SCIENTIFIC NOTE

Stimulatory MaleVolatiles for the Neotropical Brown Stink Bug, *Euschistus heros* (F.) (Heteroptera: Pentatomidae)

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Semioquímicos Produzidos Pelo Macho do Percevejo Neotropical *Euschistus heros* (Fabr.) (Heteroptera: Pentatomidae)

RESUMO – Compostos voláteis obtidos de machos do percevejo marrom Neotropical *Euschistus heros* (F.), uma das principais pragas da cultura da soja na região da América Central e Sul, foram reavaliados. A proporção dos três ésteres já identificados mostrou-se bem diferente da determinada em estudos anteriores. Uma nova combinação de ésteres metílicos é proposta como voláteis estimulatórios produzidos pelos machos, baseando-se nos resultados obtidos das análises por detecção eletroantenográfica acoplada ao cromatógrafo gasoso (GC-EAD). Os três componentes ativos detectados por CG-EAD nos voláteis coletados da aeração de machos foram os (2*E*,4*Z*)-decadienoato de metila (53%), 2,6,10-trimetildodecanoato de metila (3%), e 2,6,10-trimetiltridecanoato de metila (44%). A liberação dos voláteis pelos machos adultos alcança níveis detectáveis para análise por cromatografia gasosa capilar com detector de ionização de chamas quando os insetos estão com aproximadamente 15 dias na fase adulta. A quantidade dos três compostos estimulatórios aumentou com a idade dos percevejos e atingiu um nível máximo de produção com aproximadamente 35 dias de fase adulta. A taxa de liberação desses compostos é aproximadamente de 2,5 µg/macho/dia e a proporção desses três componentes parecem não ser afetada com a idade após a maturidade sexual.

PALAVRAS-CHAVE: Praga da soja, volátil estimulatório, (2*E*,4*Z*)-decadienoato de metila, 2,6,10-trimetildodecanoato de metila, 2,6,10-trimetiltridecanoato de metila, detecção eletroantenográfica

ABSTRACT – Volatiles compounds collected from the male Neotropical brown stink bug, *Euschistus heros* (F.), a serious Central and South American soybean pest, have been reevaluated. The proportion of three methyl esters is found to be quite different from previous study. A new blend is proposed as the male-produced stimulatory volatiles based on gas chromatographic-electroantennographic detection (GC-EAD) techniques. The three GC-EAD-active components reproducibly found in volatiles collected from males are methyl (2*E*,4*Z*)-decadienoate (53%), methyl 2,6,10-trimethyldodecanoate (3%), and methyl 2,6,10-trimethyltridecanoate (44%) respectively. Males of this hemipteran species needed to reach an age of ~15 days to produce enough male-specific compounds to be detected by capillary column GC equipped with flame ionization detector (FID). The amounts of these three stimulatory volatiles increased with age and appeared to reach a maximum at ~35 days old, with the release rate of ~2.5 µg/day/male and the ratio of these three components seemed not to be affected by aging.

KEY WORDS: Soybean pest, stimulatory volatile, methyl (2*E*,4*Z*)-decadienoate, methyl 2,6,10-trimethyldodecanoate, methyl 2,6,10-trimethyltridecanoate, electroantennographic detection

The Neotropical brown stink bug, *Euschistus heros* (Fabr.) is one of a complex of stink bugs that are serious pests of soybean, *Glycine max* (L.) Merryl, in Central and South America, especially in Brazil (Panizzi & Rossi 1991). Methyl 2,6,10-trimethyltridecanoate has been reported as the only predominant male-produced volatile of this species (Aldrich

et al. 1994, Borges & Aldrich 1994) and sex pheromone activity of this compound has also been confirmed in a laboratory olfactometer bioassay (Borges *et al*. 1998b). During a field test conducted in Brazil, however, not only *E. heros*, but also some other species of the soybean stink bug complex were caught in traps baited with the single compound, methyl 2,6,10-trimethyltridecanoate. In fact, another pentatomid, *Piezodorus guildinii* (West.), was captured in significantly greater numbers than was *E. heros* (Borges *et al*. 1998a). Thus, not only may the mating communication systems, in the family Pentatomidae, involve some common components (Borges *et al*. 1999), but also additional factors must be involved in species isolation. Additional components, or precise proportions of components may be needed to confer species specificity. Therefore, airborne volatiles of *E. heros* from individual sexes have been collected and reevaluated. Applying gas chromatographic-electroantennographic detection (GC-EAD) enabled us to focus on compounds eliciting a neural response in the bug. Although an antennal response is not necessarily a predictor of behavior, it is reasonable to expect that most, if not all, volatile compounds capable of eliciting a behavioral response would also stimulate an antennal response.

Here we report the results of aeration of virgin males and female for 40 days post-emergence, GC-EAD and GC-mass spectrometry (GC-MS) analysis. This more in-depth study has demonstrated that males of *E. heros* needed to reach an age of ~15 days to produce enough male-specific compounds to be detected by capillary column GC equipped with flame ionization detector (FID) and the proportion of male-specific components is very different from that reported in earlier work on this species.

E. heros were obtained from a laboratory colony started from adults collected near the Centro Nacional de Recuros Geneticos e Biotechnologic, Brasilia D. F., Brazil. They were reared in Beltsville laboratory on raw peanuts (*Arachis hypogaea*), soybeans (*Glycine max*), fresh green beans (*Phaseolus vulgaris*), and water at 25 ± 1.0ºC and 65% relative humidity on a 14L:10D photoperiod. To prevent olfactory interactions between the sexes, males were separated from females after their imaginal molt and cuticular hardening (ca. 24h).

Volatile collection used groups of twenty newly emerged male or female (1-day-old) *E. heros*. The bugs were gently introduced into glass containers (Zhang *et al*. 1994) to avoid emission of defensive gland secretions. Air was drawn into the container through 6-14 mesh activated charcoal (Fisher Scientific, Pittsburgh, PA), and out of the container through two traps (15 cm x 1.5-cm OD) containing Super Q (200 mg each; Alltech Associates, Inc., Deerfield, IL) by vacuum (~1 liter/min.). Male and female bugs were fed with raw peanuts, soybeans, and water (replaced weekly) and aerated continuously for 40 days, and adsorbents were changed every 24h. The adsorbents were eluted with methylene chloride (4 x 0.5 ml); the eluates (2 ml/each sample) were stored at -30ºC in a freezer until further analysis.

The steroisomeric mixture of methyl 2,6,10-trimethyltridecanoate was synthesized by Mori and Murata (Mori & Murata 1994), and methyl 2,6,10-trimethyldodecanoate was prepared by Ferreira & Zarbin (1996). Methyl (2*E*,4*Z*)-decadienoate (~85% purity, Bedoukian Research, Inc., Danbury, CT) was purified as follows: methyl (2*E*,4*Z*)-decadienoate (50.5 g, 0.28 mol) was hydrolyzed to the corresponding acid with potassium hydroxide (85% purity, 21.90 g, 0.33 mol) in 20 ml of water and 50 ml of absolute ethanol for 2 hours at 50ºC with stirring (Vliet *et al*. 1969). The mixture was concentrated under reduced pressure and resulting thick oil was dissolved in 100 ml of potassium hydroxide water solution and extracted successively with three 100 ml portions of diethyl ether, which were discarded. The water layer was acidified with 6 *N* hydrochloric acid to pH = 1. The solution was saturated with solid sodium chloride and extracted with three portions of methylene chloride (100 ml/each). The extracts were dried over anhydrous sodium sulfate and concentrated *in vacuo* to give the resultant acid (44.88 g, 0.27 mol, 96% yield) as colorless oil. A 1 M acetone solution of the resultant acid was combined with a 1 M acetone solution of cyclohexylamine (31.72 g, 0.32 mol) and the resulting solution was cooled to ~4ºC. The white solid was collected and recrystallization was carried out at same concentration. Purity of the acid in each crystallization step was monitored by GC after methylation of several crystals with diazomethane. After five recrystallizations, the acid was reesterified with thionyl chloride in methanol (Zhang *et al*. 1997) to the methyl ester (26.70 g, 0.15 mol) in 53% overall yield. Purity of methyl (2*E*,4*Z*)-decadienoate was > 98% based on FID (Fig. 1b).

A coupled GC-EAD system was as previously described (Zhang *et al*. 1997, 1999). A Hewlett Packard 6890 gas chromatograph equipped with a 60 m x 0.25-mm ID, 0.25-µm film-thickness DB-5 or DB-WAXETR capillary column (J&W Scientific Inc., Folsom, CA) in the splitless mode with hydrogen carrier gas (1.4 ml/min.) was used for GC-EAD analysis (80ºC for 2 min., then programmed to 250ºC at 15 ºC/min. and held for 10 min.). Both antennae were pulled off from a bug and positioned between two gold wire electrodes without cutting off the antennal tips, which were immersed in saline-filled (0.9% NaCl) wells (1.25 mm diameter wells about 3 mm apart) in a small acrylic plastic holder (8 cm long x 0.8 cm wide x 0.6 cm thick). The output recording electrodes were connected to a high-impedance 1:100 amplifier with automatic baseline drift compensation. The airstream flowing over the antennae (about 500 ml/min.) was humidified by bubbling through distilled water before entering the EAD interface. The antennal preparation was cooled to ~5ºC inside a condenser by circulating near 0ºC water from a benchtop refrigeration unit (RTE-100, NESLAB instruments, Inc., Portsmouth, NH) through the insulation layer of the modified condenser containing the acrylic plastic holder mounted on top of the GC. The flame ionization and electrophysiological output signals were recorded using Hewlett-Packard ChemStation software.

Electronic impact (EI) GC-MS was conducted on a Hewlett-Packard 6890 GC coupled to a HP 5973 Mass Selective Detector using an identical GC column and conditions as described above but with helium as carrier gas.

With the airborne collection conditions, only three male-specific compounds were detected by FID and EAD in the aeration extract from a group of 28-day-old male *E. heros* on a 60 m polar DB-WAXETR capillary column (Fig. 2). Females produced almost nothing at the same time and same conditions.

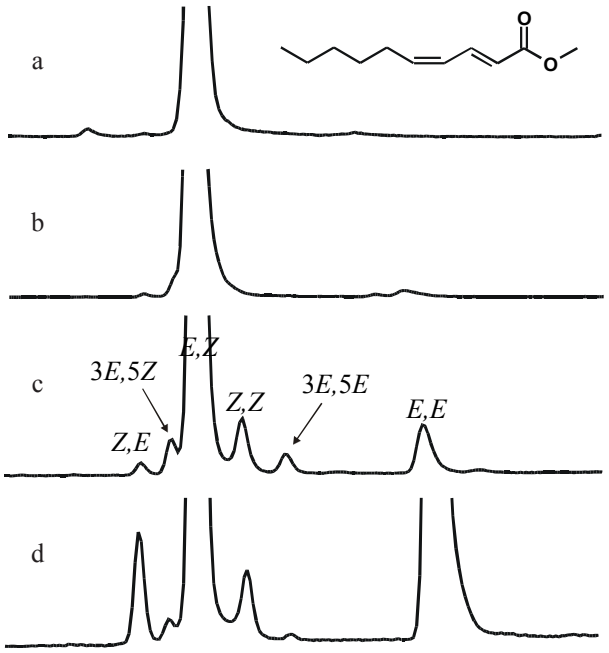

Figure 1. Reconstructed gas chromatograms (60 m of DB-WAXETR capillary column) of methyl 2,4-decadienoate from a) *E. heros* males, b) purified *E,Z* isomer, c) Newly purchased Bedoukian, Inc. sample, and d) old Bedoukian, Inc. sample.

EI-MS of compound I matches MS of methyl (2*E*,4*Z*)-decadienoate (53%) retrieved from the Wiley 275 mass spectral database. Compounds II and III have EI-MS matching those of compounds, methyl 2,6,10-trimethyldodecanoate (3%), and methyl 2,6,10-trimethyltridecanoate (44%), identified in the earlier report on *E. obscurus* (Aldrich *et al*. 1994). Identities of these three esters were confirmed by comparison of their GC retention times with those of authentic standards on both polar and non-polar capillary columns and re-confirmed by GC-EAD activity of synthetic compounds.

Geometry of natural methyl 2,4-decadienoate was verified by comparison of GC retention time with commercially available methyl (2*E*,4*Z*)-decadienoate (Bedoukian Research, Inc., Danbury, CT) and indicated as 2*E*,4*Z* isomer (Fig. 1). In addition, it was found that newly purchased commercial sample consisted not only of all four geometric isomers [different retention times, similar EI-MS; *E,Z* isomer: *m/z* (%): 182 (M^+, 39), 151 (25), 139 (7), 122 (12), 111 (100), 81 (69), 79 (41), 67 (42)], but also contained approximately 3% of another pair of 3,5-isomers [Fig. 1c, 2% (3*E*,5*Z*) and 1% (3*E*,5*E*) respectively, samilar EI-MS, *m/z* (%): 182 (M^+, 40), 150 (7), 140 (3), 126 (13), 122 (17), 108 (44), 97 (19), 93 (21), 84 (50), 79 (100), 67 (78); retention times are shorter than 2*E*,4*Z* isomer in a DB-5 capillary column; identities of 3,5-isomers were confirmed by syntheses]. The elution order (Bæckström *et al*. 1988) and percentage of the methyl 2,4-decadienoates on a DB-WAXETR capillary column were 1% (*Z,E*), 86% (*E,Z*), 4% (*Z,Z*), and 6% (*E,E*) (Fig. 1c). The base peak, *m/z* 111 are more dominant in MS of *Z,E* and *Z,Z* than other two isomers because of easily formation of this pyrylium fragment (Bæckström *et al*. 1988). Methyl 2,4-decadienoate could be further isomerized to the other geometric isomers [(3% (*Z,E*), 51% (*E,Z*), 2% (*Z,Z*), and 41% (*E,E*)], if the same Bedoukian sample was kept at room temperature (more than three years for our sample) (Fig. 1d). The most stable *E,E* isomer increased from 6% to 41% and the most unstable *Z,Z* isomer decreased from 4% to 2%. The most abundant *E,Z* isomer decreased from 86% to 51%.

Repeated aerations (n = 3) of 20 males over a period of 40 consecutive days showed that young males did not produce male-specific compounds and they needed at least ~15 days old to produce enough compounds to be detected by FID (Fig. 3). The amounts of these three EAD-active compounds increased with age and appeared to reach a maximum at approximately 35

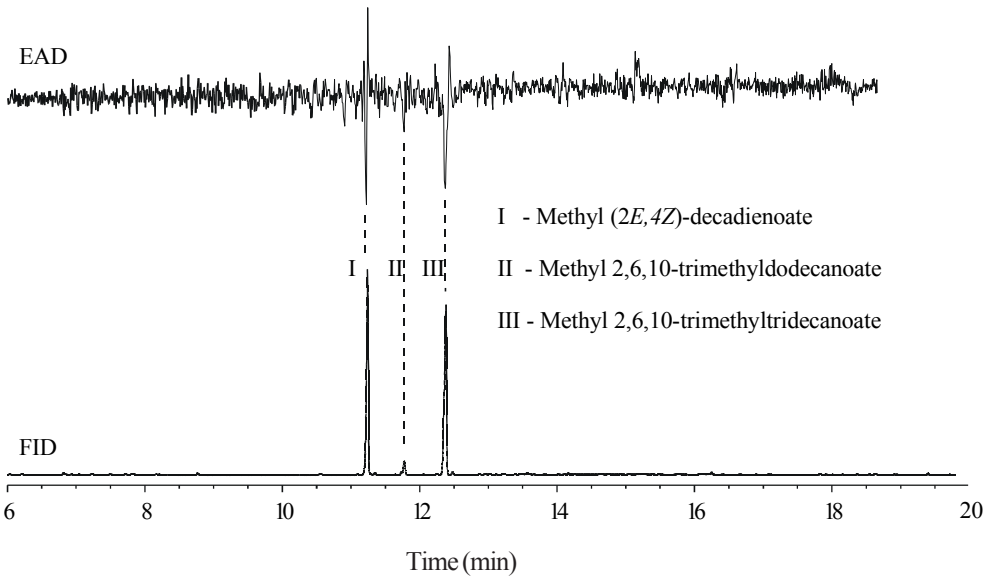

Figure 2. Simultaneous responses of flame ionization detector (FID) and electroantennographic detector (EAD) of an adult female *E. heros* to male volatiles on a 60-meter DB-WAXETR capillary column.

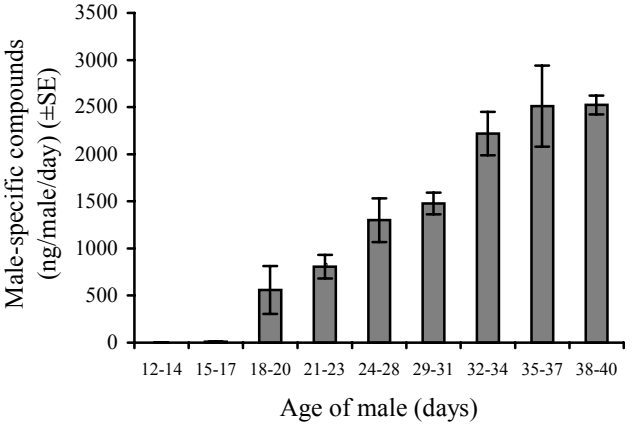

Figure 3. Combined quantity of the three male-produced compounds collected from emissions of 20 male *E. heros* over a period of 40 consecutive days (n = 3).

days old, with the approximate release rate of 2.5 µg/day/male. After about two and half weeks when high levels of male-specific compounds began to be produced, the ratio of these three components seemed not to be affected by aging. Male *E. heros* produces >99% pure *E*,*Z* isomer (Fig. 1a).

The male-produced compounds have been previously observed in the aeration extract of male *E. heros*, but methyl 2,6,10-trimethyltridecanoate was believed to be the only predominant male-produced component (Aldrich *et al*. 1994, Borges & Aldrich 1994). Males used to conduct the aeration extracts in the earlier study were prepared in Brazil using insects of unknown age, probably younger than the age when *E. heros* males are known to commence high levels of male-specific volatiles production (~18 days). In our current work, the Brazilian colony of *E. heros* was established in the Beltsville laboratory, enabling us to collect enough newly emerged male and female insects for replicated airborne collections over extended time periods. We believe that the discrepancy between the earlier and present results is likely due to the fact that the earlier study used *E. heros* males prior the age at which male-specific volatiles production had stabilized.

Volatiles collected from adult stink bugs often contain large amounts of defensive chemicals released by disturbed or dead bugs from their ventral metathoracic scent gland (MTG) (Aldrich 1988) and small amounts of secretions released from their dorsal abdominal gland (DAG) (Aldrich *et al*. 1995). However, if the insects remained calm during loading and remained alive during collection, the volatile defensive secretions could be largely avoided. With our airborne collection conditions, only three male-specific compounds were detected by FID in the aeration extract from a group of 28-day-old male *E. heros*.

As a result of the current work, we have revised our assessment of the relative ratio of the components of the blend: methyl (2*E*,4*Z*)-decadienoate, methyl 2,6,10-trimethyldodecanoate, and methyl 2,6,10-trimethyltridecanoate occur in the ratio of 53% : 3% : 44%, respectively. The biological role of methyl (2*E*,4*Z*)-decadienoate and methyl 2,6,10 trimethyldodecanoate in the *E. heros* male-specific emission still need to be addressed for mate finding in its natural habitat.

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