Volatile Compounds Emitted by the Stink Bug Antiteuchus innocens (Hemiptera: Pentatomidae)

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Volatile compounds emitted by the stink bug *Antiteuchus innocens* (Hemiptera: Pentatomidae)

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Stink bug species emit volatile compounds when disturbed; the volatiles may function as a defense mechanism to inhibit the attack of potential predators or as alarm pheromones to warn conspecifics of possible danger (Borges & Blassioli-Moraes 2017). In addition, undisturbed males of some species produced aggregation or sexual pheromones (Millar 2005; Moraes et al. 2008; Borges & Blassioli-Moraes 2017). Semiochemicals of stink bugs are produced in dorsal glands (nymphs) or metathoracic glands (adults) in the thorax, or in unicellular glands (adults) in the abdomen (Borges & Blassioli-Moraes 2017). Most of the studies on the chemical ecology of stink bugs have been performed on the subfamilies Pentatominae and Asopinae (El-Sayed 2018). Therefore, it would be valuable to extend these types of studies to other subfamilies to understand how chemical communication has evolved within this family.

The stink bug *Antiteuchus innocens* Engleman (Hemiptera: Pentatomidae) is a Neotropical species distributed from Mexico to Costa Rica (Engleman & Rolston 1983), but it is endemic to the southern Mexican Plateau (Ferrari et al. 2010). In Mexico, this species has been found infesting avocado (*Persea americana* Mill.; Lauraceae) and pine (*Pinus* [Pinaceae] spp.) trees (Ortega León 2001; G. Huerta personal observation). This stink bug has 5 nymphal instars (Ortega-León 2001), but many aspects of its natural history are not yet known, including aspects of its ecology.

This study was undertaken to investigate some aspects of the chemical ecology of *A. innocens*. Particularly, we identified the volatiles released by disturbed nymphs and adults of *A. innocens* using solid phase microextraction and gas chromatography-mass spectrometry. We also identified the compounds produced by the dorsal glands and metathoracic glands of the fifth instar nymph and adult, respectively. Finally, the biological activity of gland extracts was evaluated using electroantennography and olfactometric bioassays.

Stink bugs of all stages were collected in pine trees in the municipality of Manuel Altamirano (16.733333°N, 92.033333°W; 1,810 masl), Chiapas, Mexico. Bugs were transported to the laboratory inside plastic bags with pine branches and trunks. In the laboratory, insects were maintained at 25 to 27 °C, 60 to 70% relative humidity, and 12:12 h (L: D) photoperiod for 3 d before being analyzed or bioassayed.

Volatiles emitted by insects were sampled by solid phase microextraction fitted with fibers coated with 65 µm polydimethylsiloxane-divinylbenzene (Supelco, Bellefonte, Pennsylvania, USA). One (adult), 5 (fourth to fifth nymphs), or 10 (first to third instar nymphs) insects were gently introduced into a 7 mL glass vial (Supelco, Bellefonte, Pennsylvania, USA). The mouth of the vial was covered with aluminum foil and sealed with masking tape. Then, vials containing insects were agitated, and a solid phase microextraction fiber was introduced into the vial and exposed for 1 min to the effluvia of the insects. In all samplings, a control was performed before each test under the same conditions using an empty flask. The samples were desorbed for 1 min in the gas chromatograph injector for gas chromatography-mass spectrometry analysis. Ten replicates per stage per sex were performed. For gland extracts, insects were placed in a freezer at −20 °C for 2 min to avoid discharge of the gland contents during manipulation. The dorsal glands of fifth instar nymphs, and the metathoracic glands of females and males were dissected separately under water using a binocular microscope (Stemi 305, Carl Zeiss de Mexico, Mexico City, Mexico). Ten glands were placed into a 2 mL glass vial containing 1 mL of dichloromethane.

Volatile compounds sampled by solid phase microextraction and those from the gland extracts were analyzed by gas chromatography-mass spectrometry using a Varian CP-3800 GC coupled with a Varian Saturn 4D mass spectrometer with a nonpolar capillary column (Factor Four VF-5 ms, 30 m × 0.25 mm i.d.; Supelco, Bellefonte, Pennsylvania). The oven temperature was ramped from 50 °C (2 min hold) at 15 °C min^{-1} to 280 °C (10 min hold). Ionization was by electron impact at 70 eV. Kovats retention index of each compound was calculated. Tentative identification of compounds was based on the comparison of matching mass spectra with the NIST/EPA/NIH library (NIST 02). Identification of most of the compounds was confirmed by the comparison of mass spectra and retention times with those of synthetic compounds. The standards were purchased from Sigma-Aldrich (Toluca, Mexico), and were 97 to 99% pure according to the supplier.

Antennal response of both sexes to gland extracts from fifth instar female or male nymphs was determined by electroantennography. The electroantennograph set-up, the antennal preparations, and the experimental procedures used were similar to that conducted previously (Malo et al. 2004). In this trial, 2 µl of each extract was evaluated. Control stimuli (dichloromethane) was performed at the beginning and end of each electroantennograph analysis. Five replicates per treatment were performed.

The responses of fifth instar nymphs to volatiles emitted by disturbed nymphs and glandular extracts from the same instar were evaluated in a vertically oriented Y-tube olfactometer (SEV, Puebla, Puebla, Mexico). The olfactometer consisted of a Y-shape tube (2.5 cm diam; length of common tube = 12 cm, and 2 side arms = 10 cm) and 2 odor chambers (4.5 cm in diam, 15 cm height). Activated charcoal-filtered air was pushed into each odor chamber at 0.5 L per min.

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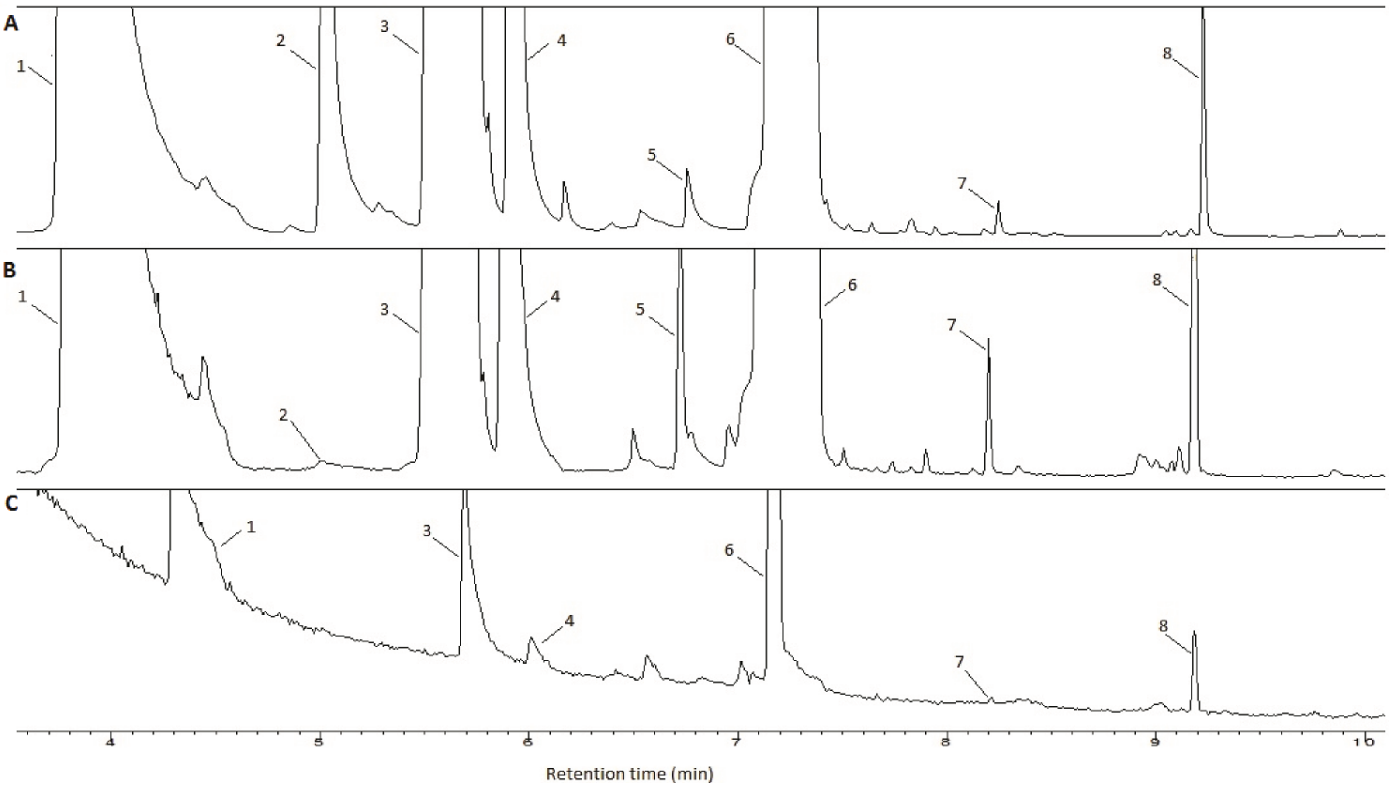

Fig. 1. Typical gas chromatograms of volatile compounds released by disturbed females (A) and fifth instar nymphs (B), or produced by the dorsal gland of fifth instar nymphs (C). For an explanation of peak numbers see Tables 1 and 2.

One chamber held the test material (i.e., 10 nymphs, 1 μl of glandular extract), and the other served as a control (clean air). The nymphs were introduced into the odor chamber followed by vigorous shaking for 30 s before starting the bioassay. A test nymph was gently introduced into the base of the Y-tube olfactometer and observed for 5 min. If the insect stayed in the common tube of the olfactometer or at the junction of the 2 arms, the test was stopped, and the insect was considered to be a non-responding individual. Ten replicates per treatment were performed.

Electroantennograph data were subjected to 1-way analysis of variance (ANOVA). Data from behavioral bioassays were analyzed using the log-likelihood ratio test (G-test) for goodness of fit with William's correction. Insects that did not show any preference were excluded from the analysis. All statistical analyses were performed in statistical software R version 3.3.3 (R Core Team 2017).

In total, 8 compounds were identified in the effluvia of disturbed nymphs and adults of *A. innocens* (Fig. 1a; Table 1). The major compounds in the blends of all nymph instars and adults were *n*-undecane (45–68%) and (*E*)-2-hexenal (16–40%) (Table 1). The gas chromatography-mass spectrometry analysis of the extracts from the abdominal glands of the fifth instar nymphs and metathoracic glands of adults showed that most compounds, except α-pinene and (*E*)-2-octenal, emitted by disturbed insects are produced in these exocrine glands (Fig. 1b; Table 2). We do not know where α-pinene and (*E*)-2-octenal originate, but one possibility is that these compounds came from the host plant. The compounds identified in *A. innocens* have been found in other stink bugs (Aldrich et al. 1978; Nagnan et al. 1994; Krall et al. 1999; Zarbin et al. 2000). For instance, Nagnan et al. (1994) found that nymphs of *Lincus spurcus* (Rolston) and *Lincus malevolus* (Rolston) (both Heteroptera: Pentatomidae) released 11 compounds, including (*E*)-2-hexenal, (*E*)-2-octenal, and *n*-undecane, compounds that also are emitted by *A. innocens*. In the present study, the same compounds were emitted by nymphs and both sexes of *A. innocens*, although nymphs and adults produce the compounds in different glands. A similar situation has been reported for 3 species of *Chlorochroa* (Hemip-

Table 1. Relative amount (%) of the volatile compounds emitted by disturbed nymphs and adults of *Antiteuchus innocens*.

Peak	RT	RI	Compound	1st	2nd	3rd	4th	5th	Female	Male
1	3.8	820	*(E)-2-Hexenal	16.4 ± 2.3	31.6 ± 8.9	18.8 ± 3.4	25.5 ± 2.3	31.7 ± 3.7	25.5 ± 5.5	40.3 ± 7.9
2	5.0	914	*α-Pinene	0.69 ± 0.3	0.14 ± 0.1	0.08± 0.03	0.46 ± 0.2	0.02 ± 0.00	0.65 ± 0.3	0.06 ± 0.2
3	5.5	954	(*E*)-2-Heptenal	9.04 ± 1.2	18.1 ± 5.7	15.6 ± 2.4	13.9 ± 1.2	13.4 ± 1.0	6.51 ± 1.1	9.54 ± 2.5
4	5.9	986	**n*-Decane	1.72 ± 0.3	3.26 ± 0.9	1.61 ± 0.3	2.29 ± 0.4	3.33 ± 0.7	4.43 ± 0.6	2.11 ± 0.5
5	6.7	1,081	(*E*)-2-Octenal	5.44 ± 0.8	0.53 ± 0.3	0.18 ± 0.02	0.27 ± 0.05	0.31 ± 0.1	0.25 ± 0.2	0.08 ± 0.02
6	7.1	1,090	**n*-Undecane	67.8 ± 1.6	44.8 ± 11.5	62.7 ± 5.2	56.9 ± 3.3	50.5 ± 3.9	61.9 ± 6.4	47.6 ± 9.2
7	8.2	1,200	**n*-Dodecane	0.17 ± 0.1	0.13 ± 0.1	0.08 ± 0.01	0.05 ± 0.00	0.08 ± 0.01	0.07 ± 0.01	0.03 ± 0.01
8	9.2	1,296	**n*-Tridecane	0.53 ± 0.1	1.37 ± 0.4	0.96 ± 0.14	0.57 ± 0.07	0.70 ± 0.1	0.57 ± 0.13	0.22 ± 0.1

Compounds indicated with an asterisk (*) were identified by the comparison of synthetic standards.

Table 2. Relative amount (%) of the volatile compounds found in the dorsal (nymphs) and metathoracic (adults) glands of *Antiteuchus innocens*, respectively.

Peak	RT	RI	Compound	5th instar nymph	Females	Males
1	4.3	859	*(*E*)-2-Hexenal	17.4 ± 2.3	22.3 ± 2.5	28.6 ± 1.8
2	5.7	970.7	(*E*)-2-Heptenal	7.32 ± 1.8	10.8 ± 0.6	11.6 ± 0.7
3	6.0	994.3	**n*-Decane	0.79 ± 0.4	1.46 ± 0.3	1.66 ± 0.5
4	7.1	1,090	**n*-Undecane	72.8 ± 3.7	64.6 ± 2.5	57.3 ± 1.2
5	8.2	1,194	**n*-Dodecane	0.18 ± 0.09	0.06 ± 0.005	0.06 ± 0.04
6	9.1	1,286	**n*-Tridecane	1.40 ± 0.04	0.74 ± 0.04	0.69 ± 0.02

Compounds indicated with an asterisk (*) were identified by the comparison of synthetic standard.

tera: Pentatomidae), where nymphs and adults released the same compounds (Ho & Millar 2001). In contrast, in some cases nymphs produced different compounds than those produced from adults, or there even may be qualitative differences among nymphal instars (Borges & Aldrich 1992; Pareja et al. 2007). Borges and Aldrich (1992) found that (*E*)-4-oxo-2-decenal was the major compound released by first instar nymphs of the Pentatominae subfamily, but this compound was absent in the older nymphs.

Nymphs did not show any preference for disturbed conspecific volatiles ($G = 0$; df = 1; $P = 1$) or glandular extracts ($G = 1.42$; df = 1; $P = 0.232$) compared to clean air. Also, there were no differences in the antennal responses of female ($F = 0.90$; df = 3, 16; $P = 0.461$) and male ($F = 0.24$; df = 3, 16; $P = 0.867$) to glandular extracts from adults and the fifth instar nymphs or the control. The electroantennograph results suggest that *A*. *innocens* does not perceive its own emitted compounds, although it is possible that antennae of this species have few receptors to these compounds, and because of the technique used we were unable to record any electrophysiological response to tested extracts and compounds. However, the fifth instar nymphs were not attracted or repelled by dorsal gland extracts when tested in the Y-tube olfactometer. Thus, both electroantennograph and behavioral results suggest that volatiles released by disturbed insects are likely involved in the *A*. *innocens* defense rather than functioning as an alarm pheromone. In fact, when the fifth instar nymphs were introduced into a nest of fire ants (*Solenopsis geminata* [F.]; Hymenoptera: Formicidae), and the ants attempted to attack the insects, they released the volatile compounds, perceived by the observer, provoking the ants to move away from the nymphs (Meneses-Arias unpublished data). The secretion of the metathoracic glands of *Cosmopepla bimaculata* (Thomas) (Hemiptera: Pentatomidae) has a defensive function, because 3 species of birds and 1 species of lizard avoided insects with metathoracic glands compared to insects with glands (Krall et al. 1999). However, in some insect species the volatiles produced by exocrine glands may function as defense or alarm pheromones, or both (Moraes et al. 2008).

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Summary

In this study, volatile compounds emitted by disturbed nymphs and adults of the stink bug *Antiteuchus innocens* were identified by gas chromatography-mass spectrometry as follows: (*E*)-hexenal, α-pinene, (*E*)-2-heptenal, *n*-decane, (*E*)-2-octenal, *n*-undecane, *n*-dodecane, and *n*-tridecane. The major components were (*E*)-2-hexenal, (*E*)-2-heptenal, and *n*-undecane. The same compounds, except α-pinene and (*E*)-2-octenal, were found in the dorsal glands and metathoracic glands of nymphs and adults, respectively. Extracts of exocrine glands did not elicit antennal responses of the adults, or affect the behavior of fifth instar nymphs, which suggests that these compounds are likely involved in the *A*. *innocens* defense rather than functioning as an alarm pheromone.

Key Words: Pentatomidae; Discocephalinae; metathoracic glands; dorsal glands; volatiles

Sumario

En este estudio, los compuestos volatiles emitidos por las ninfas y adultos molestados de la chinche apestosa *Antiteuchus innocens* fueron identificados por cromatografía de gases-espectrometría de masas como (*E*)-hexenal, α-pineno, (*E*)-2-heptenal, *n*-decano, (*E*)-2-octenal, *n*-undecano, *n*-dodecano, and *n*-tridecano. Los compuestos mayoritarios de la mezcla fueron el (*E*)-2-hexenal, (*E*)-2-heptenal, and *n*-undecano. Los mismos compuestos, excepto el α-pineno and (*E*)-2-octenal, fueron encontrados en las glándulas dorsales y metatoracicas de ninfas y adultos, respectivamente. Extractos de las glándulas exocrinas no evocaron una respuesta antenal de adultos, ni afectaron el comportamiento de ninfas de quinto instar, lo que sugiere que estos compuestos están probablemente involucrados en funciones de defensas más que como una feromona de alarma.

Palabras Clave: Pentatomidae; Discocephalinae; glándulas metatorácicas; glándulas dorsales; volátiles

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