CHARACTERISATION OF THE ALARM PHEROMONE OF *BATHYCOELIA DISTINCTA* (PENTATOMIDAE)

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ABSTRACT

Bathycoelia distincta (Pentatomidae) is the dominant pest in South African macadamia orchards, where adults are responsible for causing severe yield losses. Similar to other hemipterans, *B. distincta* release volatile compounds from scent glands that can deter natural enemies and act as an alarm signal among conspecifics. The overall aim of this study was to characterise the alarm pheromone of *B. distincta*. We: i) analysed the scent gland contents of individual adult *B. distincta* by gas chromatography-mass spectrometry (GC-MS), ii) quantified volatiles released from live stink bugs after stress, and iii) evaluated the electrophysiological and behavioural activity of alarm pheromone compounds with dose-response experiments. A blend of fourteen compounds was identified in the scent gland extracts of adult stink bugs. Of these, six compounds were detected in the effluvia of live stressed stink bugs [(*E*)-2-hexenal, (*E*)-2-decenal, tridecane, dodecane, (*E*)-4-oxohex-2-enal and (*E*)-2-decenyl acetate]. No qualitative or quantitative differences were observed between sexes. Tridecane was the most abundant compound, comprising ~50% of total secretions. Only (*E*)-2-hexenal, (*E*)-2-decenal and (*E*)-4-oxohex-2-enal elicited an antennal response in both sexes. Finally, exposure to a mixture of (*E*)-2-hexenal, (*E*)-2-decenal and (*E*)-4-oxohex-2-enal resulted in an increase in the speed and distance travelled by walking bugs and decrease in time spent resting compared to unexposed bugs. Our results show that the blend of (*E*)-2-hexenal, (*E*)-2-decenal and (*E*)-4-oxohex-2-enal can induce an alarm response in *B. distincta*.

Key Words—Stink bug, scent gland, GC-MS, antennal activity, behaviour

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INTRODUCTION

Stink bugs (Heteroptera: Pentatomidae) are well-known for producing odoriferous compounds that play a role in chemical communication and defence (Aldrich 1988; Borges and Blassioli-Moraes 2017; Millar 2005; Moraes et al. 2008; Noge et al. 2012; Weber et al. 2018). These volatile compounds, mostly represented by short-chain aliphatic aldehydes, alcohols, esters, alkanes, terpenes, and phenolics (Aldrich 1988; Millar 2005; Moraes et al. 2008; Noge et al. 2012) are produced and stored in the dorsal abdominal scent glands (DAGs) of nymphs and metathoracic scent glands (MTGs) of adults (Aldrich et al. 1984; Staddon 1979). These glands secrete volatiles through the external efferent thoracic system when insects are disturbed (Kment and Vilímová 2010; Lima et al. 2021; Pareja et al. 2007), and can act as a defence against natural enemies and microorganisms or mediate alarm or aggregation behaviours among conspecifics (Lopes et al. 2015; Noge et al. 2012; Weber et al. 2018).

In Pentatomidae, scent gland secretions can vary among species (Moraes et al. 2008; Pareja et al. 2007), genders (Aldrich et al. 1993; Ho et al. 2003) and developmental stages (Borges and Aldrich 1992; Fávaro et al. 2011; Fávaro et al. 2012; Ho and Millar 2001). For example, the secretions of adult *Chinavia impicticornis* (Stal) and *Chinavia ubica* (Rolston) contain approximately the same amount of (*E*)-4-oxohex-2-enal and tridecane, but the concentration of the (*E*)-4-oxohex-2-enal is higher than the tridecane in the nymphal secretions of both species (Pareja et al. 2007). In *Piezodorus guildinii* (Westwood) and *Dichelops melacanthus* (Dallas), (*E*)-4-oxohex-2-enal is the most abundant compound in all stages, whereas in *Euschistus heros* (F.) it is the most abundant compound in males and nymphs only (Pareja et al. 2007). In addition, tridecane and (*E*)-2-hexenal have also been observed to be produced in large quantities in both male and female *P. guildinii* (Pareja et al. 2007), while in *E. heros* the concentration of tridecane is greater in females compared to males (Lima et al. 2021; Pareja et al. 2007).

In Heteroptera, most of the hydrocarbons, aldehydes, oxo- aldehydes and esters are considered to be defensive compounds involved in dispersal (Aldrich 1988; Arif et al. 2020; Noge et al. 2012; Zhong et al. 2018). For example, behavioural experiments demonstrated that defensive secretions of *Thasus neocalifornicus* act as an alarm signal to conspecifics of the same life stage (Prudic et al. 2008). Nymphs were dispersed from their aggregation when nymph secretions, (*E*)-4-oxohex-2-enal and (*E*)-2-hexenal, were placed in the arena, while adults were dispersed after exposure to adult secretions composed of hexyl acetate and hexanal. Similar behaviours were observed with the defensive secretions of *Podisus nigrispinus* (Dallas) and *E. heros* where repellent and irritant effects occurred between species and between sexes of the same species (Lima et al. 2021). (*E*)-2-Hexenal and (*E*)-2-decenal also display strong alarm effects on both sexes of adults and nymphs of *Halyomorpha halys* (Zhong et al. 2017; Zhong et al. 2018), but nymphs showed a greater sensitivity to (*E*)-2-decenal than adults (Zhong et al. 2018). Nevertheless, defensive compounds such as the (*E*)-4-oxo-2-decenal have also been reported to act as an aggregation pheromone in the first instar nymphs of several pentatomid species (Fucarino et al. 2004).

At biologically relevant concentrations, compounds released from scent glands in pentatomids have been reported to deter predators (Noge et al. 2012), have antifungal and antibacterial properties (Lopes et al. 2015; Sagun and Collins 2016), and be used for host location by parasitoids (Laumann et al. 2009; Vieira et al. 2014; Zhong et al. 2017). For example, (*E*)-4-oxohex-2-enal has been identified as a non-specific volatile mutagen (Kasai et al. 2005) which is toxic and a deterrent to predators such as mantids or ants and other stink bugs (Eliyahu et al. 2012; Prudic et al. 2008), while (*E*)-2-hexenal can

cause temporary paralysis (Prudic et al. 2008; Noge et al. 2012). Despite their toxicity, these two compounds are used as kairomones by the egg parasitoids *Telenomus* spp. and *Trissolcus* spp. for finding *E. heros* and *Nezara viridula* (Linnaeus) (Laumann et al. 2009; Vieira et al. 2014), respectively. Finally, (*E*)-2-decenal is a repellent and irritant to predators (Noge et al. 2012) and can inhibit the growth of *Beauveria bassiana* (Lopes et al. 2015) and bacteria such as *Escherichia coli* (Sagun and Collins 2016). Repellent properties of this compound towards the parasitoid *Trissolcus japonicus* (Ashmead) have been demonstrated (Zhong et al. 2017).

Bathycoelia distincta (Distant), the two-spotted stink bug, is a major pest on macadamia in South Africa (Schoeman 2013; Schoeman 2018; Van den Berg et al. 1999). Discovered in the 1980's in the province of Limpopo, this species is now considered as one of the most damaging pests due to its long proboscis which can cause kernel damage throughout the entire season (Bruwer et al. 2021; Schoeman 2020). The annual nut damage cost attributed to stink bugs has been estimated at 200 million ZAR (Schoeman 2009; Schoeman 2013; Taylor et al. 2018). Previous studies have been conducted to determine seasonal occurrence (Schoeman 2013; Schoeman 2018) and parameters affecting the distribution and dispersal behaviour of stink bugs in South Africa (Schoeman 2014). Although the semiochemicals of Pentatomidae have been well-studied, this work constitutes the first study of semiochemicals of *B. distincta*.

The aims of this study were (a) to identify and compare the metathoracic gland contents of adult male and female *B. distincta*; (b) to quantify the volatile secretions emitted from live stink bugs in response to stress; and (c) to test the electrophysiological and behavioural responses of *B. distincta* to compounds found in gland extracts and secretions. This work provides strong chemical and behavioural evidence of the alarm pheromone system in *B. distincta*.

METHODS AND MATERIALS

Insects. The initial laboratory colony was established from wild *B. distincta* collected in Limpopo, South Africa (23°03'13''S, 30°14'02''E). Insects were reared under laboratory-controlled conditions (25 ± 2°C, 20 ± 5% relative humidity (RH), 14L : 10D photoperiod) in plastic cages (27 x 15cm) at the FABI Biological Control Centre, University of Pretoria, South Africa. The lid of the container had a mesh for ventilation and the base was covered with paper towel. Twice a week, insects were removed and placed in a clean container, and supplied with fresh corn and green beans *ad libitum*. Egg masses were collected twice a week and maintained in separate rearing cages until completion of nymphal stages.

Extraction of MTG. The MTG complex of *B. distincta* adults was removed by dissection (n = 9 per sex), and extracted in 1 ml of *n*-hexane for 30 min. To render insects less likely to discharge their gland contents and facilitate gland extractions, insects were refrigerated at 4 °C for 20 min before gland extraction (Noge et al. 2015). Extracts (1µl) were immediately analysed after collection by gas chromatography coupled to mass spectrometry (GC/MS) or stored at -20°C until analysis. A blank which consisted of 1µl of distilled *n*-hexane was also analysed.

Collection of MTG effluvia. Volatiles emitted from live stressed and unstressed male and female bugs (n = 19, 9 males and 10 females) were individually sampled by dynamic headspace collection (air entrainment). Prior to the entrainment, sampling containers made from a modified glass jar (0.58 L) sealed by a custom-made Teflon septum were cleaned with

ethanol, rinsed with distilled water, and dried out in an oven (110°C) overnight. Volatiles were sampled at room temperature. Filtered air (hydrocarbon trap, Supelco Superpure HC) was pumped into each container through Teflon tubing (Supelco, SU20532) and drawn out through the PorapakQ filled glass traps (Supelco ORBO 1103, 50/80, 150/75mg) at 40 ml min^{-1}. After sampling, PorapakQ beads were transferred to GC vials (vial N9 702293, Macherey-Nigel) and eluted with 1 ml of double distilled *n*-hexane for 1 hour. After 1 hour the solvent was removed and stored at 4°C until analysis in a separate storage vial. A total of 38 sealed PorapakQ cartridges were used to obtain extracts from dynamic headspace sampling.

A non-invasive method, adapted from Krajicek et al. (2016), was used for sampling the MTG effluvia. Male and female bugs were stressed by compression with a Pasteur pipette in the glass jar container until the appearance of droplets of liquid secreted from the metathorax of the bug. The pipette was then removed, and the glass jar immediately closed. The stressed bugs, each in a separate glass jar, were aerated for 4 hours. After the stress event, insects were removed, and the glass jar was surface rinsed with 2 ml of double distilled *n*-hexane. Before the stress event, the VOCs of each bug (non-stressed) were sampled for 4 hours under the same procedure as above except bugs were not stressed. An empty glass container was aerated as a blank each time.

Chemical analyses. The MTG extracts were analysed by GC-MS (Agilent 7890B coupled to a 5977B MSD) on a ZB-wax (30 m × 0.320 mm I.D. × 0.25 µm film thickness; 7HM-G002-11; Zebron) and ZB-5 (30 m × 0.320 mm I.D. × 0.25 µm film thickness; 7HM-G007-11; Zebron) column. The oven temperature was maintained at 50°C for 2 min, ramped at 10°C min^{-1} and then maintained for 2 min at 250°C or 300°C for the ZB-wax and ZB-5 columns, respectively. The same GC-MS machine was used to analyse the PorapakQ extracts collected from stressed and unstressed bugs but using an HP5-MS-UI (30 m × 0.250 mm i.d., 0.25 µm film thickness) and a DB-wax (30 m × 0.250 mm i.d., 0.25 µm film thickness) column. The oven temperature was maintained at 40°C for 2 min, ramped at 20°C min^{-1} and then maintained for 2 min at 300°C or 250°C for the HP5-MS-UI or the DB-wax column, respectively. All solutions were injected in splitless-mode at 250°C at constant column head pressure of 9.8 psi (helium). The mass spectrometer was operated in scan mode (m/z 45-550). Electron impact ionization (70 e-V) was used to generate ions. The ion source and MS quadrupole temperature were 230°C and 150°C, respectively. A series of *n*-alkanes (C6-C30) was run at the end of each series of analyses under the same conditions to calculate the Kovats index of each compound found in the extracts.

The data were collected and evaluated using the GCMS software MSD Chemstation, v. F.01.03.2357. Identification of compounds was done by comparing the retention times, Kovats indices (KI) and mass spectra with those obtained from reference compounds and NIST Mass Spectra Library (v 2.3). Quantitative calibration standards (1-500 ng/µl per compound) were made from the standards of the six identified compounds diluted in double distilled *n*-hexane. (*E*)-2-Hexenal, (*E*)-2-decenal, tridecane, dodecane were purchased from Sigma-Aldrich (Johannesburg, South Africa), whereas (*E*)-4-oxohex-2-enal and (*E*)-2-decenyl acetate were purchased from Molport (Riga, Latvia) and Chemspace (Monmouth, New Jersey, USA), respectively. The purity of all the standards exceeded > 95%. Standards were injected (1µl) on the GC-MS equipped with the same columns previously described using an auto-sampler (Agilent 7696 Series Liquid Sampler ALS). All the diluted standards were injected on the same day. This procedure was repeated over 3 different days. The quantities of each compound released per bug (ng per bug) were calculated using calibration linear equations.

Electrophysiological assays. Dose-response experiments using synthetic standards of the major compounds were performed on *B. distincta* antennae. Each standard was tested on 10 different male and female antennae. Electroantennograms (EAG) were conducted using 10 µL of test solution of (*E*)-2-hexenal, (*E*)-2-decenal, tridecane, dodecane, (*E*)-4-oxohex-2-enal and (*E*)-2-decenyl acetate dissolved in double distilled *n*-hexane, pipetted onto a piece of filter paper (1.5 cm^2, Whatman paper). The doses of solution tested were 10, 100, 1000 and 10 000 ng and 1 and 10 gland equivalent extract (Geq). The loaded paper was placed inside a glass Pasteur pipette. Hexane-treated and non-treated papers were used as controls. Controls were performed at the beginning and at the end of every series of stimulations. Pasteur pipettes were changed between every stimulation. Puff stimuli were blown into an airstream that passed over the antennal preparation through the stimulus delivery tube (120 mm diameter) using a flow controller (Flow Tracker 2000, Agilent) to generate a 0.5 sec stimulus (50 ml/min at puff maximum), with a constant flow rate of 150 ml/min. Air was humidified by passing it through an external glass wash bottle filled with distilled water. Each antennal preparation was centred and placed in front of the stimulus delivery tube. The signals generated by the antennae were passed through an amplifier (IDAC 4, Syntech, Hilverstrum, The Netherlands) and were recorded on software (EAGPro software, Version 2, Syntech, Hilverstrum, The Netherlands) where the antennal responses were directly measured.

For the EAG preparations, antennae were excised and mounted between two silver electrodes mounted on glass capillaries filled with Baedle-Ephrussi-Ringer solution (NaCl: 129, KCl: 4.7, $CaCl_2$: 1.9 millimoles/L) and connected to the amplifier. The silver electrodes were conditioned prior experiment through electrolysis in HCl (0.1 M) using a 9 V battery. Capillary tubes were drawn to a fine point using a capillary puller (PP-83, Narishige) to achieve a diameter enabling the insertion of the antennal tip into the capillaries.

Prior to the dose experiment, the antennal recovery time was determined based on the antennae of three males and three females. Antenna were used twice. For each antenna, 10 µL of a solution of (*S*)-cis-verbenol (Sigma-Aldrich, South Africa) at 1000 ppm was puffed randomly at 30, 45, and 60 seconds. The depolarisation after each resting time was expressed as a percentage compared to the depolarisation at T = 0.

Behavioural assays. The activity of blends of the different compounds (composition described below) found in stressed bug effluvia was evaluated with Petri dish assays adapted from Lima et al. (2021) and González-Morales et al. (2021). Glass Petri dishes (90 mm diameter x 15 mm high) were used as experimental arenas. Each Petri dish enclosed a 0.5 cm diameter filter paper (Whatman paper) placed in the centre. Adults were gently transferred into the arena and left for 3 min to acclimatize. Bugs that displayed stress behaviour during the acclimatization period (releasing of scent gland contents, buzzing) were excluded from the bioassay. After the acclimation period 10 µL of test solution was applied to the filter paper, the glass Petri dish was immediately covered to prevent insect escape, and stink bug behaviour was recorded for 10 min. A blank was also performed following the same procedure as above except that no test solution was applied to the filter paper. Prior to the experiment, the glass Petri dishes were cleaned and dried in an oven overnight at 110°C.

Three different test solutions were tested. Mixture A contained the six most abundant compounds detected in MTGs, (*E*)-2-decenal, (*E*)-2-hexenal, (*E*)-4-oxohex-2-enal, tridecane, dodecane and (*E*)-2-decenyl acetate. Mixture B contained the three compounds that elicited electrophysiological activity, (*E*)-2-decenal, (*E*)-2-hexenal and (*E*)-4-oxohex-2-enal. The mixtures A and B were prepared in *n*-hexane at gland equivalent concentration (Table 3) with 9.5 ng of (*E*)-2-hexenal, 43.7

ng of (*E*)-4-oxohex-2-enal, 20.9 ng of dodecane, 19 ng (*E*)-2-decenal, 166.9 ng of tridecane, and 6.42 ng of (*E*)-2-decenyl acetate. The third test solution was a solution of *n*-hexane. Ten males and ten females were used for each treatment (blank, Mixture A, Mixture B, *n*-hexane) in a completely randomized experimental design.

All bioassays were conducted in a custom-made white box (90 x 100 x 100 cm). The behaviour of each insect was recorded using a Logitech© C922 Pro HD stream webcam positioned approximatively 20 cm directly above the centre of the arena. The video-tracking system ToxTrac© was used to analyse the videos and to measure the distance travelled (cm), the speed (cm/sec) and resting time (sec) of the insects in the arena (Panadeiro et al., 2021; Rodriguez et al., 2018).

Statistical analyses. All data were analysed using R (version 4.1.2) and significance was accepted at $\alpha = 0.05$. Normality and homoscedasticity of the response variable was checked using a Kolmogorov–Smirnov and Bartlett's test, respectively. A linear model was used to link the peak area to the quantity of (*E*)-2-hexenal, (*E*)-2-decenal, tridecane, dodecane, (*E*)-4-oxohex-2-enal and (*E*)-2-decenyl acetate using the package "ChemCal" (Ranke, 2018). For the electrophysiological dose-response experiment, the maximal negative deflection response after the stimulus was measured for each modality and corrected by the subtraction of the response to air (non-treated papers). An ANOVA was used to compare the antennal recovery time while the electrophysiological and behavioural response data were analysed using a Kruskal-Wallis test to compare the differences between treatments, followed by a post-hoc Dunn's test. The effect of sex at each treatment was verified using a Mann-Whitney-Wilcoxon Test.

RESULTS

MTG extracts. Fourteen compounds were found in extracts from the MTGs of *B. distincta* (Table 1). By comparing the retention times, mass spectra and Kovats indexes, followed by comparisons with synthetic standards (Table 2), six of them were identified as (*E*)-2-hexenal, (*E*)-2-decenal, tridecane, dodecane, (*E*)-4-oxohex-2-enal and (*E*)-2-decenyl acetate. The eight remaining compounds were tentatively identified by comparing their mass spectra and Kovats indexes to the NIST library and with compounds from other stink bugs previously studied (Moraes et al. 2008). No differences in the relative abundance of these compounds were detected between males and females. The major compounds identified in MTG extracts were tridecane (47 ± 10 % females; 52 ± 9 % males), (*E*)-4-oxohex-2-enal (24 ± 11 % females; 17 ± 8 % males) and (*E*)-2-decenal (15 ± 8 % females; 14 ± 7 % males), followed by lower amounts of dodecane (1 ± 0 % females; 1 ± 0 % males), (*E*)-2-hexenal (3 ± 2 % females; 2 ± 1 % males) and (*E*)-2-decenyl acetate (8 ± 9 % females; 10 ± 7 % males). The abundance of each of the eight remaining compounds detected in the MTG secretions represented less than 1% of the total volume and the total volume of these eight compounds pooled together was less than 2%.

Table 1 IDENTITIES AND RELATIVE ABUNDANCE OF COMPOUNDS PRESENT IN *BATHYCOELIA DISTINCTA* MTG EXTRACTS. Retention times (RT) and Kovats retention indexes (KI) of compounds identified on the GC-MS instrument are given mean values ± std. Compounds in bold have been identified by matches with authentic standards.

		ZB-wax		ZB-5		
Sex	Compounds	RT	KI	RT	KI	Area (%)
Female (N= 9)	**(*E*)-2-Hexenal**	4.97±0.003	1221.05±0.34	4.06±0.01	845.75±3.58	3.11±2.49
	(*E*)-4-Oxohex-2-enal	-	-	5.16±0.00	955.63±0.36	23.84±10.72
	(*E*)-2-Hexen-1-ol, acetate	5.89±0.01	1336.48±1.22	5.71±0.00	1013.33±0.13	0.71±1.45
	(*E*)-2-Octenal	6.63±0.00	1435.93±0.07	6.13±0.00	1059.9±0.16	0.25±0.16
	Undecane	3.88±0.00	1097.79±0.39	6.48±0.00	1100.06±0.09	0.08±0.03
	(*E*)-2-Nonenal	7.31±0.00	1535.45±0.08	6.98±0.00	1163±0.11	0.02±0.01
	Dodecane	4.79±0.00	1199.16±0.22	7.28±0.00	1199.96±0.09	1.39±0.21
	(*Z*)-2-Decenal	7.85±0.00	1619.21±0.43	7.66±0.00	1252.07±0.06	0.12±0.06
	(*E*)-2-Decenal	8.05±0.00	1652.77±0.32	7.77±0.00	1266.39±0.42	15.44±8.13
	1-Tridecene	5.96±0.00	1344.9±1.29	7.97±0.00	1293.09±0.49	0.36±0.05
	Tridecane	5.64±0.01	1302.84±1.50	8.03±0.01	1301.63±0.77	47.00±9.79
	(*E*)-2-Decenyl acetate	8.56±0.00	1738.33±0.36	8.75±0.02	1406.83±2.61	7.70±9.01
	Tetradecane	6.38±0.00	1400.72±0.27	8.71±0.02	1400.93±2.44	0.05±0.02
	Pentadecane	7.08±0.00	1499.58±0.17	9.35±0.00	1500.02±0.05	0.14±0.05
Males (N= 9)	**(*E*)-2-Hexenal**	4.97±0.00	1221.37±0.51	4.07±0.01	845.06±0.72	2.36±1.35
	(*E*)-4-Oxohex-2-enal	7.70±0.00	1596.14±0.00	5.16±0.00	954.99±1.76	17.23±8.51
	(*E*)-2-Hexen-1-ol, acetate	5.88±0.00	1334.29±0.06	5.72±0.00	1013.48±0.15	2.20±3.46
	(*E*)-2-Octenal	6.63±0.00	1435.49±0.92	6.13±0.00	1059.99±0.13	0.24±0.16
	Undecane	3.90±0.02	1099.69±2.04	6.48±0.01	1100.06±0.14	0.1±0.07
	(*E*)-2-Nonenal	7.31±0.00	1535.51±0.11	6.98±0.02	1162.98±0.22	0.02±0.00
	Dodecane	4.79±0.00	1199.62±0.48	7.28±0.01	1199.97±0.14	1.44±0.26
	(*Z*)-2-Decenal	7.85±0.00	1619.27±0.25	7.66±0.00	1252.00±0.10	0.14±0.03
	(*E*)-2-Decenal	8.05±0.00	1652.11±1.77	7.77±0.04	1266.35±0.53	14.08±6.86
	1-Tridecene	5.96±0.00	1345.34±0.57	7.96±0.00	1292.72±0.18	0.39±0.08
	Tridecane	5.64±0.02	1303.15±2.64	8.03±0.01	1301.55±1.54	51.93±8.63
	(*E*)-2-Decenyl acetate	8.56±0.00	1738.48±0.60	8.75±0.00	1407.62±0.62	10.40±7.18
	Tetradecane	6.38±0.00	1400.59±0.54	8.70±0.00	1400.08±0.11	0.05±0.01
	Pentadecane	7.08±0.00	1499.95±0.48	9.35±0.00	1500.00±0.11	0.16±0.10

Table 2 THE STANDARD COMPOUNDS, PURITIES, RETENTION TIME (RT) AND KOVATS RETENTION INDEXES (KI) CALCULATED FOR THE DIFFERENT COLUMNS AND METHODS THAT WERE USED.

Name	Cas no	Purity (%)	ZB-wax		ZB-5		DB-wax		HP-5	
			KI	RT	KI	RT	KI	RT	KI	RT
(*E*)-2-Hexenal	6728-26-3	95	1221.80	4.91	851.62	4.13	1220.30	5.88	829.22	4.64
(*E*)-4-Oxohex-2-enal	2492-43-5	95	1595.52	7.70	954.86	5.15	1601.55	8.90	956.40	5.83
Dodecane	112-40-3	99	1200.00	4.74	1200.00	7.30	1200.00	5.71	1200.00	8.16
(*E*)-2-Decenal	3913-81-3	99	1652.02	8.03	1268.83	7.73	1657.23	9.19	1266.18	8.70
Tridecane	629-50-5	99	1300.00	5.58	1300.00	7.96	1300.00	6.58	1300.00	8.96
(*E*)-2-Decenyl acetate	19487-61-7	95	1738.19	8.56	1407.12	8.75	1742.48	9.78	1408.61	9.85

Bathycoelia distincta MTG effluvia. Comparison of effluvia from stressed adult male and female *B. distincta* identified six compounds present in both sexes (Table 3). The Kovats indexes were calculated on two different columns (ZB-wax and HP-5MS UI) and the identities were confirmed by comparison with of standards (Table 2). The amount of the six compounds released by bugs of either sex were not significantly different ($P > 0.05$). Adults of *B. distincta* released tridecane (150 ± 53 ng females; 183 ± 52 ng males), (*E*)-4-oxohex-2-enal (27 ± 9 ng females; 33 ± 11 ng males), (*E*)-2-decenal (22 ± 4 ng females; 16 ± 5 ng males), dodecane (19 ± 6 ng females; 23 ± 6 ng males), (*E*)-2-hexenal (11 ± 3 ng females; 7 ± 2 ng males) and (*E*)-2-decenyl acetate (3 ± 1 ng females; 9 ± 3 ng males). Analyses of the extracts from the unstressed stink bugs were similar to the blank.

Table 3 COMPOUNDS RELEASED BY STRESSED FEMALE AND MALE ADULTS *B. DISTINCTA*. Retention times (RT) and Kovats retention indices (KI) (mean values ± std) of compounds identified and amount emitted per bug (mean ± SE) quantified by GC-MS. No significant differences were observed between males and females (Wilcoxon-Mann-Whitney test, $P > 0.05$).

	Column		(*E*)-2-Hexenal	(*E*)-4-Oxohex-2-enal	Dodecane	(*E*)-2-Decenal	Tridecane	(*E*)-2-Decenyl acetate
Females (N=10)	DB-wax	RT	5.86±0.02	8.86±0.02	5.65±0.02	9.23±0.01	6.59±0.05	9.77±0.01
		KI	1218.28±2.44	1602.43±2.23	1194.53±1.89	1657.46±2.00	1301.74±5.50	1740.20±2.42
	HP-5	RT	4.65±0.01	5.82±0.00	8.10±0.00	8.64±0.01	8.92±0.02	9.71±0.00
		KI	833.94±0.95	956.71±0.40	1201.51±0.10	1263.61±0.69	1296.39±2.63	1406.39±0.10
Males (N=9)	DB-wax	RT	5.86±0.02	8.85±0.02	5.65±0.02	9.23±0.02	6.61±0.06	9.77±0.02
		KI	1217.70±2.82	1601.82±3.13	1194.31±2.46	1656.52±3.15	1303.14±6.73	1740.69±2.59
	HP-5	RT	4.64±0.01	5.83±0.00	8.10±0.00	8.64±0.00	8.93±0.02	9.71±0.00
		KI	833.84±1.26	956.89±0.54	1201.57±0.07	1264.04±0.52	1296.96±2.82	1406.49±0.23
Quantity (ng ± SE) per female			11.5±3.5	27.4±9.1	18.9±6.3	22±4	150.4±53.2	3.4±1.2
Quantity (ng ± SE) per male			7.5±1.9	32.7±11.3	23±6.4	16.2±5.2	183.5±52.3	9.4±3.3

Electrophysiology. No differences were observed between the size of the depolarisation events with 30, 45 and 60 seconds between successive odour puffs (Supplementary material Fig. S1, $P = 0.79$). Therefore, an interval of 30 seconds between puffs was used in the dose-response experiment. (*E*)-2-Hexenal, (*E*)-2-decenal and (*E*)-4-oxohex-2-enal elicited a significantly larger response than the other compounds tested at doses > 1000 ng ($P < 0.05$, Fig. 1a, b, f). Among all the putative pheromone compounds, (*E*)-4-oxohex-2-enal elicited the biggest relative responses for doses puffed at 10 000 ng (Fig. 1f) and (*E*)-2-decenal the second biggest relative response for the same doses (Fig. 1b). Males were more sensitive than females to *n*-hexane (Fig. 1b, $P = 0.02$; Fig. 1f, $P = 0.02$), 10 Geq (Fig. 1a, $P = 0.04$; Fig. 1f, $P = 0.01$), 10 ng of (*E*)-2-decenal (Fig. 1b, $P = 0.03$), and 10 ng ($P = 0.01$) and 100 ng ($P = 0.01$) of (*E*)-4-oxohex-2-enal (Fig. 1f). For other doses tested, males and females had similar relative responses (all $P > 0.05$). Dodecane, tridecane and (*E*)-2-decenyl acetate did not elicit responses even at the highest dose tested (Fig. 1c, d, e).

Behavioural assays. Exposure to mixture A (six most abundant compounds detected) and B (three compounds that elicited electrophysiological activity) induced a change in the behaviour of *B. distincta* compared to untreated and *n*-hexane treated controls (Fig. 2, Supplementary material Fig. S2). Both females and males travelled longer distances when exposed to mixture A (females: 3124 ± 634 cm; males: 3258 ± 384 cm) or B (females: 3419 ± 241 cm; males: 3278 ± 320 cm)

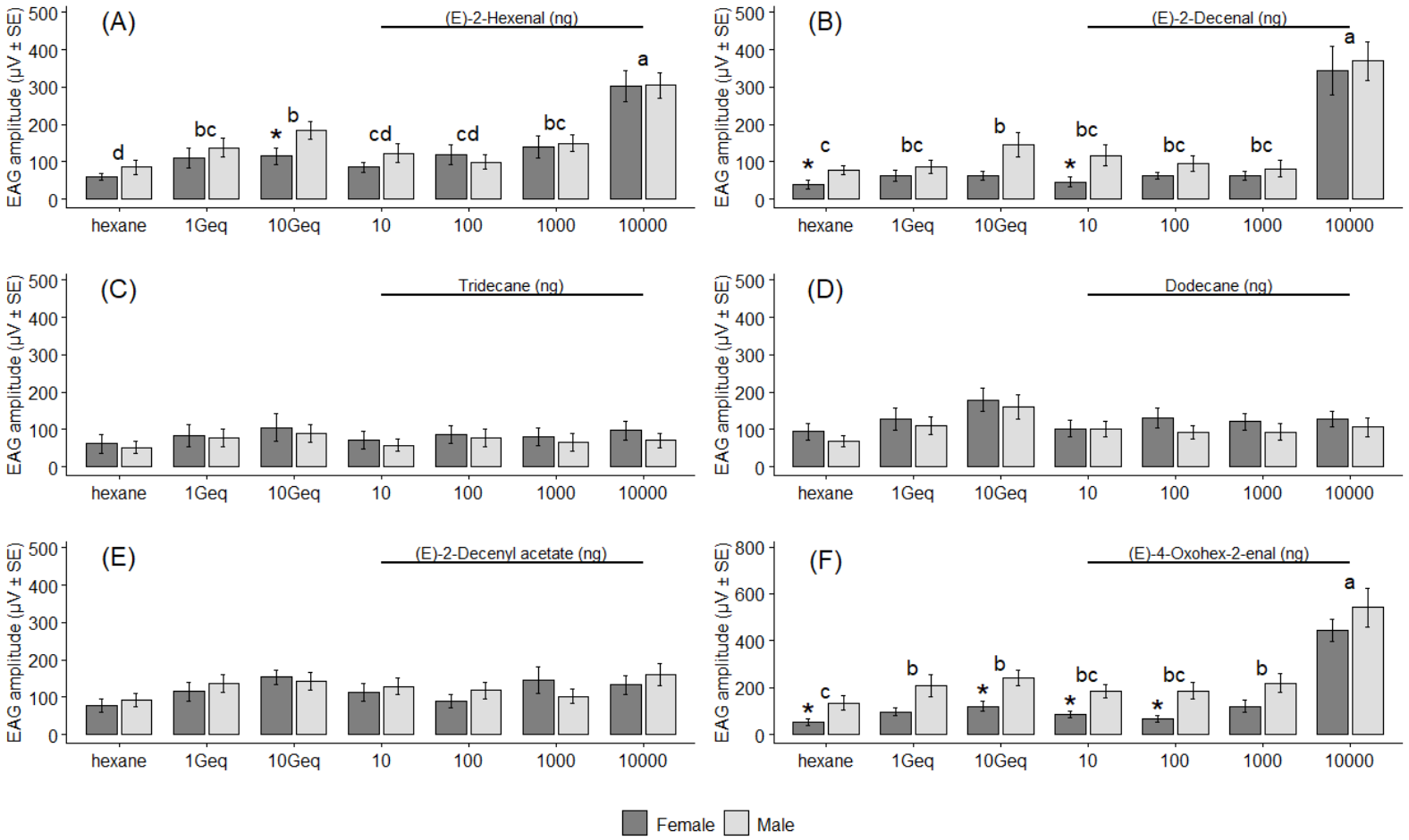

Fig. 1 Electro-antennogram dose-response profiles of major compounds found in MTGs and volatiles of stressed *B. distincta*. EAG response amplitudes (mean µV ± SE) of (*E*)-2-hexenal (A), (*E*)-2-decenal (B), tridecane (C), dodecane (D), (*E*)-2-decenyl acetate (E) and (*E*)-4-oxohex-2-enal (F) puffed on female (black, n = 10) and male (grey, n = 10) antennae. Quantity of individual compounds puffed ranged from 10 to 10 000 ng and were compared to *n*-hexane (blank), 1 and 10 MTG gland equivalent (Geq). Letters indicate significant differences between doses tested and asterisks indicate significant differences between male and females EAG responses for the same dose puffed.

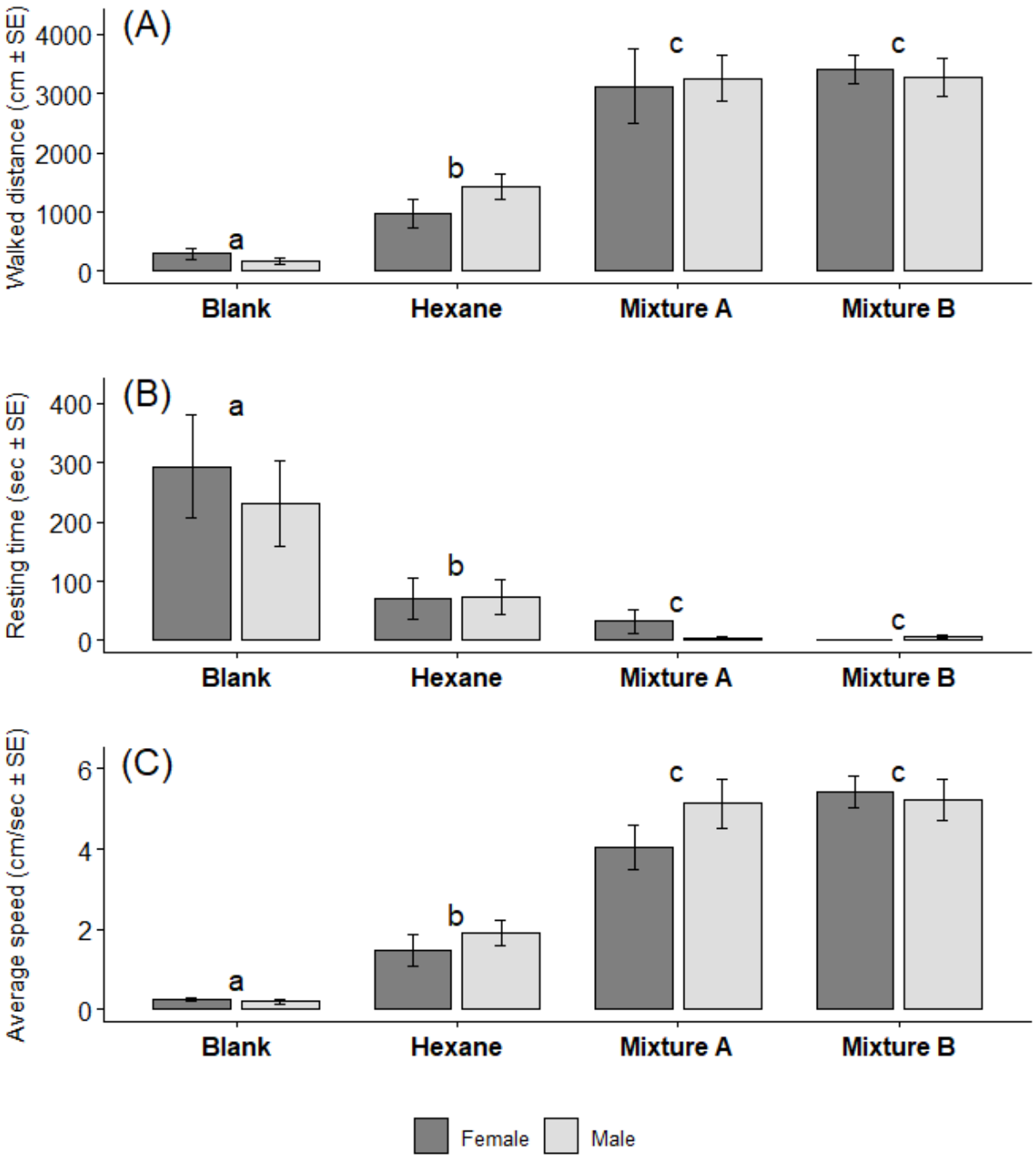

Fig. 2 Behavioural responses of *B. distincta* to putative alarm pheromone compounds. Distance walked (cm ± SE) (A), resting time (sec ± SE) (B) and average of speed (cm / sec ± SE) (C) of male (n = 10) and female (n = 10) insects after 10 min of exposure to mixture A ((*E*)-2-hexenal, (*E*)-2-decenal, tridecane, dodecane, (*E*)-2-decenyl acetate and 4-oxohex-2-enal diluted in hexane) or mixture B ((*E*)-2-hexenal, (*E*)-2-decenal and (*E*)-4-oxohex-2-enal diluted in *n*-hexane). *n*-Hexane and blank (no solvent) were used a positive and negative control respectively. Letters indicate significant differences ($P < 0.05$) among the treatments tested. No differences between males and females were found.

compared to *n*-hexane (female: 977 ± 245 cm; male: 1441 ± 213 cm) and the blank (females: 295 ± 83 cm; males: 173 ± 46 cm) (Fig. 2a, $P < 0.001$). Stink bugs exposed to the blank spent more time resting (females: 294 ± 86 sec, males: 231 ± 72 sec) compared to the *n*-hexane control (females: 71 ± 35 sec, males: 74 ± 30 sec) or mixture A (females: 32 ± 19 sec, males: 5 ± 3 sec) and B (females: 1 ± 1 sec, males: 7 ± 3 sec) (Fig. 2b, $P < 0.001$). The walking speed of male and female stink bugs significantly increased when exposed to mixture A (females: 40 ± 5 cm/sec, males: 51 ± 6 cm/sec) and B (females: 54 ± 4 cm/sec, males: 52 ± 5 cm/sec) compared to the *n*-hexane (females: 15 ± 4 cm/sec, males: 19 ± 3 cm/sec) and blank treatments (females: 2 ± 0 cm/sec, males: 2 ± 1 cm/sec) (Fig. 2c, $P < 0.001$). No statistical differences in distance travelled (Fig. 2a), resting time (Fig. 2b) and walking activities (Fig. 2c) were recorded between sexes or between mixtures.

DISCUSSION

In this study, extracts and effluvia of *B. distincta* scent glands were characterized by GC-MS analyses. Fourteen compounds were found in the MTG extracts of male and female *B. distincta*. Six compounds conclusively identified, (*E*)-2-hexenal, (*E*)-2-decenal, tridecane, dodecane, (*E*)-4-oxohex-2-enal and (*E*)-2-decenyl acetate, made up over 98% of the total MTG extract volume. Analysis of the effluvia of *B. distincta* adults confirmed that those six compounds are released in response to stress. Tridecane was the most abundant compound in effluvia, followed by (*E*)-4-oxohex-2-enal and (*E*)-2-decenal while dodecane, (*E*)-2-hexenal and (*E*)-2-decenyl acetate were released in small amounts. However, of the six compounds, only (*E*)-2-hexenal, (*E*)-2-decenal and (*E*)-4-oxohex-2-enal elicited antennal responses significantly higher than the *n*-hexane control. Laboratory bioassays demonstrated that exposure to one gland equivalent of the six compounds detected in effluvia resulted in male and female *B. distincta* walking at higher velocities, farther, and spending less time resting than control treatments. Bioassays with the three compounds that elicited antennal activity observed similar effects compared to the complete blend. This study demonstrates that the blend of (*E*)-2-hexenal, (*E*)-2-decenal and (*E*)-4-oxohex-2-enal found in the MTGs can induce an alarm response in *B. distincta*.

The compounds identified in male and female scent glands of *B. distincta* are similar to those described for other Pentatomidae species (Fávaro et al. 2011; Lima et al. 2021; Moraes et al. 2008; Pareja et al. 2007), suggesting that scent gland compounds are highly conserved across species. The pungent odour of Heteroptera scent glands is typically due to highly volatile compounds, primarily represented by carboxylic acids, alcohols, aldehydes, and saturated hydrocarbons (Aldrich 1988; Millar 2005; Moraes et al. 2008). Our results demonstrate that *B. distincta* MTG extracts contain fourteen compounds with high concentrations of tridecane followed by the (*E*)-4-oxohex-2-enal, (*E*)-2-decenal, and by lower amounts of dodecane, (*E*)-2-hexenal and (*E*)-2-decenyl acetate. These six compounds make up 98 % of the total composition of MTG extracts. In various species the alkanes are present in large quantities, where tridecane has been identified as the most abundant compound in scent glands (Borges and Aldrich 1992; Fávaro et al. 2011; Pareja et al. 2007; Zhong et al. 2017), often followed by (*E*)-4-oxohex-2-enal and (*E*)-2-decenal (Fávaro et al. 2011; Lopes et al. 2015). For example, in *Pallantia macunaima* (Grazia) thirteen compounds were identified in MTG extracts including ~60 % tridecane and ~10 % (*E*)-2-decenal. (Aldrich et al. 1978; Fávaro et al. 2011; Fávaro et al. 2012; Lockwood and Story 1987; Marques et al. 2007; Noge et al. 2012; Pareja et al. 2007). It has been suggested that the alkanes serve as solvents to facilitate the evaporation of other compounds, and as odour fixers for helping volatile compounds to penetrate the cuticles of other insects (Aldrich 1988; Gunawardena and Herath 1991; Remold 1963). These functions could explain their high quantities in the secretion of the scent gland, compared to the aldehydes and esters, which are present in lower quantities but can

induce a strong alarm effect in conspecifics and deterrent and repellent effect in heterospecifics (Lima et al. 2021; Noge et al. 2015; Noge et al. 2012; Zhong et al. 2018). The MTG extracts from adult male and female *P. macunaima* did not differ qualitatively or quantitatively, similar to what we observed in *B. distincta*.

We recorded EAG dose-response profiles to (*E*)-2-hexenal, (*E*)-2-decenal, tridecane, dodecane, (*E*)-4-oxohex-2-enal and (*E*)-2-decenyl acetate for male and female *B. distincta*. Not all the MTG compounds tested by EAG elicited a similar antennal response. Although electrophysiological tests have been widely used for the characterization of pheromone structures of many insects, few studies have measured the electrophysiological activity of semiochemicals in Pentatomidae. Sex pheromone compounds were tested by EAG in *Bagrada hilaris* (Burmeister) (Arif et al. 2020), *N. viridula* (Brézot et al. 1994), *Thyanta pallidovirens* (Stål) (Millar, 1997), *C. ubica* and *C. impicticornis* (Blassioli-Moraes et al. 2012). In *H. halys*, antennal responses have been elicited by its alarm pheromone compound tridecane and (*E*)-2-decenal in both males and females (Zhong et al. 2018; Zhong et al. 2017). (*E*)-2-Decenal even induced an electrophysiological response up to 0.5 mV in adults (Zhong et al. 2018). In our study, we obtained the highest amplitude of response with (*E*)-4-oxohex-2-enal followed by (*E*)-2-decenal and (*E*)-2-hexenal that elicited antennal responses > 300 μV in *B. distincta*. However, the alkanes tridecane and dodecane and (*E*)-2-decenyl acetate elicited no antennal responses in *B. distincta* unlike in *H. halys* (Zhong et al. 2017) and *B. hilaris* (Arif et al. 2020). Our electrophysiological results showed similar responses between *B. distincta* males and females with few exceptions.

Our video-tracking measurements show that exposure to the mixture containing the six major scent gland compounds significatively increased the walking activity and decreased the resting time of *B. distincta* compared to the controls. In addition, exposure to a mixture containing only the three EAG-active compounds induced the same behavioural responses as the blend with the six compounds. This result suggests that one or more of (*E*)-2-decenal, (*E*)-2-hexenal and (*E*)-4-oxohex-2-enal may be responsible for the alarm response in *B. distincta*. The aldehydes detected in this study are common in heteropterans and are considered as neurotoxins, repellents or irritants (Noge et al. 2012), volatile mutagens (Kasai et al. 2005) and highly reactive (Eliyahu et al. 2012) causing paralysis in some predatory insects and other stink bugs (Eliyahu et al. 2012; Prudic et al. 2008) at high concentrations. Several studies have demonstrated the toxic and repellent effects of (*E*)-2-decenal, (*E*)-2-hexenal and (*E*)-4-oxohex-2-enal (Fucarino et al. 2004; Noge et al. 2012; Prudic et al. 2008; Zhong et al., 2017; Zhong et al., 2018). The function of alkanes is poorly understood, but Gunawardena and Herath (1991) demonstrated that tridecane works synergistically with (*E*)-2-hexenal to repel insects. Esters such as (*E*)-2-hexenyl acetate and (*E*)-2-octenylacetate are part of attractant pheromones in milk-weed bugs (Aldrich et al. 1999) and *B. hilaris* (Arif et al. 2020). Finally, since a solvent effect of *n*-hexane on the behaviour of *B. distincta* was also observed additional behavioural response assays should be considered in future studies to explore the solvent effect in more detail.

In pentatomids, the volatiles secreted from MTGs appear to mediate intra- (alarm) and inter-specific (defence) behaviours and are exploited by natural enemies to locate prey (Weber et al. 2018). Therefore, the scent gland secretions of *B. distincta* could have application in integrated pest management. For example, the application of MTG compounds may attract parasitoids (Vieira et al. 2014). Alternatively, in the future it may be possible to incorporate them into push-pull tactics (Niu et al. 2022). Future research is needed to explore the development of these and other applications.

Declarations

Author Contribution All authors conceived the project. EP and QG conducted the experiments. EP conducted the analyses. EP wrote the first draft, and all authors reviewed and approved the final draft.

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Conflicts of interest The authors declare that they have no conflict of interest.

Ethics approval This article does not contain any studies with human participants or vertebrates performed by any of the authors.

Supplementary materials

Fig. S1 Antennal recovery (in %) 30, 45 and 60 sec after 1000 ng of Verbenol puffed (n = 3 males and 3 females) (ANOVA test, $P = 0.795$)

Fig. S2 Representative tracks showing the walking activity of *B. distincta* for 10 min period after exposure to different treatments. Filter papers impregnated with scent gland compounds, hexane or nothing were placed at the arena centres. Green tracks indicate the walking path realized by the female (left) and male (right) stink bugs during 10 min period.

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