Physiological Entomology (2008) **33,** 43–50 DOI: 10.1111/j.1365-3032.2007.00600.x

Food and humidity affect sex pheromone ratios in the stink bug, *Euschistus heros*

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Abstract. Male stink bugs, *Euschistus heros*, only produce the three-component sex pheromone blend consisting of methyl-(2*E*,4*Z*)-decadienoate, methyl-2,4,6-trimethyldodecanoate and methyl-2,4,6-trimethyltridecanoate in a constant ratio of 53 : 3 : 44, respectively, when provided with a food source (green beans, *Phaseolus vulgaris*). When volatiles are collected from insects with drinking water, humidified air, or under dry conditions, the insects stop producing two components (methyl-2,4,6-trimethyldodecanoate and methyl-2,4,6-trimethyltridecanoate) after 1 day and increase the amount of defensive compounds produced [e.g. (*E*)-2-hexenal, decane]. Methyl-2,4,6-trimethyltridecanoate is able to change the behaviour of females but not males. Dual-choice olfactometer bioassays with synthetic standards of all three components, as well as the ternary mixture in the correct ratio, induce a response from females but not males, indicating that all three components have a role in sexual communication. Thus, the dietary conditions under which volatile collections are carried out are crucial for determination of the precise sex pheromone blend.

Key words. Olfactometer, semiochemicals, sex pheromone, stink bug.

Introduction

Pheromones are chemical compounds released by organisms into their surrounding environment that can affect the physiology and behaviour of conspecific individuals (Nordlund & Lewis, 1976). There is great interest in sex pheromone studies from an applied perspective for use in integrated pest management. Studies show that food is important for the pheromone-producing sex, mainly as a source of precursors for pheromone synthesis and for endocrine regulation (Tamaki, 1985; Tillman *et al.*, 1999; Reddy & Guerrero, 2004). One of the first studies on the influence of food on pheromone production reveals that the male boll weevil (*Anthonomus grandis*) reduces pheromone production by approximately 50% 1 h after the removal of food and by approximately 90% after 24 h (Hardee, 1970). Meola *et al.* (1977) show that female stable flies, *Stomoxys calcitrans*, produce the sex pheromone when they are fed with bovine blood but stop producing when they are fed with a solution of glucose and salts. Compounds from the diet can have synergistic effects, increasing the response of the pheromone to receiver or inducing the production and release of the pheromone (Reddy & Guerrero, 2004).

Despite these known effects of food on pheromone production and release, there is no information on the importance of diet in the production of sex pheromones of pentatomids. The male-produced sex pheromone of the Neotropical brown stink bug, *Euschistus heros* (F.) (Hemiptera: Pentatomidae), is reported as methyl-2,6,10-trimethyltridecanoate, with two other components: methyl-(2*E*,4*Z*)-decadienoate and methyl-2,4,6-trimethyldodecanoate (Aldrich *et al.*, 1994; Borges & Aldrich, 1994; Borges *et al.*, 1998a; Costa *et al.*, 2000; Zarbin *et al.*, 2000a). Zhang *et al.* (2003) show that male *E. heros* produce the three components in the sex pheromone in the ratio: 53% methyl-(2*E*,4*Z*)-decadienoate, 3% methyl-2,4,6-trimethyldodecanoate and 44% methyl-2,4,6-trimethyltridecanoate. In laboratory bioassays, methyl-2,4,6-trimethyltridecanoate is confirmed as the main component of the pheromone blend (Borges *et al.*, 1998b) and Costa *et al.* (2000) show that the racemic mixture of methyl-2,4,6-trimethyltridecanoate is efficient in attracting female *E. heros*.

The present study aims to understand the importance of feeding during the period of volatile collection and to evaluate

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the function of the other two male specific components [methyl-(2*E*,4*Z*)-decadienoate, and methyl-2,4,6-trimethyldodecanoate] in the behavioural response of male and female *E. heros*. Accordingly, air entrainments are carried out with males under different food and humidity conditions and bioassays are carried out using the synthetic sex pheromone components as stimuli.

Materials and methods

Insects

Euschistus heros individuals were obtained from a laboratory colony started from adults collected from soybean fields near the Embrapa Genetic Resources and Biotechnology Laboratory, Brasília, DF, Brazil (15°47′S, 47°55′W). Bugs were reared on raw peanuts (*Arachis hypogaea*), soybeans (*Glycine max*), sunflower seeds (*Helianthus annuus*), fresh green beans (*Phaseolus vulgaris*), and water at 26 ± 1 °C and 65% RH under an LD 14 : 10 h photoperiod (lights on 06.00 h). Insects were reared in 8-L plastic containers (100–150 insects/container) with the food supply being renewed three times per week. A 15-cm^2 piece of plastic mesh (approximately 40 mesh) was placed against the inner wall of each container as an oviposition substrate.

Egg masses were collected daily and incubated in 9-cm plastic Petri dishes until hatching. When the resulting nymphs moulted to second instar, they were transferred to plastic containers and reared as above. To prevent interactions between the sexes, males were separated from females after their imaginal moult and cuticular hardening (approximately 24 h after moulting). To evaluate the age when the males start to release the blend of sex pheromone, volatile collection was carried out using young insects (7–8 days old). Only sexually mature adults were used for experiments and bioassays.

Collection of volatiles

Preliminary studies using 20 males per air entrainment (eight replicates with green beans as a food source) were carried out with males from 7–8 days old to determine the age at which they begin to release pheromone components. The results showed that the insects start to release the three components in the ratio reported by Zhang *et al.* (2003) only after 9 days. Therefore, fully replicated volatile collections were carried out for groups of 20, 9-day-old male *E. heros*. The insects were carefully introduced into 1-L glass containers to minimize emission of defensive secretions (Zhang *et al.*, 1994). Air was drawn into the container through a bed of 4–12 mesh activated charcoal (Fisher Scientific, Pittsburgh, PA), and out of the container by vacuum (approximately 1 L min^{-1}) through two traps (15 × 1.5 cm outer diameter), each containing 200 mg of Super Q (Alltech Associates Inc., Deerfield, IL). Volatile collections were carried out with five treatments: (i) food-insects: insects fed with fresh green beans (the green beans were replaced three times per week); (ii) starved with water-insects: insects air entrained with water in a glass tube placed inside the chamber; the insects drank water through a piece of cotton wool that was immersed in the water; (iii) starved with humidity-insects: insects air entrained with a flow of humidified air; (iv) starved insects: insects air entrained without food and in dry air; and (v) three pods of green beans without insects to evaluate if green beans release detectable volatiles. Each treatment was replicated 12 times in three sets with four replicates per set.

All chambers were entrained continuously over 7 days, changing the adsorbent traps daily. The traps of each chamber were eluted with 2 mL of hexane and the eluates were combined and stored at −30 °C until needed for chemical analysis. The extracts were concentrated under nitrogen to yield a concentration of approximately 0.1 bug-equivalent μL^{-1} of solution to be tested. For quantitative analysis, the volume of the extract was reduced to approximately 200 μL and an internal standard (1.1 μg 4-ethyl-dodecanoate per 50 μL) was added to the solution.

Survival analysis

Insects maintained in the entrainment chambers were observed daily to calculate the number dying each day. Dead insects were removed from the containers. Data on daily mortality were used to construct and analyse survivorship curves.

Analysis of extracts

Volatile extracts were analyzed by gas chromatography using gas chomatography (GC) (Perkin-Elmer, Überlingen, Germany) equipped with a flame ionization detector (FID) at 250 °C, using a DB-5 column (30 m × 0.25 mm inner diameter, 0.25 μm film; J&W Scientific, Folsom, California), which was maintained at 50 °C for 2 min, then programmed at 15 °C min^{-1} to 250 °C (10 min).

Extracts were also analysed by coupled GC-mass spectrometry using a Shimadzu QP-2010 mass selective detector (Schimadzu Corp., Kyoto, Japan), with electron impact ionization (70 eV). A TRB-5 column (30 m × 0.25 mm inner diameter, 0.25 μm film thickness; Teknokroma, Barcelona, Spain), was maintained at 50 °C for 1 min then programmed at 15 °C min^{-1} to 250 °C (10 min), splitless injection at 250 °C, and transfer line 250 °C. The compounds were identified by comparing the retention time and mass spectra with those of authentic standards.

Chemicals

The stereoisomeric mixture of methyl-2,6,10-trimethyltridecanoate was synthesized by Mori & Murata (1994), and methyl-2,6,10-trimethyldodecanoate was prepared by Ferreira & Zarbin (1996). Methyl-(2*E*,4*Z*)-decadienoate (approximately 85% purity, Bedoukian Research, Inc., Danbury, Connecticut) was purified as described in Zhang *et al.* (2003).

Synthetic 4-oxo-(*E*)-2-hexenal and (*E*)-2-decenyl acetate were provided by J. R. Aldrich (USDA, Beltsville, Maryland). All other authentic standards were purchased from Sigma-Aldrich (St Louis, Missouri) or Fluka (Buchs, Switzerland).

Bioassay

A two-choice olfactometer modified from Borges & Aldrich (1994) was used to test the biological activity of odours from live males, male air entrainments and synthetic compounds. The olfactometer consisted of a 500-mL three-neck, round-bottom flask (24/40 joints, Kontes, Vineland, New Jersey), used as the release chamber. Two 250-mL rotary evaporator trap adapters (24/40 joints) were attached to the two outside arms to provide treatment and control chambers. A charcoal filter (20/40 mesh, 130-mm × 10-mm inner diameter) connected in series to a water bubbler to humidify the incoming air was attached to the inlets of the two chambers with silicon rubber tubing (Silastic, 4.8 mm inner diameter; VWR Scientific, Darmstadt, Germany). Air was pulled through the system (400 mL min^{-1}) by connecting the middle neck of the flask to a regulated vacuum source, with the flow measured with a Clear Flow Rotameter (Accura Flow Products, Warminster, Pennsylvania). The olfactometer was positioned horizontally on a countertop in a room with bright fluorescent 'day' lights (4 × 40 W, 50–60 Hz). Temperature in the bioassay room was maintained at 26.0 ± 1.0 °C. Positions of control and treatment arms were alternated between replicates to avoid any positional bias. The apparatus was cleaned after each five replicates with fragrance-free liquid soap, rinsed thoroughly with water, and dried in a convection oven at 80 °C.

Bioassays with male entrainment extracts

The response of females to male extracts were tested using 5 μL of crude entrainment extract of 20 males from the food treatment applied to a 2.0 × 0.5 cm strip of filter paper against the control (5 μL of hexane). The solvent was allowed to evaporate for 20 s before placing the strips in the connectors of the olfactometer. Ten sexually mature virgin females (> 20 days old) were introduced into the release chamber of the olfactometer and the number entering the arm with the extract was recorded. The response of females to one of the treatments was registered by counting the number of insects that had entered at least 5 cm inside the arm at the end of the experiment. Each experiment lasted 10 min. Ten replicates of this bioassay were carried out. Bioassays with extracts obtained from the starved, starved with water and starved with humidity were not carried out because of the high quantity of defensive compounds present in these extracts, which could mask the attraction to sex pheromone components. Therefore, the influence of the three sex pheromone components, individually and in mixture, were evaluated using synthetic compounds.

Biossays with synthetic compounds

Effects of individual compounds [methyl-(2*E*,4*Z*)-decadienoate, methyl-2,6,10-trimethyldodecanoate, methyl-2,6,10-trimethyltridecanoate] as well as their ternary mixture (53 : 3 : 44% by volume, respectively) were determined by recording female and male response to 5 μL of 1 μg mL^{-1} hexane solution of each on filter paper strips. Ten replicates using the females introduced simultaneously into the olfactometer were tested towards each treatment. For males, the number of replicates was higher (30 replicates of ten sexually mature virgin males > 15 days old), and different concentrations of the standards were used (5 μL and 50 μL of 1 μg mL^{-1} hexane solution) because the response was more ambiguous than that of females. The response was registered as a number of insects inside of each arm after 10 min of the experiment.

Statistical analysis

Bioassay data (responses of individuals to treatments versus controls) were analysed using chi-square tests. The proportions of each component of the sex pheromone were compared between days and between treatments by nultivariate analysis of variance after arcsine transformation of the data. The difference in quantity of the sex pheromone and defensive compounds in each treatment and between the treatments were analysed using analysis of variance. The mortality of the insects in the different treatments were analysed using Kaplan–Meier survival analysis followed by pairwise multiple comparison procedures (Holm–Sidak method).

Results

Volatile collection and identification

The preliminary studies revealed that production of pheromone components in insects 7 and 8 days old was highly variable (Fig. 1) and, often, only one component was produced (data not shown).

Comparison of gas chromatograms and mass spectra of volatiles collected on the first day of entrainment from the four different replicated treatments assembled with sexually mature virgin males of *E. heros* showed the presence of the three components of the sexual pheromone, methyl-(2*E*,4*Z*)-decadienoate, methyl-2,4,6-trimethyldodecanoate and methyl-2,4,6-trimethyltridecanoate, as well as a number of defensive compounds that are common to other stink bug species (Borges & Aldrich, 1992; Zarbin *et al.*, 2000b; Pareja *et al.*, 2007), including (*E*)-2-hexenal, 4-oxo-(*E*)-2-hexenal, decane, (*E*)-2-octenal, undecane, dodecane, tridecene, tridecane, tetradecane, (*E*)-2-decenyl acetate and (*E*)-2-hexenyl acetate. All these compounds are produced by the insects because the aerations carried out with green beans did not release any compound detectable by GC-FID (data not shown).

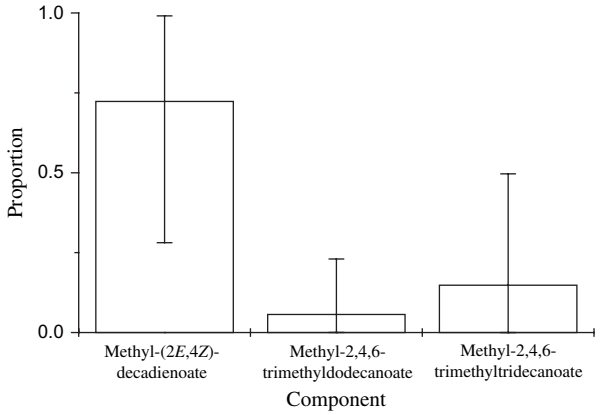

Fig. 1. Proportion of the three sex pheromone components (mean and 95% confidence interval; CI) produced by male *Euschistus heros* aged 7–8 days. The mean and 95% CI were calculated for the arcsine-transformed data and back-transformed for presentation.

All aerations carried out in the food treatment ($n = 12$) showed that the total release rate of sex pheromone components by males 9 days old or older, was constant over 7 days and it was approximately 6.75 ± 3.14 μg male^{-1} day^{-1} (mean ± SE).

The chemical analysis of extracts obtained from airborne collections where the insects (> 9 days old) were kept with food revealed three male-specific compounds in the ratio: 53% methyl-(2*E*,4Z)-decadienoate, 3% methyl-2,4,6-trimethyldodecanoate and 44% methyl-2,4,6-trimethyltridecanoate, which is the same ratio reported by Zhang *et al.* (2003) (Fig. 2A). The proportion of each component released did not vary between days [methyl-(2*E*,4*Z*)-decadienoate, $F_{6,24} = 3.16$, $P = 0.922$; methyl-2,4,6-dodecanoate, $F_{6,24} = 0.441$, $P = 0.898$; methyl-2,4,6-tridecanoate, $F_{6,24} = 0.227$, $P = 0.964$] (Fig. 2A).

The proportion of each sex pheromone component released depended on the treatment ($F_{9,183} = 15.24$, $P < 0.001$), on the day ($F_{18,213} = 4.46$, $P < 0.001$) and also on the treatment-by-day interaction ($F_{54,224} = 3.25$, $P < 0.001$), indicating that the proportions changed differently comparing the treatments. In the food treatment, the proportion was maintained at approximately 53 : 3 : 44 during the 7 days of aeration whereas, in the starved, starved with humidity and starved with water treatments, this proportion was obtained on the first day, but there was a progressive increase in the relative amount of methyl-(2*E*,4*Z*)-decadienoate over subsequent days (Fig. 2B–D). In the starved with water treatment, the sex pheromone components showed high variability, and methyl-(2*E*,4*Z*)-decadienoate was produced in higher quantities compared with the other two components. In the starved with humidity treatment, there also was a great variability in the ratio between the three components and, even though the overall production of sex pheromone components increased, after the third day, only methyl-(2*E*,4*Z*)-decadienoate was released in any quantity, with the other two components being released only in trace quantities (Figs 2C, 3C). In the starved treatment, release of all volatile compounds decreased due to mortality.

In the food treatment, the quantity of defensive compounds was constant over the 7 days ($F_{6,22} = 1.780$; $P = 0.106$) and in lower quantity than sexual pheromone production (Fig. 3A). On the first day, there was a higher production of defensive compounds due the manipulation of the insects when introducing them into the glass chambers (Borges *et al.*, 2007).

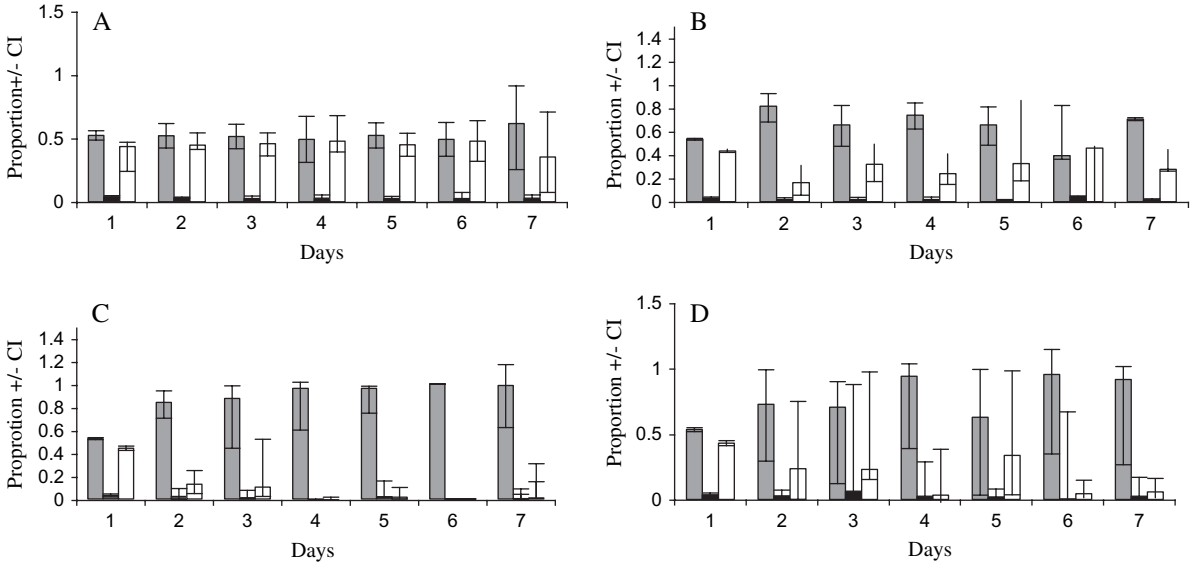

Fig. 2. Proportions (± 95% confidence interval; CI) of the sex pheromone components released by *Euschistus heros* males during aerations in each treatments. (A) Food treatment, (B) starved with water, (C) starved with humidity and (D) starved. ■, Methyl-(2*E*,4*Z*)-decadienoate; □, methyl-2,6,10 trimethyldodecanoate; ■, methyl-2,6,10 trimethyltridecanoate. Collections began with groups of 20 9-day-old male *E. heros*.

The insects in the starved treatment stopped producing the sex pheromone in significant quantity from the second day onwards and increased the production of defensive compounds until the fifth day, when most of the insects had died and the production of defensive compounds started to decrease (Fig. 3D). In the starved with water and starved with humidity treatments, the defensive compounds also remained high.

Survival

The mortality of insects in the food and starved with water treatment was lower compared with the other treatments, with less than one insect dying per chamber during the 7-day experiment (Fig. 4). The highest mortality occurred in the starved treatment, with 90% of the insects dying after 5 days (Fig. 4). The starved with humidity treatment also showed a high mortality rate, with 40% of the insects dying after 5 days (Fig. 4). In the starved with water treatment, the survival over 7 days was similar to the food treatment (log-rank test = 298.71 d.f. = 3, $P < 0{,}001$; Holm–Sidak multiple comparison procedure, $P = 0.05$) (Fig. 4).

Bioassays with male extracts and synthetic compounds

Significantly more females were found in the arm containing strips with extracts of male volatiles from the food treatment, and on arms treated with all individual synthetic active components and their ternary blend, compared with the control arm (hexane) (Fig. 5), indicating that the extract male volatiles and the three components were behaviourally active.

Bioassays testing males with synthetic sex pheromone components showed that these compounds were not active (Fig. 6).

Discussion

The results of the present study demonstrate the importance of diet on sex pheromone production in *E. heros*, and it is likely that this is the case in other pentatomid species. It is well known that insects convert chemical components from host plants into pheromones, or that plant compounds are necessary for endocrine regulation or even as the pheromone itself (Seybold & Vanderwel, 2003; Reddy & Guerrero, 2004). Beetles in the genera *Oryzaephilus* (Silvanidae) and *Cryptolestes* (Laemophloeidae) have a low production of aggregation pheromone in the absence of food, and it is likely that, for these beetles, rather than serving to provide precursors for pheromone synthesis, the effect of feeding could be involved in hormonal regulation because pheromone production can be stimulated by a juvenile hormone analogue (Seybold & Vanderwel, 2003). In Lepidoptera, the most common type of pheromones, which are polyunsaturated hydrocarbons and the epoxy derivatives with a long

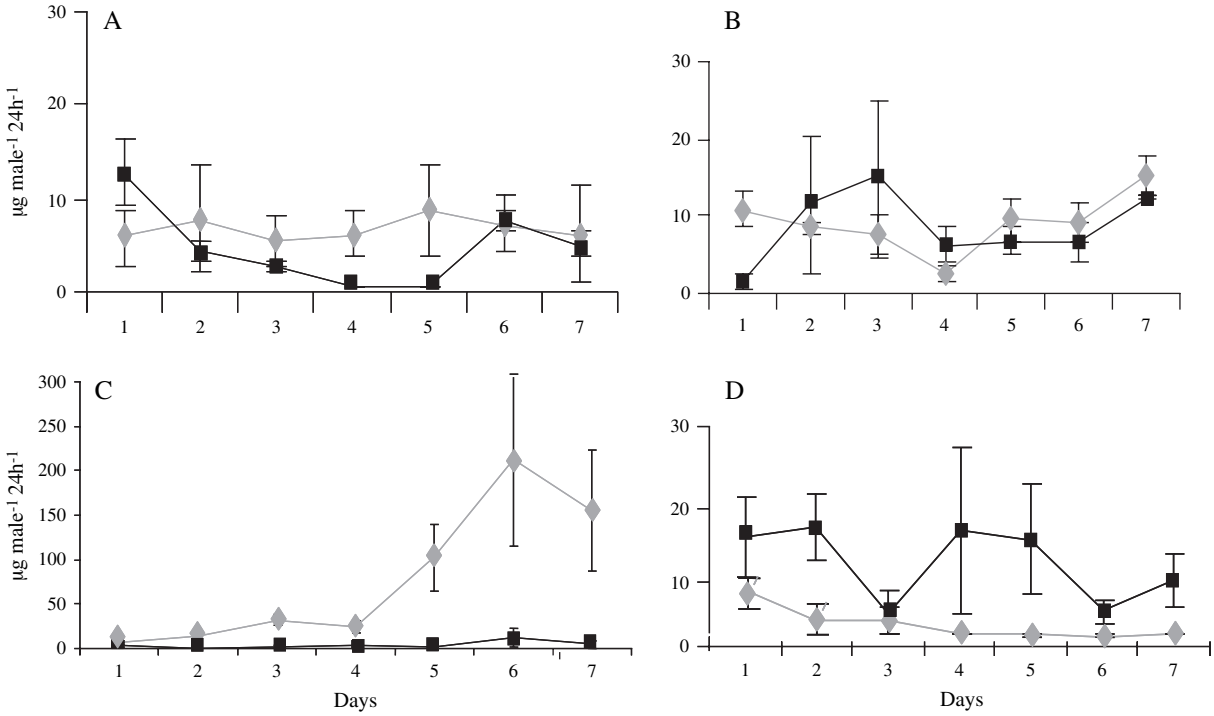

Fig. 3. Quantity in µg male^{-1} 24 h^{-1} of sex pheromone components and defensive compounds released by male *Euschistus heros* during the 7 days of volatile collections for each treatment: (A) Food; (B) starved with water; (C) starved with humidity; (D) starved. □, Defensive compounds; ◆, sex pheromone. Note that (C) is shown with a different scale for the *y*-axis for clarity.

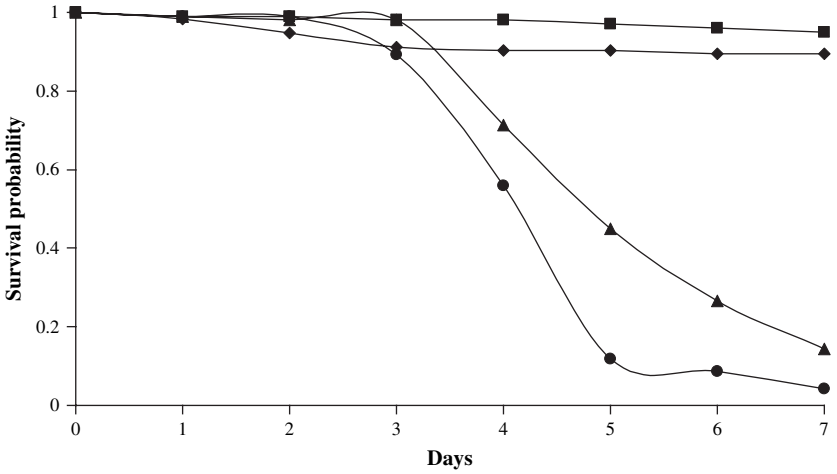

Fig. 4. Probability of surviving during the volatile collection period in different treatments: □, Food; ●, starved; ◆, starved with water; ▲, starved with humidity. Treatments with the same letter are not statistically different (log rank test = 298.71, d.f. = 3, $P < 0.001$ and Holm–Sidak multiple comparison procedure $P = 0.05$).

straight chain (C_{17}–C_{23}), often have linoleic and linolenic acid from plants as precursors. In contrast to other insect families (Schulz, 2005), there is no evidence for the biosynthetic pathways leading to sex-pheromone production in stink bugs. Thus, it is difficult to speculate whether *E. heros* uses plant components in pheromone production directly, or in endocrine regulation. However, the results presented here suggest that diet plays a major role in one of these processes, and that it is essential in maintaining pheromone production.

The first two studies on *E. heros* pheromones report methyl-2,4,6-trimethyltridecanoate as the major sex pheromone component, with methyl-2,4,6-trimethyldodecanoate and methyl-(2*E*,4*Z*)-decadienoate present in lesser quantities (Aldrich *et al.*, 1994; Borges & Aldrich, 1994). Zhang *et al.* (2003) show that methyl-(2*E*,4*Z*)-decadienoate is produced in a similar amount to methyl-2,4,6-trimethyltridecanoate, and that methyl-2,4,6-trimethyldodecanoate is a minor component. These results are confirmed by the present study, which also shows that this ratio is constant over 7 days when food is available. Despite the report by Zhang *et al.* (2003) that *E. heros* males achieve maximum sex pheromone production (2.5 μg male^{-1} day^{-1}) at approximately 35 days of age, in the present study, males produce sex pheromone components from 9 days onwards in a constant ratio and in higher amounts (6.75 μg male^{-1} day^{-1}). These discrepancies may be due to different rearing conditions and the quality and quantity of nutrients provided in the diet during entrainment because, without nutrients obtained from the diet, *E. heros* males do not maintain production of sex pheromone components at a consistent ratio. Furthermore, young male insects (7–8 days old) with food show variability in the ratio of the three components, suggesting that the different ratios obtained in different studies are due to studying immature males. The contrast between the results of the present study and those obtained by Zhang *et al.* (2003) suggests that different rearing conditions can affect the age of sexual maturity of *E. heros*, and this should be taken into account when studying stink bug pheromone blends.

This pattern of release of pheromone components highlights an important methodological point. For example, from the pattern of release of compounds in the treatment without food and the olfactometer in bioassays, it could be concluded, erroneously, that methyl-(2*E*,4*Z*)-decadienoate is the sex pheromone of *E. heros*, and the importance of the other two components might not be discovered. These methodological considerations therefore have important implications in pheromone identification.

The importance of methyl-(2*E*,4*Z*)-decadienoate to *E. heros* species is unknown, but it is the main sex pheromone component released by males of five species of Nearctic stink bugs (*Euschistus conspersus*, *Euschistus tristigmus*, *Euschistus servus*, *Euschistus politus* and *Euschistus ictericus*) and in the Neotropical species *E. heros*, and it is a minor component in *Euschistus obscurus* (Aldrich *et al.*, 1991; Borges & Aldrich, 1994). This compound is reported to capture females, males and nymphs of some Neartic species in field tests (Aldrich *et al.*, 1991) and, more recently, predominantly female *E. servus*, *E. tristigmus* (Leskey & Hogmire, 2005) and *E. conspersus* (Cullen & Zalom, 2006) are captured in fields tests with this compound. In those studies, the authors suggest that this compound has an aggregation function, rather than a sex-pheromone function because both sexes were captured.

In contrast to other species of *Euschistus*, there is little evidence of an aggregation function of methyl-(2*E*,4*Z*)-decadienoate for *E. heros*. The bioassay results of the present study show that females respond to the three components of the male-produced sex pheromone individually and to the mixture with the three components in the ratio of 53 : 3 : 44, contrasting with a previous report that 2,6,10-trimethyldodecanoate is not active to females on its own (Zarbin *et al.*, 2000a).

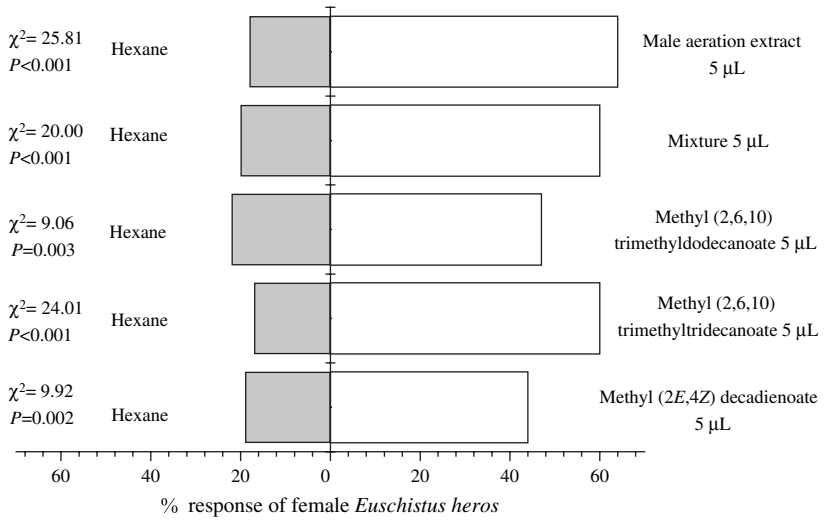

Fig. 5. Percentage response of female *Euschistus heros* (outline) towards individual synthetic pheromone components (5 µl of 1 µg mL^{-1} solution), a mixture of the three components in the ratio: 53% methyl-(2*E*,4*Z*)-decadienoate, 3% methyl-2,6,10-trimethyldodecanoate and 44% methyl-2,6,10-trimethyltridecanoate (fixed at 1 µg mL^{-1}) and male aeration extract in a two-choice olfactometer test in laboratory bioassays. Hexane was used as the control (grey). Statistical analysis were conducted using the chi-square test ($P = 0.05$).

In this study, males do not respond to any one of the compounds or to the mixture, suggesting that methyl-(2*E*,4*Z*)-decadienoate could have a function in sexual communication, although further work is necessary to determine the exact role of this compound.

In conclusion, diet is essential for the production of the complete blend of the components in the sex pheromone blend of *E. heros*, and it is likely that this is the case for other stink bugs, and not providing nutrients during collections could lead to erroneous sex pheromone identification. Further work is needed to elucidate whether nutrition plays a role directly in pheromone biosynthesis or is important in the endocrine regulation of pheromone production. Furthermore, the results of the present study demonstrate that the green beans used for nutrition are not only essential, but do not produce volatiles that interfere with interpretation of results.

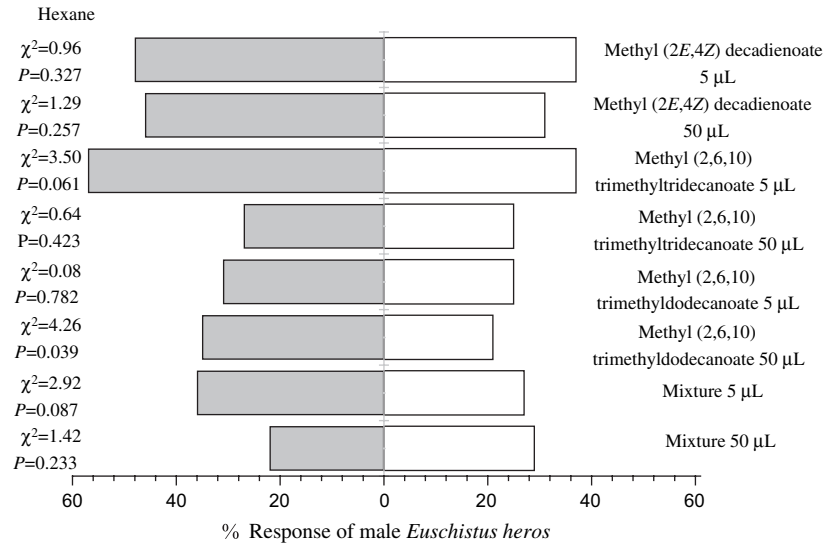

Fig. 6. Percentage of response of male *Euschistus heros* (outline) towards individual synthetic pheromone components (5 µl and 50 µL of 1 µg mL^{-1} solution) and 5 and 50 µL of a mixture of the three components in the ratio: 53% methyl-(2*E*,4*Z*)-decadienoate, 3% methyl-2,6,10 trimethyldodecanoate and 44% methyl-2,6,10 trimethyltridecanoate (fixed at 1 µg mL^{-1}) in a two-choice olfactometer test in laboratory bioassays. Hexane was used as the control (grey). Statistical analysis were conducted using the chi-square test ($P = 0.05$).

Acknowledgements

We thank Hélio Santos and Diva Tiburcio, for helping with the field collection and laboratory rearing of the insects used in this study. This work received financial support from the Brazilian Council for Scientific and Technological Development (CNPq), Distrito Federal Research Foundation (FAPDF) and EMBRAPA. This research was also supported by the International Foundation for Science, Stockholm, Sweden, through a grant to M.C.B.M.

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Accepted 14 September 2007
First published online 14 January 2008

