

# Artifacts and Pheromone Blends from *Nezara* spp. and Other Stink Bugs (Heteroptera: Pentatomidae)

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Isomeric compounds reportedly released by mature males of *Nezara viridula* as attractants for conspecific females are evidently artifacts formed by dimerization of (*E*)-4-oxo-2-hexenal. Additional analyses of the male-specific volatiles from Italian, Australian, Brazilian, and Japanese populations of *N. viridula* verify that at least two distinctive pheromone strains exist, but an active synthetic pheromone has not yet been developed. Analyses of volatiles from *N. antennata* and *Acrosternum aseasonum* males are also reported showing that the native Japanese *Nezara* sp., and species in the sister genus *Acrosternum*, produce species-specific blends based on the same compounds as *N. viridula*: (*Z*)- $\alpha$ -bisabolene (1-methyl-4-(1,5-dimethyl-(*Z*)-1,4-hexadienyl)-cyclohexene), and *trans*- and *cis*-1,2-epoxides of (*Z*)- $\alpha$ -bisabolene. The *trans*-/ *cis*-1,2-epoxide ratio of *N. antennata* is within the range found for most *N. viridula* populations, but the blend from Japanese *N. viridula* males deviates radically from those of other conspecific populations.

## Introduction

*Nezara viridula* (Heteroptera: Pentatomidae) is a native of the Ethiopian Region [1, 2], but is now a serious pest in most subtropical areas of the world. This insect, known by various common names, is called the southern green stink bug (SGSB) in the United States. As the common name suggests, stink bugs have highly developed scent glands that produce irritating secretions to defend themselves against predators [3]. The imma-

tures (nymphs) possess two large dorsal abdominal glands, whereas the adults emit their allomones from metathoracic scent glands. In addition to their repugnant defensive secretion, males release a pheromone that attracts females and males, as well as conspecific nymphs [4]. Although the glandular source of the pheromone has not been positively identified, aeration extracts of laboratory-reared SGSB males (U.S. strain) were active in the field, and attraction of the tachinid parasite, *Trichopoda pennipes* (Diptera), indicated that this fly uses the pheromone as a host-finding kairomone [5]. The major male-specific volatiles of *N. viridula* [5–7], and species in the sister genus *Acrosternum* [7], include (*Z*)- $\alpha$ -bisabolene (MW = 204) (1-methyl-

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4-(1,5-dimethyl-(*Z*)-1,4-hexadienyl)-cyclohexene and 1,2-epoxides of (*Z*)- $\alpha$ -bisabolene (MW = 220). The abundance of the *cis*-epoxide isomer released by *N. viridula* males varies, depending on the geographic origin of the bugs, from being undetectable [6] to being present at an equivalent concentration to that for the *trans*-epoxide isomer [7]. However, French researchers reported that the 220 MW volatiles from SGSB males were not attractive to females in a laboratory bioassay, whereas a pair of unidentified 224 MW isomers from males did attract females [8, 9]. Here we report on the identity of these 224 MW compounds, and on additional pheromone analyses for *N. viridula* from Brazil, Italy, Australia, and Japan. We also report the results of pheromone analyses for the native Japanese species, *N. antennata*, and the South American species, *A. aseasonum*.

## Materials and Methods

*Nezara viridula* used for this study were from colonies started from insects collected near Stoneville, Mississippi (JRA); Brasilia and Londrina, Brazil (MB); Perugia, Italy (FB); Nambour, Queensland, Australia (GKW); and Kamitondacho, Wakayama, Japan (HN). *Acrosternum aseasonum* and *Podisus connexivus* were collected near Brasilia (MB), and *N. antennata* was collected in Kyoto City, Japan (HN) [10]. *Nezara*, *Acrosternum*, and *Podisus* colonies were maintained after previously described methods [5, 10, 11] in the laboratories of the countries of origin. *Euschistus servus* adult males were collected in pheromone-baited traps near the Beltsville Agricultural Research Center [12].

Groups of 4–40 sexually mature males of *Nezara* spp., *A. aseasonum*, and *E. servus* were aerated in the respective laboratories as previously described [5, 7]. Heptane or hexane were used instead of  $\text{CH}_2\text{Cl}_2$  for extraction of most *Nezara* and *Acrosternum* entrained samples because bisabolene derivatives are unstable in acidic solvents, and the relatively low volatility of heptane reduced the risk of evaporation during shipment.

Pentatomid nymphs possess two large dorsal abdominal glands [3] whose contents are shed with the exuviae at ecdysis [11, 13]. The exuviae ( $\leq 24$  h after ecdysis) from *ca.* 50 *P. connexivus* nymphs (first-to-fifth-instar) were extracted in 1 ml of

$\text{CH}_2\text{Cl}_2$ , and the secretion from the posterior dorsal abdominal gland in the exuviae from one fifth-instar *N. viridula* nymph was collected in a micropipette and extracted in 100  $\mu\text{l}$  of  $\text{CH}_2\text{Cl}_2$  for the analyses reported here.

Samples prepared in the U.S., Japan, Italy, and Brazil were analyzed by gas chromatography (GC) in the U.S. on a bonded methyl silicone column (0.25  $\mu\text{m}$  film, 14 m  $\times$  0.25 mm ID; DB-1<sup>TM</sup>, J&W Scientific, Folsom, CA) in a Varian 3700 GC with helium as carrier (40 cm/sec), a temperature program from 45 °C for 2 min to 230 °C at 15°/min, a flame ionization detector (FID), and a Shimadzu C-R3A recorder. Some Brazilian samples were analyzed by GC in the Brasilia laboratory under conditions identical to those for U.S. analyses except that a 30 m DB-1 column was used. The Australian samples were analyzed on a DB-5<sup>TM</sup> column (0.25  $\mu\text{m}$  film; 30 m  $\times$  0.25 mm ID; J&W Scientific) in a Hewlett-Packard 5890 GC/3392A Integrator with helium as carrier, programmed from 45 °C for 2 min to 260 °C at 15°/min, and a FID.

Electron impact mass spectra (EI-MS) were obtained in the U.S. laboratory for samples prepared in the U.S., Japan, Italy, and Brazil. Most extracts were analyzed using a Finnigan 4510 GC-MS equipped with an INCOS Data System, at 70 eV, and a 30 m DB-1 column, programmed from 60 °C for 2 min to 250 °C at 5°/min. Samples of *N. antennata*, and *N. viridula* from Londrina, Brazil, were analyzed using a Hewlett Packard 5971 GC-MS instrument at 70 eV, with a HP-5<sup>TM</sup> column (0.11  $\mu\text{m}$  film; 25 m  $\times$  0.2 mm ID), programmed from 50 °C for 2 min to 250 °C at 15°/min. Aeration extracts of Australian *N. viridula* were analyzed at 70 eV using a Finnigan 1020 GC-MS with a 30 m DB-5 column (0.11  $\mu\text{m}$   $\times$  0.2 mm), programmed from 40 °C for 2 min to 260 °C at 10°/min.

Compounds identified by mass spectral data were cross-checked by GC and MS comparisons to authentic standards. Linalool, *n*-tridecane, and *n*-nonadecane were obtained from Aldrich Chemical Co. (Milwaukee, WI); (*E*)-2-decenal was purchased from Bedoukian Research Inc. (Danbury, CN). (*Z*)- $\alpha$ -Bisabolene was synthesized according to a published procedure [14], and the corresponding *cis*- and *trans*-1,2-epoxides were synthesized as part of an earlier investigation [7, 15].

(*E*)-4-Oxo-2-hexenal was synthesized according to Ward and Van Dorp [16]. To produce dimers of (*E*)-4-oxo-2-hexenal *ca.* 20  $\mu$ l of the neat material was flame-sealed in a capillary tube, placed in an oven at 95 °C/22 h, and the heat-treated material was dissolved in CH<sub>2</sub>Cl<sub>2</sub> for GC and GC-MS analyses.

## Results

After Pavis and Malosse [8] reported that a pair of 224 MW isomers from *N. viridula* males were attractive to conspecific females, we (JRA & WRL) manually entered artificial EI-MS matching the reported spectra for these compounds [9] into the computerized mass spectral library of our Finnigan 4510 GC-MS. A computer search of the EI-MS recorded for a pooled aeration sample of *ca.* 250 SGSB males failed to produce a match for either EI-MS reported by Pavis [5], but the artificially created spectra were never deleted from the library.

Later, in the course of other semiochemical investigations [10–13], best-fit matches for the artificial spectra of these 224 MW isomers were re-

trieved from the library for compounds eluting at retention time (RT) = 9.5 min and RT = 9.6 min in extracts of exuviae from *N. viridula* and *P. connexivus* nymphs (Fig. 1 A and B; compounds **4** and **5**), and in an aeration extract of adult *E. servus* males (not shown). The exuvial extracts contained substantial amounts of (*E*)-4-oxo-2-hexenal (Fig. 1; compound **1**, MW = 112) [11, 13], and the *E. servus* aeration extract was contaminated with metathoracic scent gland secretion also containing (*E*)-4-oxo-2-hexenal [unpublished data]. Therefore, it was suspected that the presence of 224 MW isomers might be due to dimerization of (*E*)-4-oxo-2-hexenal. Analysis of heat-treated synthetic (*E*)-4-oxo-2-hexenal showed that the relative abundance of **1** was greatly reduced by heating, with the concomitant appearance of later eluting compounds, including components **4** and **5** (Fig. 1 C). The EI-MS of components **4** and **5** derived from (*E*)-4-oxo-2-hexenal are virtually identical to those for the compounds of matching RT in the nymphal *N. viridula* extract (Fig. 2), and the corresponding components in the *P. connexivus* and *E. servus* extracts (not shown). The mass spectra reported here (Fig. 2) for the (*E*)-4-oxo-2-

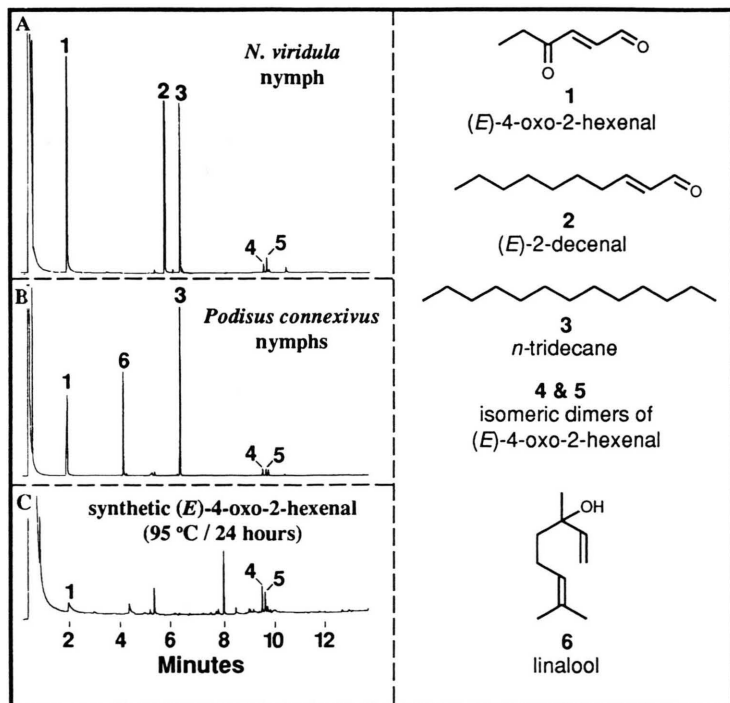


Fig. 1. Gas chromatograms of (A) *Nezara viridula* posterior dorsal abdominal secretion extract from the exuviae of a single fifth-instar nymph, (B) *Podisus connexivus* exuvial extract from *ca.* 50 first- to fifth-instar nymphs and, (C) an extract of heat-treated (*E*)-4-oxo-2-hexenal. (1 = (*E*)-4-oxo-2-hexenal, 2 = (*E*)-2-decenal, 3 = *n*-tridecane, 4 and 5 = isomeric dimers of (*E*)-4-oxo-2-hexenal, and 6 = linalool).

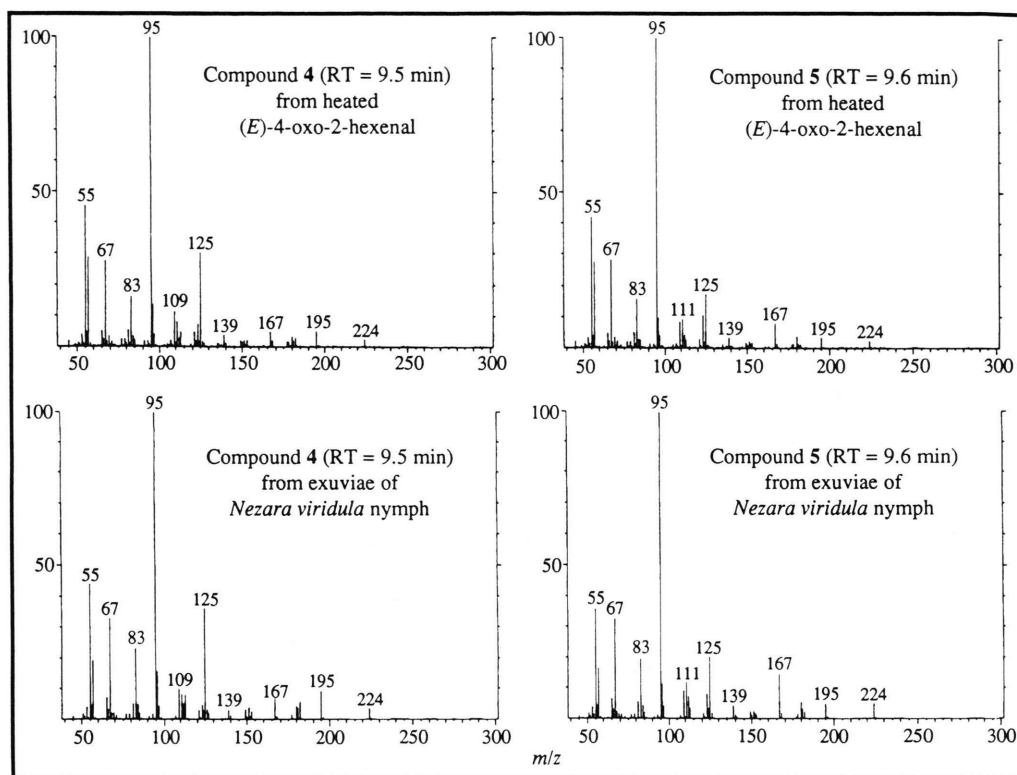


Fig. 2. Electron impact mass spectra of isomeric dimers (Fig. 1, compounds 4 and 5) from heat-treated (*E*)-4-oxo-2-hexenal (top), and *Nezara viridula* posterior dorsal abdominal gland secretion (bottom).

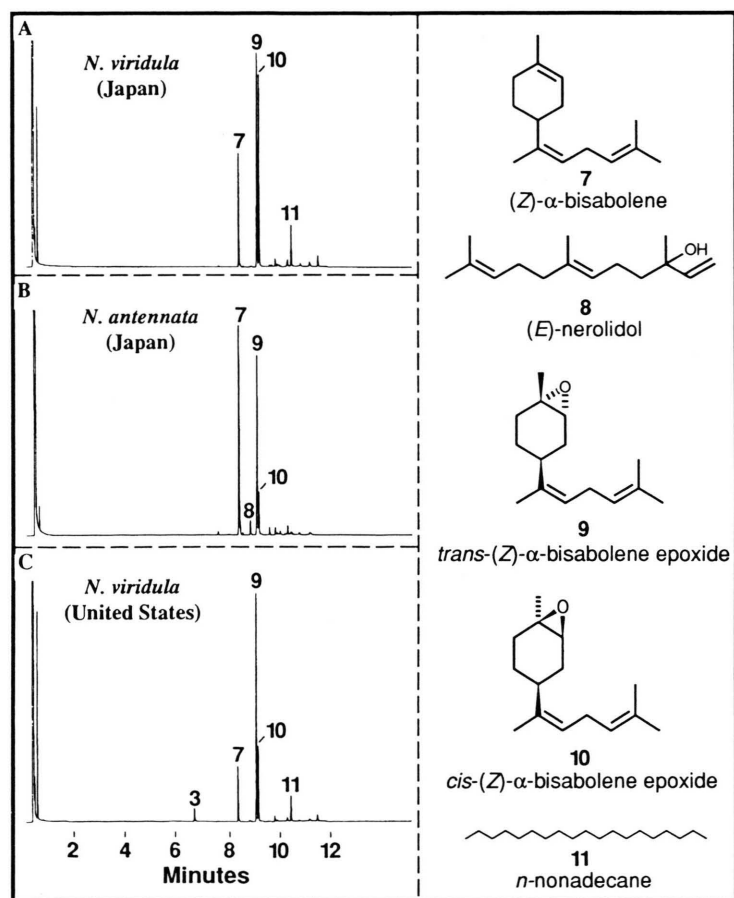
hexenal dimers have less intense low molecular weight ions than the mass spectra reported by Pavis [9], but this discrepancy is due to a feature of the Finnigan INCOS software whereby the recorded intensities of ions are a function of the square root of ion mass times intensity.

Identified compounds that are produced exclusively by adult males of *Nezara* and *Acrosternum* spp. are listed in Table I. An earlier investigation verified that the blend of male-specific volatiles from a U.S. strain of *N. viridula* is an attractant pheromone [5]. Pheromone blends for 5 additional geographically isolated populations of *N. viridula*, and for *N. antennata* and *A. aseadum*, are reported here. Samples used to calculate the percentages of compounds reported in Table I contained up to 24% contamination from metathoracic scent gland secretion so that sums of the male-specific components for the species range from 95.2% (Brasilia *N. viridula*) to 75.6% (*A. aseadum*). However, the proportions of the sesquiterpenoid components

within each population are relatively uniform. For *N. viridula* populations from Italy, Australia and Brazil, ratios of *trans*-/*cis*-(*Z*)- $\alpha$ -bisabolene-1,2-epoxides ranged from 2.16–4.67. *Nezara viridula* males from Wakayama, Japan, produced a significantly different blend, containing equivalent amounts of the epoxide isomers (*trans*-/*cis*-epoxide ratio = 0.82). The concentration of *n*-nonadecane in samples from *N. viridula* populations (2.2–20.6%) was much more variable than were concentrations of sesquiterpenoids. The volatiles from *N. antennata* males resembled the blends from the non-Japanese populations of *N. viridula* (Fig. 3), with a *trans*-/*cis*-epoxide ratio = 4.08; however, the concentrations of (*Z*)- $\alpha$ -bisabolene and (*E*)-nerolidol from *N. antennata* males were higher than for *N. viridula* blends, and *n*-nonadecane was barely detectable in *N. antennata* extracts. Finally, the single aeration sample for *A. aseadum* males contained the same sesquiterpenoids as for *Nezara* spp., but in distinctly different proportions: (*Z*)-

Table I. Pheromone blends (%  $\pm$  SEM) of five *Nezara viridula* populations, *Nezara antennata*, and *Acrosternum aseedum*.

Species (Source)	<i>n</i>	Total Males	( <i>Z</i> )- $\alpha$ -Bisabolene	( <i>E</i> )-Nerolidol	<i>trans</i> -Epoxide	<i>cis</i> -Epoxide	<i>n</i> -Nonadecane	<i>trans/cis</i> Ratio
<i>N. viridula</i> (Perugia, Italy)	6	162	29.3 $\pm$ 2.3	—	33.1 $\pm$ 2.0	15.3 $\pm$ 1.2	13.7 $\pm$ 2.1	2.16
<i>N. viridula</i> (Nambour, Australia)	2	30	22.7 $\pm$ 1.0	0.4 $\pm$ 0.1	42.1 $\pm$ 0.4	10.8 $\pm$ 0.8	2.2 $\pm$ 0.5	3.90
<i>N. viridula</i> (Brasilia, Brazil)	5	66	21.8 $\pm$ 3.2	—	45.3 $\pm$ 3.3	19.9 $\pm$ 2.6	8.2 $\pm$ 1.4	2.28
<i>N. viridula</i> (Londrina, Brazil)	2	4	28.2 $\pm$ 0.8	—	31.3 $\pm$ 1.3	6.7 $\pm$ 2.1	20.6 $\pm$ 2.1	4.67
<i>N. viridula</i> (Wakayama, Japan)	7	183	23.6 $\pm$ 4.3	1.7 $\pm$ 0.4	23.0 $\pm$ 3.0	28.0 $\pm$ 2.5	5.0 $\pm$ 0.8	0.82
<i>N. antennata</i> (Kyoto, Japan)	10	250	46.7 $\pm$ 2.0	3.5 $\pm$ 0.4	31.0 $\pm$ 1.0	7.6 $\pm$ 0.2	—	4.08
<i>A. aseedum</i> (Brasilia, Brazil)	1	38	7.0	—	6.4	62.2	—	0.10

Fig. 3. Gas chromatograms of male-specific volatiles entrained from (A) *Nezara viridula* from Kamitondo-cho, Wakayama, Japan, (B) *Nezara antennata* from Kyoto, Wakayama, Japan, and (C) *Nezara viridula* from Stoneville, Mississippi, United States. (30 males/sample; 7 = (*Z*)- $\alpha$ -bisabolene, 8 = (*E*)-nerolidol, 9 = *trans*-(*Z*)- $\alpha$ -bisabolene epoxide, 10 = *cis*-(*Z*)- $\alpha$ -bisabolene epoxide, and 11 = *n*-nonadecane).

$\alpha$ -bisabolene occurred at a lower concentration and the ratio of bisabolene epoxide isomers was greatly skewed in favor of the *cis*-isomer.

## Discussion

Investigations by two of us (JRA & WRL) indicate the male-specific 224 MW isomers reported by French investigators to be attractive to *N. viridula* females [8, 9] are formed by dimerization of (*E*)-4-oxo-2-hexenal. Although the exact structure of these dimers has not been determined, the mass spectra and GC retention times of the two main 224 MW compounds from heat-treated (*E*)-4-oxo-2-hexenal are virtually identical to compounds detected in extracts of two other pentatomids (*P. connexivus* and *E. servus*), and in an extract from the exuviae of a fifth-instar *N. viridula* nymph, all of which contained (*E*)-4-oxo-2-hexenal as a major component. Electron-impact mass spectra created to match those reported by Pavis [9], and entered into the computer library of our Finnigan GC-MS, were retrieved as the best-fit matches to the mass spectra for the (*E*)-4-oxo-2-hexenal dimers (Fig. 2, compounds **4** and **5**; 94.8% and 93.6%, respectively, INCOS software processing).

Isolation of male-specific pheromone blends without contamination from the metathoracic scent gland secretion is difficult, even by the aeration method, because if just one bug emits its allomone during loading into the apparatus or dies during the sampling period, the sample will be overwhelmingly contaminated. The *N. viridula* extracts analyzed by the French researchers were prepared either by rinsing the walls of glass bottles that had contained bugs for 24 h or by rinsing the bugs themselves with hexane [9]. The cuticular rinses were most active in olfactometer tests [9], but were highly contaminated by metathoracic scent gland secretion containing (*E*)-4-oxo-2-hexenal [9, 17].

Hexenal in metathoracic scent gland secretions of coreid bugs (Heteroptera: Coreidae) gradually formed aldol condensation and trimerization products after extraction [18, 19]. Similarly, we suggest that the (*E*)-4-oxo-2-hexenal dimers are artifacts produced spontaneously from the primary secretory components. The greater abundance of the 224 MW isomers in extracts from sexually mature *N. viridula* males versus immature males may

be due to higher (*E*)-4-oxo-2-hexenal concentrations in the scent glands of mature males [8, 9]. It seems unlikely that dimers of (*E*)-4-oxo-2-hexenal are part of the attractant pheromone of *N. viridula* since these compounds occur in conspecific nymphs, as well as other pentatomid nymphs and adults. Moreover, (*E*)-4-oxo-2-hexenal dimers were not detected in uncontaminated aeration extracts of *N. viridula* males that were attractive to the bugs and their tachinid parasite in the field [5].

The additional analyses of pheromone blends for *N. viridula* reported here substantiate earlier reports [5, 7] that geographically isolated pheromone strains of the insect exist. The determination that SGSB males from the Wakayama location express an epoxide ratio of 0.82 is consistent with previous determinations for a population from the southerly island Kyushu (*trans*-/*cis*-epoxide = 0.97 [7]), confirming that a Japanese strain exists whose males produce equivalent amounts of the bisabolene epoxide isomers. Contrary to an earlier report [6], the *N. viridula* males we sampled from Londrina, Brazil, produced *cis*-(*Z*)- $\alpha$ -bisabolene-1,2-epoxide (6.7%), as well as the *trans*-epoxide isomer (31.3%, *trans*-/*cis*-epoxide = 4.67). Males from a colony originating near Brasilia (ca. 1200 km north of Londrina) produced relatively much more of the *cis*-epoxide isomer (19.9%, *trans*-/*cis*-epoxide = 2.28), suggesting that even within Brazil there may be different *N. viridula* strains. The *trans*-/*cis*-epoxide ratio of 3.90 for Australian *N. viridula* males is within the range of ratios determined for those populations exhibiting a clear excess of *trans*-(*Z*)- $\alpha$ -bisabolene-1,2-epoxide (all populations except Japanese), and males from the Italian population of *N. viridula* exhibited the lowest ratio (2.16) for this group of populations.

While it seems clear that two or more different pheromone strains of *N. viridula* exist, the significance of the distinctive ratios of the major epoxide isomers is unclear. Field-tests of synthetic *trans*-/*cis*-(*Z*)- $\alpha$ -bisabolene-1,2-epoxide blends have been conducted in the U.S. and Brazil on a small scale, but very few bugs or tachinid parasites were attracted (Aldrich, unpublished data). The discovery that males of three *Acrosternum* spp., including *A. aseadum*, release (*Z*)- $\alpha$ -bisabolene-1,2-epoxides in ratios essentially the reserve of those for most *N. viridula* populations, and that one U.S. species

(*A. pennsylvanicum*) liberates a blend like Japanese *N. viridula* males [7], is evidence that unique ratios of these sesquiterpenoids might be involved in species isolation.

The native Japanese species, *N. antennata*, produces a blend of male-specific volatiles similar to those of most *N. viridula* populations, whereas *N. viridula* males from Japan express the most clearcut deviation from other conspecific populations. Geographically isolated populations of *N. viridula* produce somewhat different acoustical courtship signals [10, 20], and the songs of *N. antennata* are much different than the songs of *N. viridula* [10]. Nevertheless, interspecific mating occurs naturally between *N. antennata* and *N. viridula* in sympatric areas even though sperm is not transferred to the females [21, 22]. One interpretation of this situation is that pheromones and acoustical signals of these *Nezara* spp. are still diverging.

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