parr apparatus. The aldehydes (*E*)-2-hexenal, (*E*)-2-decenal, and tetradecanal were made by the Ferreira and Zarbin (1996) method using a mixture of their precursor alcohols and PCC. Methyl 2,6,10-trimethyltridecanoate was previously synthesized by Zarbin et al. (2000).

Olfactometer Bioassays with Synthetic Sex Pheromone Behavioral responses of *A. griseus* to the synthetic pheromone were tested in a Y-tube olfactometer using humidified, charcoal-filtered air flowing at 2.5 L/min. The olfactometer consisted of a Y-shaped glass tube (4 x 40 cm) with two 20 cm arms. The odorant source was placed at the end of the arms, consisting of a piece of filter paper (2 x 2 cm) impregnated

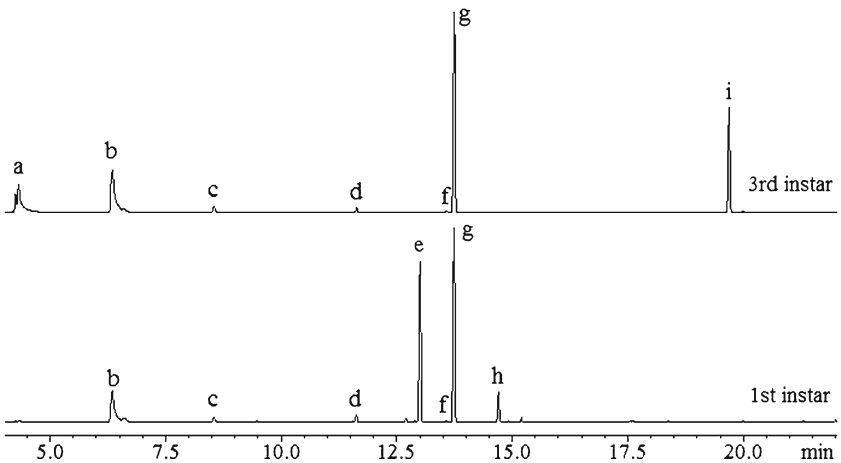

Fig. 1 Gas chromatogram of exuvial extracts from the first- and third-instar nymphs of *Agroecus griseus*. Compounds: (*E*)-2-hexenal **(a)**, (*E*)-4-oxo-2-hexenal **(b)**, (*E*)-2-octenal **(c)**, dodecane **(d)**, (*E*)-2-decenal **(e)**, 1-tridecene **(f)**, tridecane **(g)**, (*E*)-4-oxo-2-decenal **(h),** and tetradecanal **(i)**

Table 1 Relative abundances (%) (mean±SD) of compounds present in *Agroecus griseus* exuvial extracts from first to fifth-instars. Tukey test, $P<0.05$

Compound		1st	2nd	3rd	4th	5th
a	(*E*)-2-Hexenal[a]	0a	15.9±0.7b	13.9±1.3b	16.5±1.9b	14.0±0.3b
b	(*E*)-4-Oxo-2-hexenal[a]	18.2±1.8a	18.3±2.8a	10.7±2.5b	15.9±9.0ab	3.9±1.9c
c	(*E*)-2-Octenal[a]	1.3±0.5a	1.4±0.1a	3.9±0.5ab	6.5±1.2b	7.5±1.5b
d	Dodecane[a]	1.1±0.4ab	0.9±0.1a	1.5±0.1ab	1.5±0.2ab	2.1±0.3b
e	(*E*)-2-Decenal[a]	31.8±2.2a	0b	0b	0b	0b
f	1-Tridecene	0.2±0.1a	0.4±0.1a	0.8±0.1a	0.7±0.0a	0.7±0.2a
g	Tridecane[a]	43.9±2.8a	43.0±1.9a	48.4±4.9ab	41.3±8.5a	63.3±2.9b
h	(*E*)-4-Oxo-2-decenal[a]	3.5±1.6a	0b	0b	0b	0b
i	Tetradecanal[a]	0a	20.2±1.1b	20.8±1.8b	17.6±4.0bc	8.6±5.5 ac

[a]Compounds that showed significant differences between the instars

with the synthetic compounds or hexane (control). Two experiments were conducted to test the biological activity of the synthetic male-specific compound by measuring the response of females to the compound vs. control, and the response of males to the same compound vs. control.

An insect was introduced into the base of the olfactometer, and its behavior was observed for 15 min. A positive response was defined as the insect walking against the airflow more than 5 cm into an arm toward the odor source and remaining there for more than 2 min. No response was defined as the insect not leaving the main tube. Each insect was counted as one data point, and was tested only once. The odor source was replaced after each test. Insects that did not choose either of the arms were excluded from statistical analysis. The olfactometer was moved after every three tests to cancel positional effects.

Statistical Analyses Analysis of variance (ANOVA) followed by the Tukey test was used to compare the percentages of compounds present in exuviae and MTG extracts. Response data of olfactometer bioassays with the synthetic male-specific compound were analyzed using a *Chi-square* test. All tests were performed using the BioEstat program (version 5.0) (Ayres et al., 2003).

Results and discussion

A maximum of nine compounds were found in secretions from the DAGs of *A. griseus* nymphs (Fig. 1). By comparing the mass spectra of these compounds with those for other stink bugs previously studied (Fávaro et al., 2011; Fávaro and Zarbin, 2012), followed by coinjection with synthetic standards, the nine abundantly encountered compounds were identified as (*E*)-2-hexenal **(a)**, (*E*)-4-oxo-2-hexenal **(b)**, (*E*)-2-octenal **(c)**, dodecane **(d)**, (*E*)-2-decenal **(e)**, 1-tridecene **(f)**, tridecane **(g)**, (*E*)-4-oxo-2-decenal **(h),** and tetradecanal **(i)**.

The GC trace for the exuvial extract of the first instar nymphs was significantly different from that for the second to fifth instars according to analysis of variance, while the composition of exuvial extracts for the second to fifth instars did not differ significantly (Table 1). Tridecane **(g)** and (*E*)-2-decenal **(e)** were detected as the major components from the first instar followed by lower amounts of (*E*)-4-oxo-2-hexenal **(b)** and (*E*)-4-oxo-2-decenal **(h)**. The major component from the second instar was tridecane **(g)** followed by significant amounts of (*E*)-2-hexenal **(a)**, (*E*)-4-oxo-2-hexenal **(b),** and tetradecanal **(i)**.

Almost all compounds varied in relative abundances among the different exuvial extracts (Table 1). However, the main difference was the appearance of two compounds only in the first instar, specifically, (*E*)-2-decenal **(e)** and (*E*)-4-oxo-2-decenal **(h)**, and the absence of two others in this instar, specifically, (*E*)-2-hexenal **(a)** and tetradecanal **(i)**. Other studies of DAG secretory compounds showed that first-instars of some, but not all, pentatomid species contain (*E*)-4-oxo-2-decenal, which is totally absent in the secretions of later instars (Borges and Aldrich, 1992). (*E*)-4-Oxo-2-decenal mediates the aggregation behavior of first-instars

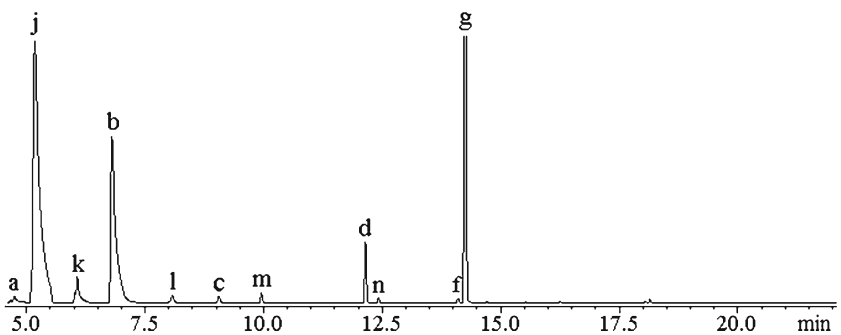

Fig. 2 Gas chromatogram of an MTG extract of an *Agroecus griseus* adult. Compounds: (*E*)-2-hexenal **(a)**, (*E*)-4-oxo-2-hexenal **(b)**, (*E*)-2-octenal **(c)**, dodecane **(d)**, 1-tridecene **(f)**, tridecane **(g)**, (*S*)-2-methylbutyl acetate **(j)**, 3-methyl-2-butenyl acetate **(k)**, (*E*)-2-hexenyl acetate **(l)**, undecane **(m),** and (*E*)-2-octenyl acetate **(n)**

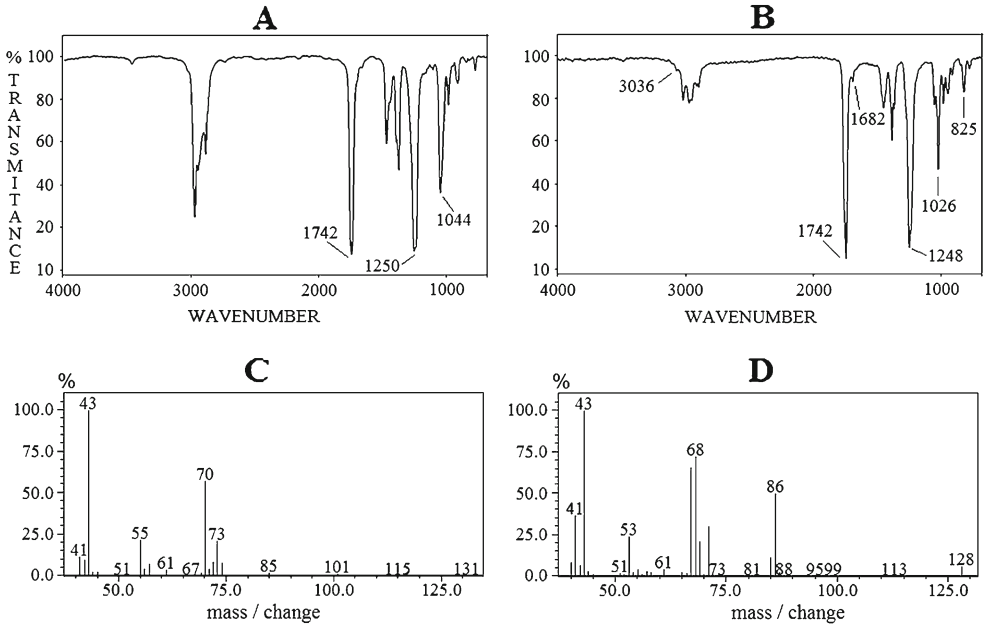

Fig. 3 Infrared and mass spectra of compounds **j** (**A** and **C**) **k** (**B** and **D**)

(Pavis et al., 1994; Fucarino et al., 2004), behavior that is strongly expressed by *A. griseus* nymphs.

As in other stink bugs, the MTG of *A. griseus* adults is well-developed with an orange-colored reservoir easily recognizable upon dissection (Aldrich, 1988). Analysis of the MTG extract showed the same compounds were present as found in late-instars of the *A. griseus* nymphs, with the exception of tetradecanal **(i)**, along with five new components (**j-n**, Fig. 2).

Three *A. griseus* MTG components exhibited mass spectra that matched those from other stink bugs we have recently studied (Fávaro et al., 2011; Fávaro and Zarbin, 2012), and these compounds were confirmed by GC coinjection with synthetic standards as (*E*)-2-hexenyl acetate **(l)**, undecane **(m),** and (*E*)-2-octenyl acetate (**n**). However, matching mass spectra for compounds **j** and **k** were not found, and their structures were identified by a combination of GC-MS and GC-FTIR, as well as micro-derivatization.

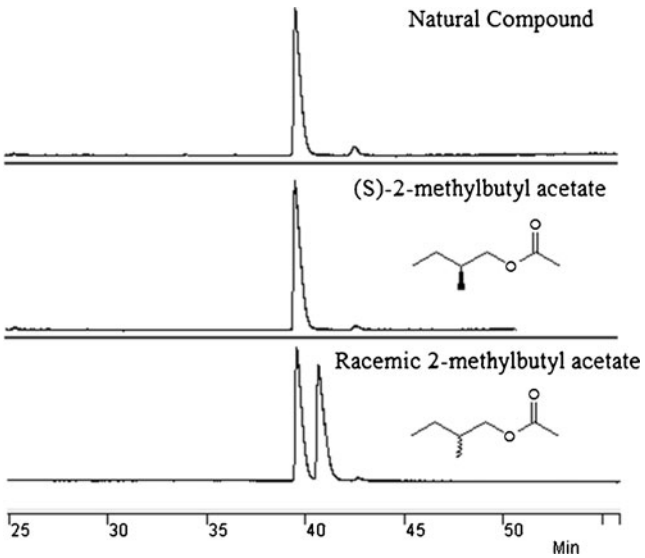

Fig. 4 Resolution of 2-methylbuthyl acetate (**j**) enantiomers on a cyclodextrin-based column

The GC-FTIR spectrum of compound **j** (Fig. 3A) exhibits three characteristic ester stretching frequencies at 1044 cm^{-1}, 1250 cm^{-1}, and 1742 cm^{-1}, which are due to O-C-C, C-O-C, and C=O stretching, respectively (Smith, 1999). The mass spectrum of this compound (Fig. 3C) showed a molecular ion ($M^+ + 1$) at *m/z* 131, a fragment at *m/z* 70, due to the loss of an acetic acid molecule ($M^+ - 60$) characteristic of acetate compounds, and a base peak at *m/z* 43 resulting from the cleavage of the C-O single bond of the ester (Silverstein et al., 2005). Comparison of the mass spectrum of this

Table 2 Amount (%) (mean±SD) of compounds present in the metathoracic gland (MTG) extracts of *Agroecus griseus* adults. Values in % by MTG

Compound		Mean (%)±SD
a	(*E*)-2-Hexenal	2.28±1.64
j	(*S*)-2-Methylbutyl acetate	30.28±2.46
k	3-Methyl-2-butenyl acetate	4.11±0.22
b	(*E*)-4-Oxo-2-hexenal	14.95±2.24
l	(*E*)-2-Hexenyl acetate	6.64±4.28
c	(*E*)-2-Octenal	0.52±0.02
m	Undecane	1.71±0.21
d	Dodecane	3.95±0.08
n	(*E*)-2-Octenyl acetate	0.70±0.20
f	1-Tridecene	1.42±0.06
g	Tridecane	33.48±1.40

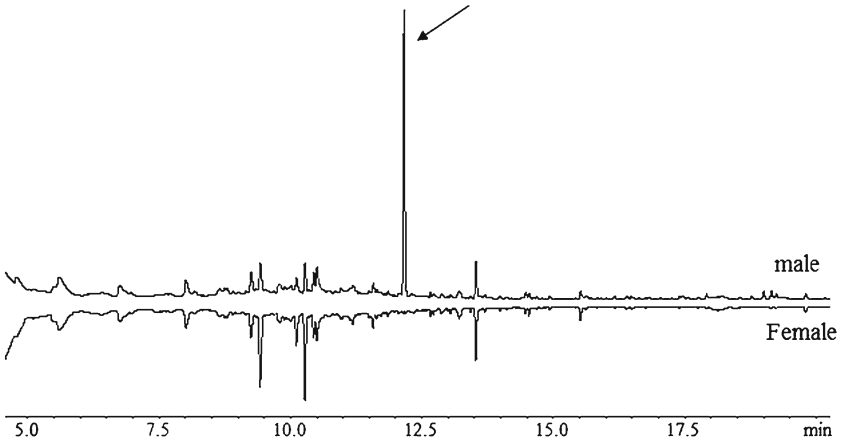

Fig. 5 Comparison of gas chromatograms of volatile compounds obtained from *Agroecus griseus* males and females showing the male-specific compound

compound with the NIST computer database, along with infrared data, suggested two possible chemical structures, specifically, 2- or 3-methylbutyl acetate. From the coinjection of the extract with the synthetic standards of these two acetates on three different columns (EC-1, RTX-5, and RTX-WAX), the identity of the compound **j** was determined to be 2-methylbutyl acetate.

To establish the absolute configuration of this molecule, GC analysis employing a chiral column was conducted. Racemic **j** was baseline resolved on a cyclodextrin-based capillary column and showed two peaks with retention times of 39.5 and 40.6 min (Fig. 4). The synthetic (*S*)-enantiomer co-eluted with the earlier eluting peak, and the purified natural product shared the same retention time as this compound. Therefore, the acetate **j** present in the *A. griseus* MTG content was fully characterized as enantiopure (*S*)-2-methylbutyl acetate.

Besides the ester absorbances (Fig. 3B) at 1026 cm^{-1} (O-C-C), 1248 cm^{-1} (C-C-O), and 1742 cm^{-1} (C=O), compound **k** presented three bands at 825 cm^{-1} (C-H bend), 1682 cm^{-1} (C=C stretch), and 3036 cm^{-1} (C-H stretch) that are characteristic of a non-terminal, trisubstituted alkene (Smith, 1999). In the mass spectrum of this compound (Fig. 3D), a base peak at *m/z 43*, and a molecular ion peak at *m/z* 128 (M^+) were observed. Another intense fragment at *m/z* 68 also was detected resulting from the loss of acetic acid (M^+ - 60) (Silverstein et al., 2005). From the data described above, and by comparison with the NIST mass spectra database, two different chemical structures were proposed: 2-methyl-2-butenyl acetate and 3-methyl-2-butenyl acetate.

To clarify further the structure of this compound, the purified natural product was hydrogenated over Pd/C, and the resulting product was co-injected on three different GC columns with authentic samples of 2-methylbutyl acetate and 3-methylbutyl acetate, which were available in our laboratory. These analyses unambiguously identified compound **k** as 3-methyl-2-butenyl acetate.

Thus, the MTG scent blend of *A. griseus* contains high concentrations of tridecane (**g**), which is also the major component in nymphs, (*S*)-2-methylbutyl acetate (**j**) and (*E*)-4-oxo-2-hexenal (**b**). All the other components appear only at trace levels (Table 2).

Most of the defensive compounds identified for *A. griseus* have been found previously for other Pentatomidae species (Moraes et al., 2008). However, our identification of 2-methylbutyl acetate and 3-methyl-2-butenyl acetate is the first time these compounds have been identified from the Pentatomidae. 2-Methylbutyl acetate also is found as a volatile plant component of several Orchidaceae species and in certain Fabaceae. 3-Methyl-2-butenyl acetate has been reported as a defensive compound only in a Hemiptera species, *Spilostethus rivulari* (Lygaeidae) (Staddon et al., 1985; El-Sayed, 2011).

Besides defensive compounds, attractant pheromones also can be produced in and released from the MTGs of

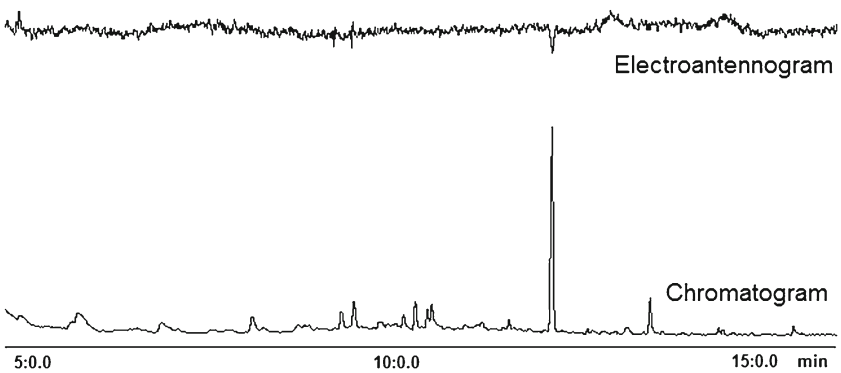

Fig. 6 Response of an antenna from an *Agroecus griseus* female to the male-specific compound released by conspecific males

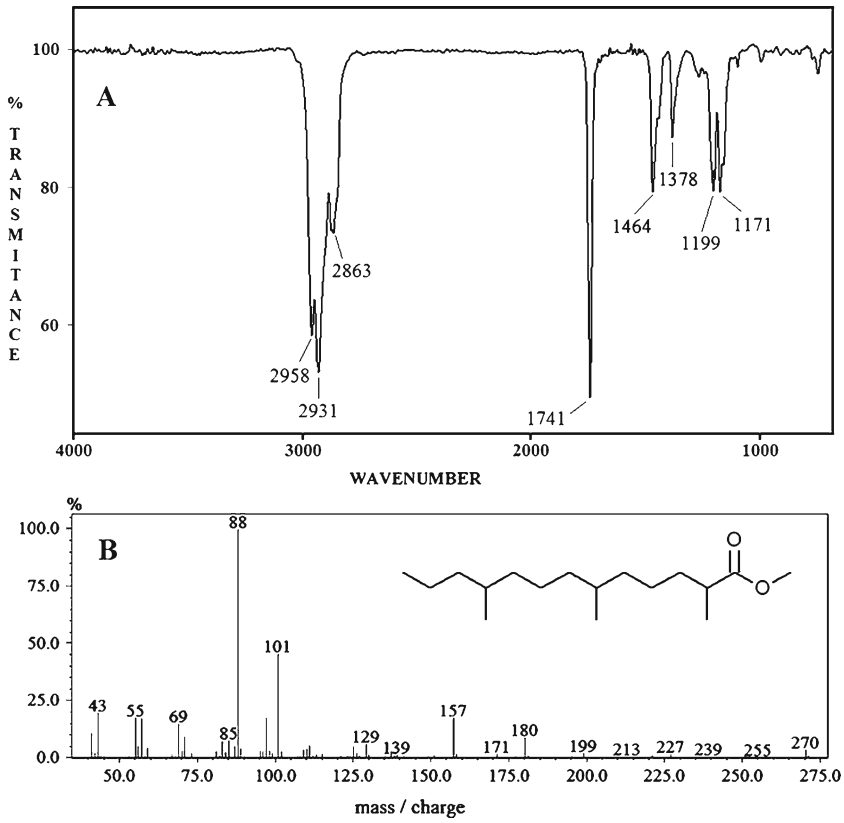

Fig. 7 Infrared (**A**) and electron impact mass spectra (**B**) of the *Agroecus griseus* male-specific compound

certain Heteroptera (Millar, 2005), probably from the lateral accessory glands attached to the MTG reservoir (Aldrich et al., 1997, 1999, 2000). MTG-derived attractant pheromones can be produced by either males, as in some Lygaeidae and Alydidae (Leal et al., 1995; Aldrich et al., 1997,1999), or females, as in Miridae and some Alydidae (Smith et al., 1991; Leal et al., 1996; Millar et al., 1997; Aldrich et al., 2000; Zhang and Aldrich, 2003, 2008). We recently described that males of *P. picta* produce a specific sex pheromone in the lateral accessory glands (Soldi et al., 2012). However, no difference was observed between the MTG secretions of *A. griseus* males and females; therefore, we decided to use the aeration method to study the sex pheromone system of the species (Zarbin et al., 1999).

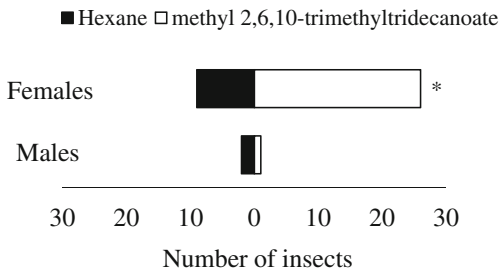

Fig. 8 Total number of *Agroecus griseus* males and females attracted to the synthetic compound, methyl 2,6,10-trimethyltridecanoate, in a Y-tube olfactometer. *Chi-square* test, $P<0.001$

Comparison of the gas chromatograms of aeration extracts collected from male and female *A. griseus* adults showed the presence of a male-specific compound, along with other compounds that were common for both sexes (Fig. 5). The Kovats index from this compound was calculated on two different columns: the RTX-WAX, which showed a value of 1889, and the RTX-5, with a value of 1752 being observed.

Electrophysiology analysis of the male volatile extract on the antennae of males and females was performed to determine whether the male-specific compound or other compounds in the extract showed biological activity for this species. Only antennae of females responded to the male-specific compound (Fig. 6), suggesting a sex pheromone mediating the behavior of *A. griseus*.

For the structural elucidation of the *A. griseus* sex pheromone, the male volatile extracts were analyzed by GC-MS and GC-FTIR. The GC-FTIR spectrum (Fig. 7A) showed a band at 1741 cm^{-1}, which is consistent with the C=O stretching of esters, and two other bands at 1171 cm^{-1} and 1199 cm^{-1}, corresponding to the C-O-C stretching of this functional group (Smith, 1999). Among the main fragments present in the mass spectrum (Fig. 7B), a molecular ion of *m/z* 270, a base peak of *m/z* 88, and a second most intense peak at *m/z* 101 were observed, fragments characteristic of a methyl branch bonded to the α-carbon of a methyl ester. Two peaks at *m/z* 157 and *m/z* 227 showed a high relative

intensity, suggesting the presence of two other methyl branches (Silverstein et al., 2005). This same fragmentation pattern was described previously for methyl 2,6,10-trimethyltridecanoate, which was identified as a component of the sex pheromone of the stink bugs, *Euschistus heros* (Aldrich et al., 1991) and *E. obscurus* (Borges and Aldrich, 1994).

Several similarities between *Agroecus* and *Euschistus* had already been reported, but only morphologically (Rider and Rolston, 1987). It is common to find compounds that are repeated in the pheromonal mixture of related insect genera. For example, *cis* and *trans-Z*-bisabolene epoxides are present in the sex pheromone mixtures of two *Nezara* species (*N. viridula* and *N. antennata*), and four *Chinavia* species (formerly *Acrosternum,* Schwertner and Grazia, 2007; *A. aseadum*, *A. hilare*, *A. marginatum,* and *A. pennsylvanicum*) (Millar, 2005).

Methyl 2,6,10-trimethyltridecanoate was first synthesized by Mori and Murata (1994a), using a cyclopropane cleavage by a Julia rearrangement as the key reaction. The same authors later showed the synthesis of all of the eight possible stereoisomers of this compound (Mori and Murata, 1994b). More recently, Zarbin et al. (2000) described an alternative six-step synthetic route to this compound, starting from (±)-citronellol with a 16 % overall yield.

The synthetic compound (Zarbin et al., 2000) was co-injected with the male volatile compound extracts, and co-elution with the male-specific compound was observed on two different columns (RTX-5 and RTX-WAX). The mass and infrared spectra of the synthetic compound also were identical to those of the natural product, confirming methyl 2,6,10-trimethyltridecanoate as the male-specific compound of *A. griseus*.

The biological activity of the synthetic compound was analyzed with GC-EAD and behavioral tests using male and female *A. griseus* in a Y-shaped olfactometer. The synthetic compound elicited antennal responses in females but not in males, as did the natural compound. Methyl 2,6,10-trimethyltridecanoate was highly attractive to females in olfactometer bioassays when tested against the solvent control [insects used=40, response (N)=35, treatment 26 (74 %), control 9 (26 %), $P<0.001$], while the males show no such effect [insects used=10, response (N)=3, treatment 1 (33 %), control 2 (67 %)] (Fig. 8). These bioassay results implicate methyl 2,6,10-trimethyltridecanoate as a sex pheromone of *A. griseus*.

Methyl 2,6,10-trimethyltridecanoate has eight stereoisomers, and the importance of chirality on a compound's bioactivity as an insect pheromone has been demonstrated previously (Mori, 2007). Although the mixture of all the eight isomers of this pheromone was attractive toward *E. heros* females, the (2*R*,6*R*,10*S*)-isomer proved to be more effective than the stereoisomeric mixture in olfactometer bioassays (Costa et al., 2000). *Agroecus griseus* females also were attracted to a racemic mixture of the pheromone; however, testing whether they exhibit a similar increased attraction to the enantiopure compound has yet to be completed. Nor has the possibility that the unusual MTG esters, (*S*)-2-methylbutyl acetate and 3-methyl-2-butenyl acetate, are involved in pheromonal attraction been eliminated.

In summary, this work describes all of the defense compounds present in the DAGs of nymphs and MTG of adults of *A. griseus*, as well as a sex pheromone produced by males of this species. Besides some typical defense compounds found in stink bugs, two components that have not been described previously for pentatomids also were discovered: (*S*)-2-methylbutyl acetate and 3-methyl-2-butenyl acetate. A sex pheromone of *A. griseus* was identified as methyl 2,6,10-trimethyltridecanoate, and its biological activity was demonstrated by behavioral and EAD tests. Field experiments employing the synthetic molecules are now underway.

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References

ALDRICH, J. R., BLUM, M. S., HEFETZ, A., FALES, H. M., LLOYD, H. A., and ROLLER, P. 1978. Proteins in a nonvenomous defensive secretion: biosynthetic significance. *Science* 201:452–454.

ALDRICH, J. R. 1988. Chemical ecology of the Heteroptera. *Annu. Rev. Entomol.* 33:211–238.

ALDRICH, J. R., LUSBY, W. R., MARRON, B. E., NICOLAOU, K. C., HOFFMANN, M. P., and WILSON, L. T. 1989. Pheromone blends of green stink bugs and possible parasitoid selection. *Naturwissenschaften* 76:173–175.

ALDRICH, J. R., HOFFMANN, M. P., KOCHANSKY, J. P., LUSBY, W. R., EGER, J. E., and PAYNE, J. A. 1991. Identification and attractiveness of a major pheromone component for Nearctic *Euschistus* spp. stink bugs (Heteroptera: Pentatomidae). *Environ. Entomol.* 20:477–483.

ALDRICH, J. R., LEAL, W. S., NISHIDA, R., KHRIMIAN, A. P., LEE, C.-J., and SAKURATANI, Y. 1997. Semiochemistry of aposematic seed bugs. *Entomol. Exp. Appl.* 84:127–135.

ALDRICH, J. R., OLIVER, J. E., TAGHIZADEH, T., FERREIRA, J. T. B., and LIEWEHR, D. 1999. Pheromones and colonization: Reassessment of the milkweed bug migration model (Heteroptera: Lygaeidae: Lygaeinae). *Chemoecology.* 9:63–71.

ALDRICH, J. R., ZHANG, A., and OLIVER, J. E. 2000. Attractant pheromone and allomone from the metathoracic scent gland of a broad-headed bug (Hemiptera: Alydidae). *Can. Entomol.* 132:915–923.

ATTYGALLE, A. B. 1998. Microchemical techniques, pp. 207–294, *in* J. G. Millar and K. F. Haynes (eds.), Methods in chemical ecology. Chapman & Hall, New York.

AYRES, M., AYRES, M. J., AYRES, D. L. and SANTOS, A. S. 2003. BioEstat3.0, Aplicações estatísticas nas áreas das ciências biológicas e médicas. Sociedade Civil Mamirauá, Belém.

BORGES, M. and ALDRICH, J. R. 1992. Instar-specific defensive secretions of stink bugs (Heteroptera: Pentatomidae). *Experientia* 48:893–896.

BORGES, M. and ALDRICH, J. R. 1994. Attractant pheromone for Nearctic stink bug, *Euschistus obscurus* (Heteroptera: Pentatomidae): insight into a Neotropical relative. *J. Chem. Ecol.* 20:1095–1102.

BORGES, M., MORI, K., COSTA, M. L. M., and SUJII, E. R. 1998. Behavioural evidence of methyl-2,6,10-trimethyltridecanoate as a sex pheromone of *Euschistus heros* (Het., Pentatomidae). *J. Appl. Entomol.* 122:335–338.

BORGES, M., ZARBIN, P. H. G., FERREIRA, J. T. B., and DA COSTA, M. L. M. 1999. Pheromone sharing: Blends based on the same compounds for *Euschistus heros* and *Piezodorus guildinii*. *J. Chem. Ecol.* 25:629–634.

BUNDY, C. S. and MCPHERSON, R. M. 2000. Dynamics and seasonal abundance of stink bugs (Heteroptera: Pentatomidae) in a cotton-soybean ecosystem. *J Econ Entomol* 93:697–706.

COSTA, M. L. M., BORGES, M., VILELA, E. F., MARCO JR, P. M., and LIMA, E. R. 2000. Effect of stereoisomers of the main component of the sex pheromone of *Euschistus heros* (F.) (Hemiptera: Pentatomidae) in the attractiveness of females. *An. Soc. Entomol Brasil* 29:413–422.

EL-SAYED, A. M. 2011. The Pherobase: database of insect pheromones and semiochemicals. < http://www.pherobase.com >.

FÁVARO, C. F., RODRIGUES, M. A. C. D. M., ALDRICH, J. R., and ZARBIN, P. H. G. 2011. Identification of semiochemicals in adults and nymphs of the stink bug *Pallantia macunaima* Grazia (Hemiptera: Pentatomidae). *J. Braz. Chem. Soc* 22:58–64.

FÁVARO, C. F. and ZARBIN, P. H. G. 2012. Identification of the defensive compounds in the metathoracic and dorsal abdominal glands of the stink bugs *Loxa deducta* and *Pellaea stictica* (Hemiptera: Pentatomidae). *Quim. Nova* in press.

FELDLAUFER, M. F., DOMINGUE, M. J., CHAUHAN, K. R., and ALDRICH, J. R. 2010. 4-oxo-Aldehydes from the dorsal abdominal glands of the bed bug (Hemiptera: Cimicidae). *J. Med. Entomol.* 47:140–143.

FERREIRA, J. T. B. and ZARBIN, P. H. G. 1996. Pheromone syntheses: A tropical approach. Enantioselective synthesis of the (2R,6 S,10 S) and (2 S,6 S,10 S) isomers of methyl 2,6,10-trimethyldodecanoate. *Bioorg. Med. Chem.* 4:381–388.

FUCARINO, A., MILLAR, J. G., MCELFRESH, J. S., and COLAZZA, S. 2004. Chemical and physical signals mediating conspecific and heterospecific aggregation behavior of first instar stink bugs. *J. Chem. Ecol.* 30:1257–1269.

GASSEN, D. N. 1996. Manejo de pragas associadas a cultura do milho. Aldeia Norte, Passo Fundo.

HO, H.-Y. and MILLAR, J. G. 2001. Compounds in metathoracic glands of adults and dorsal abdominal glands of nymphs of the stink bugs, *Chlorochroa uhleri*, *C. sayi*, and *C. ligata* (Hemiptera: Pentatomidae). *Zool. Stud.* 40:193–198.

LEAL, W. S., HIGUCHI, H., MIZUTANI, N., NAKAMORI, H., KADOSAWA, T., and ONO, M. 1995. Multifunctional communication in *Riptortus clavatus* (Heteroptera: Alydidae): Conspecific nymphs and egg parasitoid *Ooencyrtus nezarae* use the same adult attractant pheromone as chemical cue. *J. Chem. Ecol.* 21:973–985.

LEAL, W. S., UEDA, Y., and ONO, M. 1996. Attractant pheromone for male rice bug, *Leptocorisa chinensis*: Semiochemicals produced by both male and female. *J. Chem. Ecol.* 22:1429–1437.

LEAL, W. S., KUWAHARA, S., SHI, X., HIGUCHI, H., MARINO, C. E. B., ONO, M., and MEINWALD, J. 1998. Male-released sex pheromone of the stink bug *Piezodorus hybneri*. *J. Chem Ecol.* 24:1817–1829.

LUBECK, A. J. and SUTTON, D. L. 1983. Kovats retention indexes of selected hydrocarbons through C10 on bonded phase fused-silica capillaries. *J. High Res. Chromatog.* 6:328–332.

MILLAR, J. G., RICE, R. E., and WANG, Q. 1997. Sex pheromone of the mirid bug *Phytocoris relativus*. *J. Chem. Ecol.* 23:1743–1754.

MILLAR, J. G. 2005. Pheromones of true bugs. *Top. Curr. Chem.* 240:37–84.

MORAES, M. C. B., PAREJA, M., LAUMANN, R. A., and BORGES, M. 2008. The chemical volatiles (Semiochemicals) produced by neotropical stink bugs (Hemiptera: Pentatomidae). *Neotrop. Entomol.* 37:489–505.

MOREIRA, J. A. and MILLAR, J. G. 2005. Short and simple syntheses of 4-oxo-(E)-2-hexenal and homologs: Pheromone components and defensive compounds of hemiptera. *J. Chem. Ecol.* 31:965–968.

MORI, K. and MURATA, N. 1994a. Synthesis of methyl 2,6,10-trimethyltridecanoate, the male-produced pheromone of the stink bugs, *Euschistus heros* and *E. obscurus*, as a stereoisomeric mixture. *Liebigs Ann. Chem.* 1994:637–639.

MORI, K. and MURATA, N. 1994b. Synthesis of All of the Eight Stereoisomers of Methyl 2,6,10-Trimethyltridecanoate, the Male-produced Pheromone of the Stink Bugs. Euschistus heros and E. obscurus. *Liebigs Ann. Chem.* 1994:1153–1160.

MORI, K. 2007. Significance of chirality in pheromone science. *Bioorg. Med. Chem.* 15:7505–7523.

PAVIS, C., MALOSSE, C., DUCROT, P. H., and DESCOINS, C. 1994. Dorsal abdominal glands in nymphs of southern green stink bug, *Nezara viridula* (L.) (Heteroptera: Pentatomidae): chemistry of secretions of five instars and role of (E)-4-oxo-2-decenal, compound specific to first instars. *J. Chem. Ecol.* 20:2213–2227.

RIDER, D. A. and ROLSTON, L. H. 1987. Review of the genus *Agroecus* Dallas, with the description of a new species (Hemiptera: Pentatomidae). *J. N. Y. Entomol. Soc.* 95:428–439.

SCHWERTNER, C. R. and GRAZIA, J. 2007. The genus *Chinavia* Orian (Hemiptera, Pentatomidae) in Brazil, with a pictorial key to adults. *Revista Brasileira Entomologia* 51:416–435.

SILVERSTEIN, R. M., WEBSTER, F. X., and KIEMLE, D. J. 2005. Spectrometric Identification of Organic Compounds. John Wiley & Sons, New York.

SMITH, B. C. 1999. Infrared spectral interpretation: a systematic approach. CRC Press, New York.

SMITH, R. F., PIERCE JR, H. D., and BORDEN, J. H. 1991. Sex pheromone of the mullein bug, *Campylomma verbasci* (Meyer) (Heteroptera: Miridae). *J. Chem. Ecol.* 17:1437–1447.

SOLDI, R. A., RODRIGUES, M. A. C. D., ALDRICH, J. R., and ZARBIN, P. H. G. 2012. The sex pheromone of the true bug *Phthia picta* (Heteroptera: Coreidae) is an unusual hydrocarbon. *J. Chem. Ecol*. 38:814–824. doi:10.1007/s10886-012-0147-1.

STADDON, B. W., GOUGH, A. J. E., OLAGBEMIRO, T. O., and GAMES, D. E. 1985. Sex dimorphism for ester production in the metathoracic scent gland of the lygaeid bug *Spilostethus rivularis* (German) (Heteroptera). *Comp. Biochem. Physiol.* 80B:235–239.

ZARBIN, P. H. G., FERREIRA, J. T. B., and LEAL, W. S. 1999. Metodologias gerais empregadas no isolamento e identificação estrutural de feromônios de insetos. *Quím. Nova* 22:263–268.

ZARBIN, P. H. G., RECKZIEGEL, A., PLASS, E., BORGES, M., and FRANCKE, W. 2000. Synthesis and biological activity of methyl 2,6,10-trimethyldodecanoate and methyl 2,6,10-trimethyltridecanoate: Male produced sexual pheromones of stink bugs *Euschistus heros* and *Piezodorus guildinii*. *J. Chem. Ecol.* 26:2737–2746.

ZARBIN, P. H. G., FÁVARO, C. F., VIDAL, D. M., and RODRIGUES, M. A. C. M. 2012. Male-produced sex pheromone of the stink bug *Edessa meditabunda*. *J. Chem. Ecol*. 38:825–835. doi:10.1007/s10886-012-0144-4.

ZHANG, Q.-H. and ALDRICH, J. R. 2003. Pheromones of milkweed bugs (Heteroptera: Lygaeidae) attract wayward plant bugs: *Phytocoris* mirid sex pheromone. *J. Chem. Ecol.* 29:1835–1851.

ZHANG, Q.-H. and ALDRICH, J. R. 2008. Sex pheromone of the plant bug, *Phytocoris calli* Knight. *J. Chem. Ecol.* 34:719–724.