

**ATTRACTANT COMPOUNDS OF THE SOUTHERN GREEN
STINK BUG, *Nezara viridula* (L.) (HETEROPTERA:
PENTATOMIDAE)**

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ABSTRACT

By comparing responses of female *Nezara viridula* (L.) to live males, male aeration extracts and extracts of body parts of males in olfactometers, it was found that an attractant pheromone is produced from the abdominal sternites. Combined gas chromatographic/mass spectrometric analysis confirmed the presence of *n*-dodecane, *n*-tridecane, and (*Z*)-*trans*-epoxy- α -bisabolene in both types of extract. Bioassays of synthetic enantiomers showed that females respond strongly only to (*Z*)-(1'S)-*trans*-epoxybisabolene. Addition of (*Z*)-(1'R)-*trans*-epoxybisabolene to the active antipode significantly reduced responsiveness. (*Z*)-(1'S)-*trans*-epoxybisabolene attracts females to the odor source, but does not initiate courtship behavior.

KEY WORDS: Insecta, (*Z*)-(1'S)-*trans*-epoxybisabolene, behavior, olfactometer, attraction, volatile, pheromone.

RESUMO

Compostos Atraentes do Percevejo Verde, *Nezara viridula* (L.) (Heteroptera: Pentatomidae)

Comparando respostas das fêmeas de *Nezara viridula* (L.) para machos vivos, extrato de aeração dos machos e extratos de partes do corpo de machos em olfatômetros, foi encontrado que um composto atraente é produzido nos esternitos abdominais. Análises combinadas de cromatografia gasosa/espectômetro de massa confirmou a presença de *n*-dodecane, *n*-tridecano, e (*Z*)-*trans*-epoxy- α -bisabolene em dois tipos de extratos. Bioensaios com enantiômeros sintéticos demonstraram que as fêmeas respondem prontamente somente para o (*Z*)-(1'S)-*trans*-epoxybisabolene. A adição do (*Z*)-(1'R)-*trans*-epoxybisabolene com o composto ativo significativamente reduziu as respostas. (*Z*)-(1'S)-*trans*-epoxybisabolene atrai fêmeas para a fonte de odor, mas não induz comportamento de acasalamento.

PALAVRAS-CHAVE: Insecta, (*Z*)-(1'S)-*trans*-epoxybisabolene, comportamento, olfatômetro, atração, volátil, feromônio.

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INTRODUCTION

Reproductive behavior of the southern green stink bug, *Nezara viridula* (L.) was described by Borges *et al.* (1987) and divided into two phases: mate location and courtship. Long-range behavior includes those components leading females to contact males. Courtship is the sequence of interactions between the sexes that eventually results in copulation. Extracts from males were attractive in the field (Aldrich *et al.* 1987) and laboratory (Borges *et al.* 1987), allowing the sequence of *N. viridula* courtship behavior to begin (Borges *et al.* 1987). Chemical extracts from male *N. viridula* consist of *n*-dodecane, *n*-tridecane, *n*-nonadecane, (*Z*)- α -bisabolene, and (*Z*)-*trans*-epoxybisabolene (Baker *et al.* 1987), plus (*Z*)-*cis*-epoxybisabolene in a U.S. strain of the species (Aldrich *et al.* 1987). There are at least two distinctive strains for *N. viridula* populations based on the ratios of the *trans*- and *cis*-epoxides isomers (Aldrich *et al.* 1987, 1994). Pavis & Malosse (1986) found that sexually mature southern green stink bugs produce a pheromone from unicellular glands in the ventral abdominal epithelium. However, Aldrich *et al.* (1993) showed that the 224 molecular weight (MW) pheromone components reported by Pavis & Malosse (1986) were actually artifacts formed by dimerization of (*E*)-4-oxo-2-hexenal.

Tomioka & Mori (1992) describing a facile synthesis of a racemic mixture of the southern green stink bug epoxides, speculated that this mixture might work as an attractant for this polyphagous, world-wide pest.

The aim of the present study was to verify that sex pheromone is produced from the ventral abdominal region in *N. viridula*, and to determine which chemical components lead the insects to the vicinity of the lure.

MATERIAL AND METHODS

Insects. Eggs of *N. viridula* from Brazil (Londrina, Paraná) were sent to Southampton (UK) and cultured on green beans (*Phaseolus vulgaris* L.), broad beans (*Vicia fabae* L.), raw shelled peanuts (*Arachis hypogaea* L.), and soaked soybean seeds (*Glycine max* (L.) Merrill) (Harris & Todd 1980). To prevent olfactory interactions between the sexes, males were separated from females after the imaginal molt and cuticular hardening and reared in separate culture rooms (26.0 \pm 3.0°C, 45-60% r.h., 14:10h L.D. and a light intensity of ca. 2000 Lux).

Olfactometers. The olfactometer termed "single-choice" was constructed from one large (27.5 x 15.5 x 9.5cm) and one small (13.5 x 7.5 x 5.5cm) clear plastic container, termed respectively the release and treatment chambers. A piece of glass tubing (5.5cm ID x 30cm long) was used to connect the two chambers. A plastic mesh funnel prevented males from moving into the release chamber but permitted females access to males (Fig. 1A). An air current through the treatment chamber was produced and regulated by two single-phase fans controlled by double "variac" voltage regulators. The air current speed at the center of the olfactometer was 17.0cm s⁻¹. The upwind end of the treatment chamber and downwind end of the release chamber were pierced with small holes (10 and 20 respectively) with a heated No. 2 cork borer. A border of polytetrafluoroethane was painted onto the vertical surfaces of the olfactometer to restrict insects to the lower floors of the chamber.

The olfactometer termed "two-choice", used to test the biological activity of the dissected samples, was constructed from one large and two small clear plastic containers (dimensions as quoted above), two pieces of glass tubing (3.1cm ID) each 20cm long, two plastic mesh

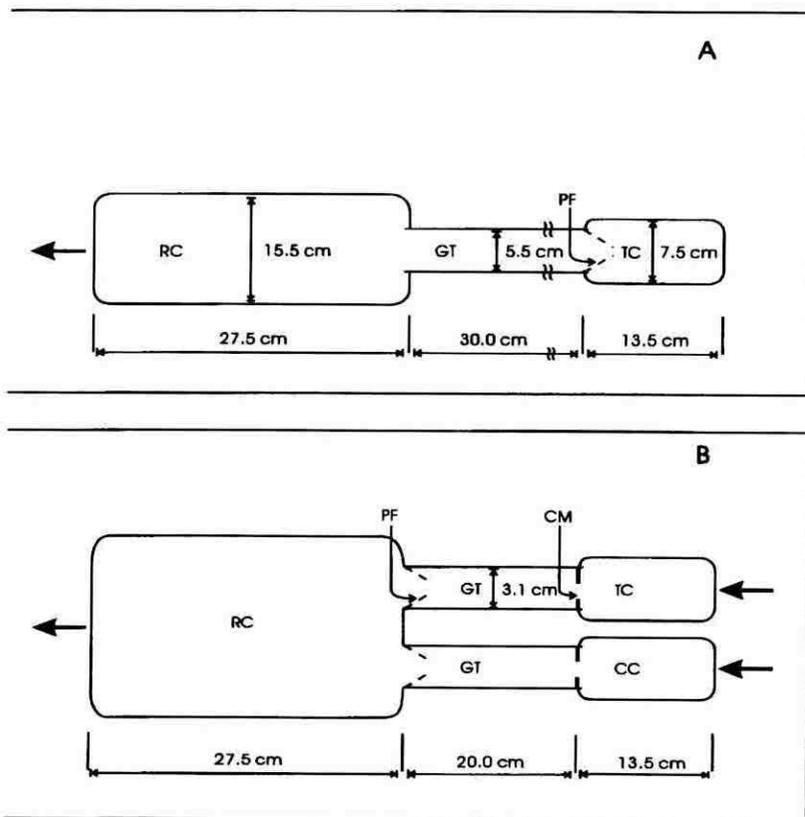


Figure 1. A) Single-choice olfactometer (lateral view). TC = treatment chamber, RC = release chamber, GT = glass tubing, PF = plastic funnel. Air flows from the treatment chamber to the release chamber (arrows). B) Dual-choice olfactometer (top view), key as above.

funnels and two pieces of cotton mesh. The large container was the release chamber and one of the small chambers was used for the treatment and the other for the control (Fig. 1B). The air current through the "two-choice" olfactometer was generated in the same way as in the "single-choice" olfactometer. The plastic mesh funnels (downwind side of the tube) allowed the insects in the assay to pass easily into the tube but made it difficult for them to escape, and the cotton mesh on the other (upwind) side of the tube prevented direct contact with males and/or the odor source.

Aeration. A batch of 20 sexually mature virgin males and females were aerated continuously for a period of four days. A purified airstream was passed over live males kept in a glass chamber and volatile compounds were trapped on activated granular charcoal (20-40 mesh, 6cm³). Subsequent extraction of these compounds by solvent desorption resulted in the crude aeration extract.

The aeration apparatus consisted of an aeration chamber (30.0cm x 6.0cm ID), prefilter chamber (30.0cm x 3.0cm ID), and a small filter chamber (6.0cm x 1.0cm ID) for collection of the crude pheromone aeration extract. Activated granular charcoal (20-40 mesh) filling the prefilter chamber was used for the purification of the airstream and also used in the filter chamber as an adsorbent for trapping the pheromones. The air flow (at $< 2\text{cm s}^{-1}$) for the aeration was generated by a water aspirator.

The volatile compounds trapped on the activated charcoal column were extracted with 10ml of redistilled dichloromethane. Excess solvent was removed in a stream of dry nitrogen, to yield 1 ml of extracted solution which was separated by gas chromatography (GC), (PYE model GCD, 3m x 3mm I.D., 5% OV-101 column programmed from 80°C to 300°C at 16°C min⁻¹, carrier gas = N₂, 25 ml min⁻¹). The identity of pheromone components was verified by GC/mass spectrometry (MS) (Baker *et al.* 1987).

Dissection. Ten sexually mature virgin males were kept overnight in the freezer (10°C) and then dissected into the following parts by separating the abdomen and cutting through the pleura. Ventral abdominal: abdominal sternites, including the 7-8th genital abdominal segments. Dorsal abdominal: anterior part containing the III-IVth dorsal abdominal glands (Aldrich *et al.* 1978), and the posterior part containing the remaining tergites. Viscera: the internal organs, including the metathoracic scent gland.

Each of the samples was placed in a small glass vial with 1 ml of solvent (dichloromethane) and kept overnight at -10°C, after which the extract was filtered through glass wool. Excess solvent was evaporated under nitrogen to 0.25ml for bioassay. The sample that induced behavioral activity in the females was then analyzed by GC.

The biological activity of the extracted sample was monitored and compared with the activity induced by live male insects, and with the activity of the aeration extract from males.

Bioassay of Extracts. There was no observable variation in the level of responsiveness of females to males throughout the light period. The bioassays were therefore run throughout the light period, between 8:30 am and 4:30 pm.

The natural stimulus was provided by ten sexually mature virgin males released into the treatment chamber. Ten sexually mature virgin females were placed in the release chamber. Prior to testing, the insects were allowed to acclimate for about two minutes in the release chamber. The olfactometer sections were then connected and the number of females responding to the odor source was recorded. Unmated males and females were used exclusively in order to maintain a constant level of response and avoid the refractory period which may occur after mating (Brennan *et al.* 1977; McLain 1992). The bioassay was run for ten minutes for each replicate.

The optimum dosage of the aerated crude extract for the bioassay was determined by recording responses of females to a range of doses in the "two choice" olfactometer. The optimum dose was then substituted for males in the olfactometer bioassay and used as the standard for calibration of the dissected sample solution. A strip of filter paper (2.0cm x 0.5cm) was used as a substrate for extracts. Solvent was allowed to evaporate for about 20 seconds prior to the bioassay.

Synthetic Compounds. In preliminary bioassays, no sex pheromone activity was detected in response to mixtures of *n*-dodecane, *n*-tridecane, and *n*-nonadecane. Attention was therefore switched to the sesquiterpenes, but only the mono-oxygenated sesquiterpene (220 MW) could be separated in sufficient quantity for analysis. These chemical analyses led to the conclusion

that the compound was an epoxide of (*Z*)- α -bisabolene and a synthesis of the eight stereoisomers was then proposed (Baker *et al.* 1987). Ultimately, six out of eight stereoisomer were available for biological evaluation.

The Optimum Dose of Pheromone Extract. Maximum responsiveness was obtained using 15 μ l of male aeration extract (Borges *et al.* 1987). This dose gave a significantly higher response than any other doses, i.e., 5, 25, and 50 μ l ($P < 0.002$, Mann-Whitney U-test; Siegel 1956). Therefore, the 15 μ l dose was used as a standard for calibration of the concentrations of the new samples to be tested (15 μ l extract = 1.2 individual equivalent). Females aeration extracts totally lacked the sesquiterpenes compounds.

Bioassay of Synthetic Sesquiterpenes. For each dosage of chemical or mixture of chemicals tested, three controls were run: air current alone, 10 males, and 0.94 μ g of material from aerated males. Twenty adult virgin females of *N. viridula* were exposed to one of the following treatments: air current in olfactometer with no treatment; ten virgin *N. viridula* males; 0.94 μ g of material from extract from male aeration; five doses of (*Z*)-(1'S)-*trans*-epoxybisabolene (6, 12, 18, 24, and 30ng); five doses of (*Z*)-(1'R)-*trans*-epoxybisabolene (6, 12, 18, 24, and 30ng); three treatments of a 12ng mixture containing (*Z*)-(1'S)-*trans*-epoxybisabolene and (*Z*)-(1'R)-*trans*-epoxybisabolene in the following ratios: 25:75, 50:50, and 75:25; 12ng of (*Z*)-(1'R)-*cis*-epoxybisabolene; 12ng of (*E*)-(1'R)-*trans*-epoxybisabolene; 12ng of (*E*)-(1'S)-*trans*-epoxybisabolene; 12ng of (*E*)-(1'R)-*cis*-epoxybisabolene.

Virgin *N. viridula* females submitted to the above treatments were placed in the release chamber and used only once, and the ten virgin males as treatment were used only once. chemicals and mixtures of chemicals were dissolved in dichloromethane and applied to a strip of filter paper and, after solvent evaporation, introduced into the treatment chamber. Three replicates of the experiment were carried out for each treatment.

Responses to the stimuli were recorded during a ten-minute period. A count was made of the number of females moving from the release chamber to the last section of the olfactometer, closest to the treatment chamber. The results were submitted to analysis of variance (one way analysis) and then Duncan's multiple range test (D.M.R.T.) was applied (Steel & Torrie, 1960). The rejection level for the null hypothesis was set at 0.01.

Analysis of Courtship Behavior. Sequences of courtship behavior, i.e., antennal elevation (AE), orientation towards the odor source (O), walking toward the odor source (W), wing fanning (F), and arrive in treatment chamber (TC), were evaluated in the single-choice olfactometer with 12ng of (*Z*)-(1'S)-*trans*-epoxybisabolene and 12ng of (*Z*)-(1'R)-*trans*-epoxybisabolene offered separately to single mature virgin females. The results were transformed to percentages and compared with results using live males and male aeration extract.

RESULTS

Dissected Body Parts. Preliminary tests with the five dissected samples were carried out in the two-choice olfactometer. The results indicated that only the whole abdominal sternites sample was attractive to virgin females (60% response), compared with 6 and 3% of abdominal intersegmental membrane and dorsal abdominal glands respectively. No responses were obtained with the remaining samples, i.e., the remaining tergites and viscera. Comparison with previous mass spectral data of the aeration sample showed that three chemical

compounds were abundantly present, i.e., *n*-dodecane, *n*-tridecane, and (*Z*)-*trans*-epoxy- α -bisabolene.

Responses of Females to Aerated and Dissected Samples. Both aeration and whole abdominal sternite samples elicited responses from the females similar to those of the standard concentration (15 μ l aeration extract). There were no significant differences from the responses elicited by live males (Table 1) ($P < 0.05$, χ^2 analysis). Extracts of females failed to induce responses in males or females (M. Borges, unpublished data).

Table 1. Number of females ($m=20$) attracted in the single-choice olfactometer to live males, aeration extract, and whole abdominal sternite extracts.

Samples	Females (Mean \pm SD)
Live males	6.3 \pm 1.1
Aeration extract	5.8 \pm 1.1
Whole abdominal sternite	5.2 \pm 0.7
χ^2 ($\alpha = 0.05$)	NS

Bioassays of Synthetic Compounds. Figure 2 shows the data for the set of bioassays using 12ng of (*Z*)-(1'S)-*trans*-epoxybisabolene and 12ng of (*Z*)-(1'R)-*trans*-epoxybisabolene. The mean number of females responding to (*Z*)-(1'S)-*trans*-epoxybisabolene was not significantly

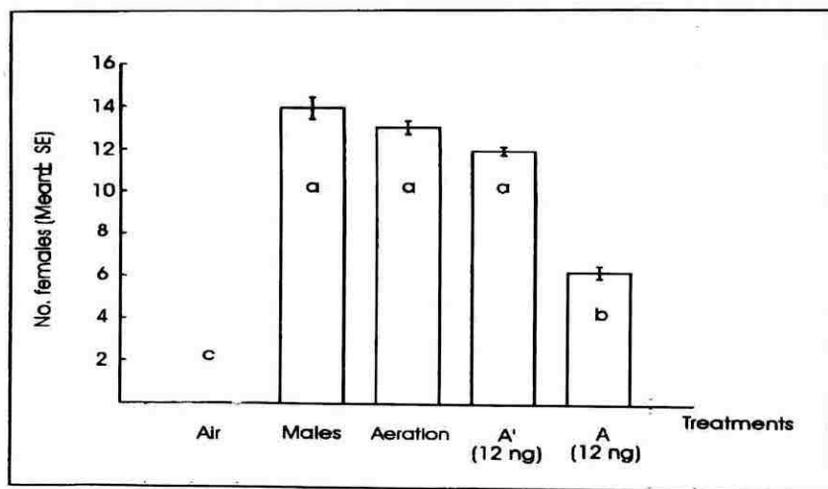


Figure 2. Number of females (mean \pm SE) that had moved from the release chamber to the last section of a single-choice olfactometer after 10 minutes. A = (*Z*)-(1'R)-*trans*-epoxybisabolene, A' = (*Z*)-(1'S)-*trans*-epoxybisabolene. Means followed by the same letters do not differ significantly ($\alpha = 0.01$, D.M.R.T.).

different from the number responding to males or natural male aeration extract. When the dosages of this epoxide were increased to 30ng, levels of response were similar to those of male aeration extract, but did not induce levels of response as high as those induced by live males.

Increasing the dose of (*Z*)-(1*R*)-*trans*-epoxybisabolene considerably reduced the number of females responding, while the other diastereomer, (*Z*)-(1*S*)-*trans*-epoxybisabolene, was only slightly less active with increasing concentration (Fig. 3). However, (*Z*)-(1*S*)-*trans*-epoxybisabolene induced similar levels of response in females to those induced by males and

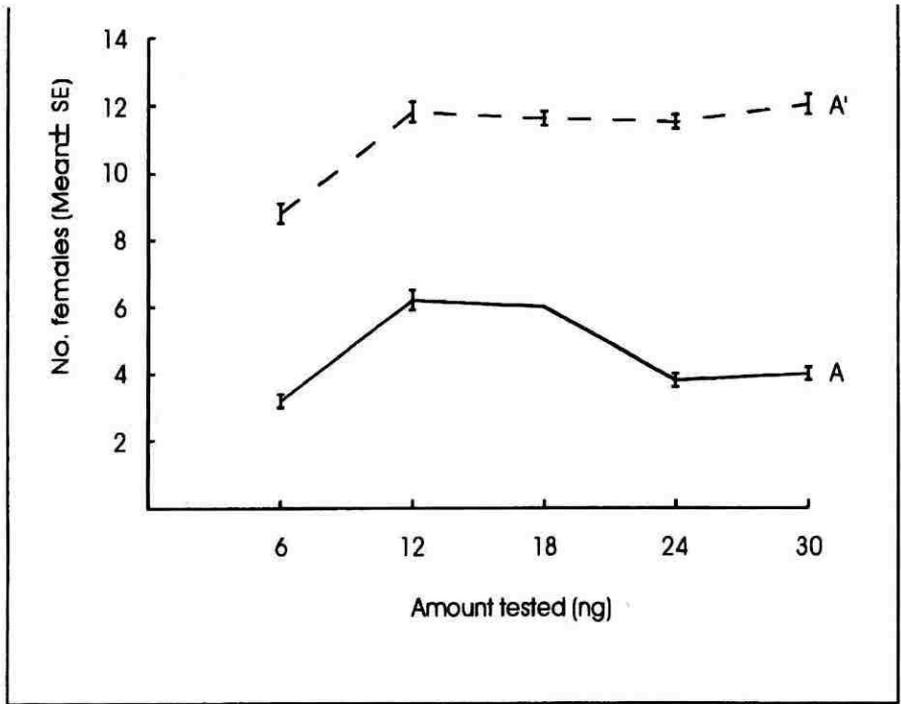


Figure 3. Variation with concentration of female responses to compound (*Z*)-(1*R*)-*trans*-epoxybisabolene (A) and (*Z*)-(1*S*)-*trans*-epoxybisabolene (A'). Means \pm SE of n° of females that had moved from the release chamber to the last section of the olfactometer after 10 min.

male aeration extract. Mixtures of (*Z*)-(1*S*)-*trans*-epoxybisabolene and (*Z*)-(1*R*)-*trans*-epoxybisabolene in various ratios attracted significantly fewer females than did (*Z*)-(1*S*)-*trans*-epoxybisabolene alone or live males (Fig. 4A). Responses to the five treatments of the

other enantiomers (*Z*)-(1'R)-*cis*-epoxybisabolene; (*E*)-(1'R)-*trans*-epoxybisabolene; (*E*)-(1'S)-*trans*-epoxybisabolene, and (*E*)-(1'R)-*cis*-epoxybisabolene were insignificant (Fig. 4B).

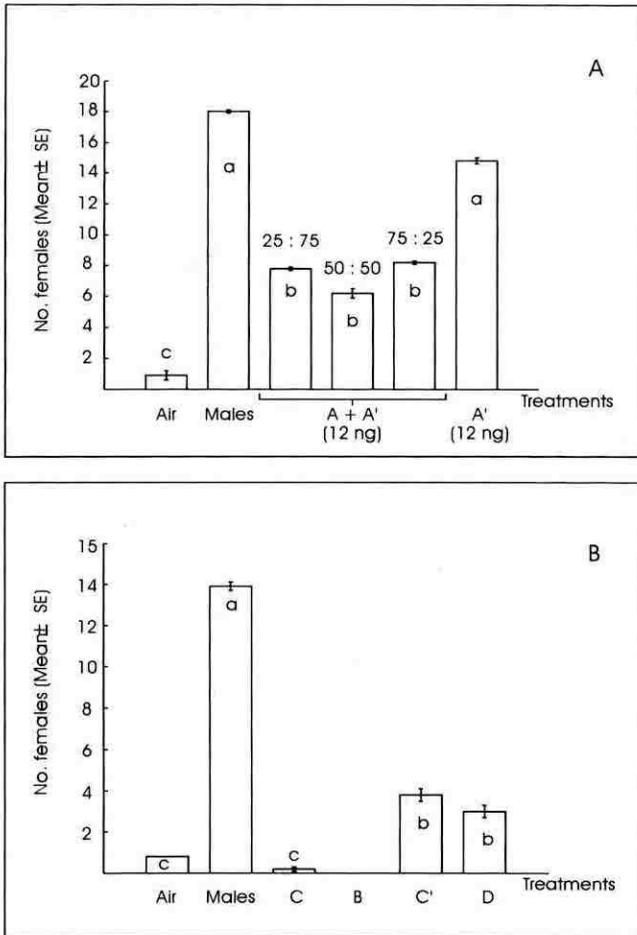


Figure 4 (A and B). Number of females (mean \pm SE) that had moved from the release chamber to the last section of a single-choice olfactometer after 10 minutes. A = (*Z*)-(1'R)-*trans*-epoxybisabolene; A' = (*Z*)-(1'S)-*trans*-epoxybisabolene; B = (*Z*)-(1'R)-*cis*-epoxybisabolene; C = (*E*)-(1'R)-*trans*-epoxybisabolene; C' = (*E*)-(1'S)-*trans*-epoxybisabolene, and D = (*E*)-(1'R)-*cis*-epoxybisabolene. Means followed by the same letters do not differ significantly ($\alpha = 0.01$, D.M.R.T.).

Analysis of the courtship behavior (Table 2) showed that significantly fewer females approached (*Z*)-(1'R)-*trans*-epoxybisabolene than the antipode, and responses to (*Z*)-(1'S)-*trans*-epoxybisabolene were similar to those of live males and male extracts.

DISCUSSION

This work corroborates the finding of Pavis and Malosse (1986) that the attractant pheromone of *N. viridula* originates from the abdominal sternum. The (*Z*)-(1'S)-*trans*-epoxybisabolene was abundantly present in aeration extracts of live males and in extracts of the abdomen of males (Baker *et al.* 1987). The two extracts are similarly attractive to females in olfactometers. However, we found that (*Z*)-(1'S)-*trans*-epoxybisabolene (220 MW) is attractive to female *N. viridula*. To the contrary, Pavis & Malosse (1986) claimed that the 220 MW components were perhaps involved in courtship, but not in long-range attraction. Moreover, they reported that a pair of 224 MW compounds were attractive, and assumed that these components came from the abdominal sternum. Aldrich *et al.* (1993) showed that these 224 MW compounds are dimerization products of the metathoracic stink gland constituent, (*E*)-4-oxo-2-hexenal. Our results confirm and extend those of Baker *et al.* (1987) and Aldrich *et al.* (1987 and 1993) showing that 220 MW compounds are part of the attractant pheromone for *N. viridula*.

Table 2. Components of courtship behavior (%) exhibited by single females *Nezara viridula* in a single-choice olfactometer. (AE) = Antenal Elevation; (O) = Orientation to Source; (W) = Walking Toward the Odor Source; (F) = Wing Fanning; (TC) = arrive in Treatment Chamber. A' = (*Z*)-(1'S)-*trans*-epoxybisabolene; A = (*Z*)-(1'R)-*trans*-epoxybisabolene (number of females in parentheses).

Stimulus	% Response				
	AE	O	W	F	TC
Live males (33)	91	79	73	9	70
Male extract (27)	100	93	93	4	93
12ng A' (20)	100	95	90	35	75
12ng A (20)	85	70	65	15	25

The females *N. viridula* selectively respond to the single enantiomer, (*Z*)-(1'S)-*trans*-epoxybisabolene. (*Z*)-(1'R)-*trans*-epoxybisabolene inhibited the response to (*Z*)-(1'S)-*trans*-epoxybisabolene, suggesting that the racemic mixture will not attract *N. viridula*. The response of females to (*Z*)-(1'R)-*cis*-epoxybisabolene was negligible, while (*Z*)-(1'S)-*cis*-epoxybisabolene was not available for testing.

The (*Z*)-(1'S)-*trans*-epoxybisabolene attracts females to the odor source, but it does not initiate behavior. It is possible that the (*Z*)-*cis*-epoxybisabolene produced by *N. viridula* males (Aldrich *et al.* 1993) is the (1'S)-enantiomer that was unavailable for testing. Possibly the *cis*-enantiomer plays a role in short-range attraction and courtship. This may explain the absence of courtship behavior when females were attracted into the treatment chamber by (*Z*)-(1'S)-*trans*-epoxybisabolene (Borges *et al.* 1987). In fact, males of the Londrina strain of *N. viridula* do produce a small amount of the *cis*-epoxide isomer (Aldrich *et al.* 1993). For the purpose of using a synthetic pheromone in a control program for *N. viridula*, the failure to stimulate

copulation is unlikely to be significant. Further is needed to determine the complete pheromone blend.

A long-range attractant compound has been identified from the III-IVth dorsal abdominal glands (DAG) of another pentatomid, the spined soldier bug, *Podisus maculiventris* (Say) (Aldrich *et al.* 1984). The present study of *N. viridula*, in which the pheromone glands have been confirmed to be on the ventral abdomen, indicates that long-range pheromone-producing glands evolved independently in these two species of pentatomid bugs. The pheromone system of *N. viridula* may be typical of phytophagous pentatomids, most of which probably release attractant pheromones, yet lack sexually dimorphic DAGs (e.g. Carayon 1981, Aldrich *et al.* 1991, Borges & Aldrich 1994). Nevertheless, at least one exception exists for this generalization: the attractant pheromone for the phytophagous spined citrus bug, *Biprorulus bibax* (Breddin), is produced in the enlarged DAGs of males (James *et al.* 1994).

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