

**Ecology and management of *Bathycoelia distincta* (two-spotted stink bug)
in Macadamia orchards in South Africa**

by

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SUMMARY

Ecology and management of *Bathycoelia distincta* (two-spotted stink bug) in Macadamia orchards in South Africa

by

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Keywords: stink bugs, Pentatomidae, macadamia, IPM, population genetics,
pheromone, insecticide

The aim of this PhD was to investigate the ecology and management of *B. distincta* (Pentatomidae) associated with macadamia orchards in South Africa.

In chapter 1, the available literature of the life history traits of the Pentatomidae and their potential for use in management programmes was reviewed. The Pentatomidae are hemimetabolous with egg, nymphal and adult life stages, which differ in morphological, ecological, and behavioural traits. Some of these traits, such as the pheromone mediated aggregation and the disruption of symbiont acquisition are important targets for Pentatomidae control. Other traits such as the alarm pheromone and substrate-borne

vibration also deserve further consideration. Finally, colour vision and flight ability are still poorly studied in Pentatomidae despite their potential importance for stink bug management.

In chapter 2, a population genetic analysis was conducted to determine the genetic diversity of *B. distincta* in South Africa. Individuals were sampled over three consecutive seasons from the three main macadamia regions of South Africa. Two gene regions, namely Cytochrome c Oxidase Subunit 1 (COI) and Cytochrome b (Cytb) were sequenced and subjected to various population genetic analysis. The analyses revealed a high haplotype diversity, an absence of cryptic species and a low genetic differentiation, despite the important geographic distances separating the various growing regions. These results suggest that this species is not currently structured among the various regions in South Africa which might be explained by its high dispersal capacity, and the rapid expansion of macadamia orchards in South Africa over the past decade.

In chapter 3, the composition of *B. distincta* alarm pheromone, antennal sensitivity and behavioural responses were studied. Gland extractions and dynamic headspace sampling revealed the presence of fourteen compounds, with only six of them that are released when stink bug adults are stressed. The quantification and antennal sensitivity of these six major compounds were determined and showed differences in doses and compound tested, but no differences were observed between sexes. Finally, the behavioural bioassays revealed that the alarm pheromone response observed in *B. distincta* is induced by a blend of at least (*E*)-2-hexenal, (*E*)-2-decenal, and (*E*)-4-oxohex-2-enal.

In chapter 4, the lethal and sublethal effects of insecticides used in the management of *B. distincta* in macadamia orchards were investigated. Firstly, the toxicity of four commercial products (i.e., acephate, lambda-cyhalothrin, beta-cyfluthrin and tau-fluvalinate) was assessed on adult stink bugs and behavioural tests were conducted to determine the effects of these products on the mobility of this pest. Thereafter, the acute toxicity of the lambda-cyhalothrin was determined and sublethal effects of this insecticides at LC10 and LC30 were analysed to determine their effect on the biological parameters of parent *B. distincta* and offspring generations. The results showed that pyrethroids lambda-cyhalothrin, beta-cyfluthrin and tau-fluvalinate are less toxic for *B. distincta* than the organophosphate acephate, which was also visible in the mobility bioassays. Sublethal effects were observed

when *B. distincta* adults were exposed to sublethal concentrations of lambda-cyhalothrin, including a reduction in male longevity and fecundity with LC30, and a shorter developmental time of the offspring. These results suggest that *B. distincta* is not susceptible to pyrethroids and could explain why the macadamia industry is struggling to control this pest.

In chapter 5, various commercial lures and traps were evaluated in order to assess their effectiveness to capture *B. distincta* and other stink bugs in macadamia orchards. The commercial pheromone products were tested since cross-attraction with these commercial lures has been observed within Pentatomidae and may occur between stink bugs present in macadamia orchards in South Africa as well. Two trials in Limpopo and Mpumalanga were conducted over two periods, corresponding to phenological stages of macadamia nut development (from nut set to harvest). Overall, low numbers of stink bug species were captured during both the Limpopo and Mpumalanga trials, especially on clear sticky traps. However, considering the low density of bugs present in both orchards during the trials, the effectiveness of these pheromone-baited traps is not clear. More research, including the identification of pheromones emitted by the species present in orchards, needs to be conducted before pheromone baited traps can be implemented as part of the IPM program of the macadamia industry.

To summarize, this thesis showed that the ecology and management of *B. distincta* is complex and specific to South Africa. Since its discovery in the 80's in the Limpopo province, this pest has spread extensively over South Africa as the genetic analyses showed. Its management mainly relies on the chemical utilisation of insecticides, especially pyrethroids and organophosphates. We showed there are some indications that this species might be less susceptible to pyrethroids than organophosphate. The pheromone-based management program is still in development, but the characterization of its alarm pheromone might help the industry to develop it.

RESEARCH OUTPUTS

Journal articles

Pal E., Allison J. D., Hurley B. P., Slippers B. and Fourie G. (2023). Life history traits of the Pentatomidae (Hemiptera) for the development of pest management tools. *Forests* 14(5), 861. <https://doi.org/10.3390/f14050861>

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Pal E., Allison J., Guignard Q., Hurley B. P., Slippers B. and Fourie G. (2022). Characterisation of the alarm pheromone of *Bathycoelia distincta* (Pentatomidae). *Journal of Chemical Ecology* 48, 791–801 (2022). <https://doi.org/10.1007/s10886-022-01388-w>

Conference presentations

Oral presentations

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Pal E., Allison J. D., Hurley B. P., Slippers B. and Fourie G. (2021). Characterization of the alarm pheromone of *Bathycoelia distincta* (Pentatomidae), a major pest of macadamia. 36th Annual meeting of the International Society of Chemical Ecology (ISCE), Stellenbosh, South Africa, 5 - 10 September.

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orchards in South Africa. 22nd Biennial Entomological Society of Southern Africa (ESSA), Limpopo, South Africa, 28 June - 1 July.

Poster presentations

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LIST OF ABBREVIATIONS

IPM	Integrated Pest Management
COI	Cytochrome c Oxidase Subunit 1
Cytb	Cytochrome b
DAG	Dorsal abdominal scent glands
MTG	Metathoracic scent glands
GC	Gas chromatography
MS	Mass spectrophotometry
EAD	Electroantennogram detection
LT50	Mean lethal time
LC50	Mean lethal concentration

DECLARATION

I declare that the dissertation/thesis, which I hereby submit for the degree of Doctor of Philosophy in Entomology at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

ETHICS STATEMENT

The author, whose name appears on the title page of this dissertation/thesis, has obtained, for the research described in this work, the applicable research ethics approval.

The author declares that s/he has observed the ethical standards required in terms of the University of Pretoria's Code of Ethics for Researchers and the Polict guidelines for responsible research.



Elisa Pal

May 2023

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Chapter 1

Life history traits of the Pentatomidae
(Hemiptera) for the development of
pest management tools

Review

Life History Traits of the Pentatomidae (Hemiptera) for the Development of Pest Management Tools

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Abstract: Knowledge of the biology of a pest is essential for building sustainable management programmes. Pentatomidae have a hemimetabolous life cycle with egg, nymphal, and adult life stages, which differ in morphological, ecological, and behavioural traits. Some of these traits, such as mating behaviour, pheromones (alarm and aggregation pheromones) and the acquisition of gut symbionts can be targeted for pest management strategies. Here, we review the available literature on these life history traits of the Pentatomidae with potential for use in management programmes. Pheromone-mediated aggregation and the disruption of symbiont acquisition are two important targets for Pentatomidae control. Other traits such as the use of alarm pheromones for enhancing natural enemies and substrate-borne vibration for mating disruption deserve further consideration. Colour vision and flight ability are still poorly studied, despite their potential importance for stink bug management.

Keywords: stink bugs; behaviour; pheromones; symbionts; IPM



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1. Introduction

Pentatomidae, commonly known as shield or stink bugs, is the largest family in the Pentatomoidea, which is known to contain a considerable number of economically important pest species [1–3]. Divided into eight subfamilies, the Pentatomidae represents the third-largest Heteropteran family [1,4]. Pentatomidae are mostly phytophagous and polyphagous, with the exception of predatory species of the subfamily Asopinae [1–4]. Phytophagous pentatomids are a major concern for many agricultural systems, damaging crops through direct feeding and by transmitting plant pathogens [1,3], whereas predatory pentatomids are considered to be beneficial, as they prey on various insect pest species [5]. This review focuses on phytophagous stink bugs.

Chemical control programs have been the principal approach for managing stink bugs [6,7]. Despite these management efforts, populations continue to surpass damage thresholds, and the development of insecticide resistance has been observed [6]. To reduce the use of insecticides, behaviourally based management and monitoring programs need to be further developed. These require knowledge of the biological and behavioural traits of stink bugs [5–8].

The Pentatomidae have hemimetabolous development with an egg, nymphal, and adult stage, and can have multiple generations per year (Figure 1). Five nymphal instars occur in the Pentatomidae. The first instars are generally gregarious, inactive, and remain on top of or near the eggshells. The first instars do not feed on host plants but acquire

specific symbionts by ingesting secretions covering the shells. These bacterial symbionts are thus vertically transmitted from females via the egg surface [9–11]. The acquisition of these symbionts plays a beneficial role in the fitness of the insect host, especially in survival and development, and detoxification of their food [12–14]. As the nymphs pass through instars, they gradually and progressively disperse. The size of the nymphs increases throughout the different stadia and the growth of the wing pads starts during the fourth and fifth instar. During the adult stage, the bug possesses fully developed wings; reproduction begins after the passage to adulthood and completion of sexual maturation, with typical precopulatory and copulatory behaviours combining chemical and vibrational signals [6].

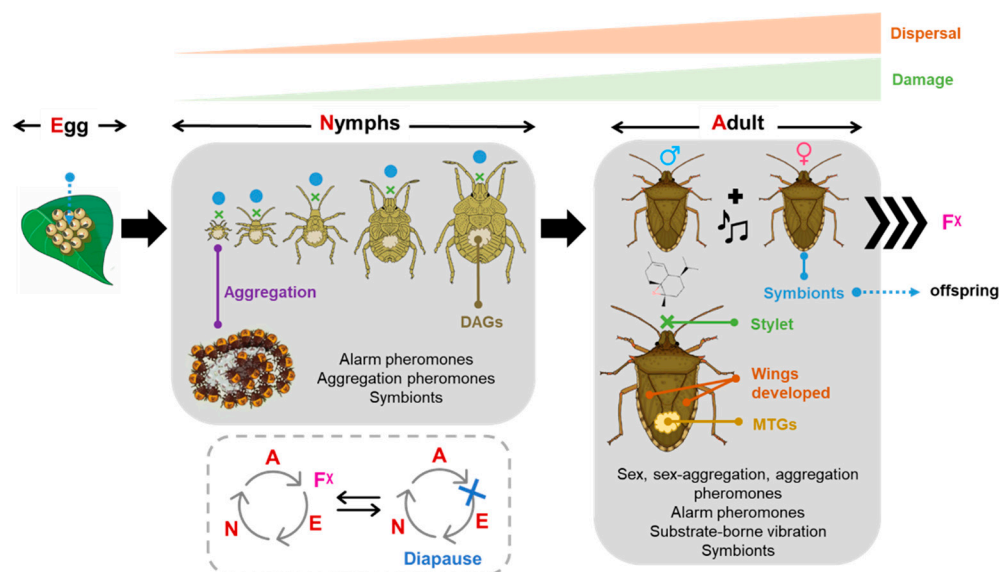


Figure 1. Schematic drawing showing various traits associated with the life cycle of the Pentatomidae, which can be targeted for pest management. The chemical molecule represents the sex, sex–aggregation or aggregation pheromones, and the musical note represents the vibrational signals (F^x: number of generations; DAGs: dorsal abdominal scent glands; MTGs: metathoracic scent glands).

Pentatomids are well known for producing a wide variety of chemicals during their life cycle, such as sex, alarm, and aggregation pheromones [15–17]. Sex–aggregation pheromones are defined as compounds that are produced by one sex that attract both sexes for mating purposes, whereas sex pheromones attract only the opposite sex. Compounds that are produced by one sex that attract both sexes, including nymphs, for behaviours other than mating are considered aggregation pheromones [18]. In all pentatomid species for which pheromones (sex, sex–aggregation, aggregation) have been identified, males are responsible for production [16,17]. However, the distinction between sex–aggregation and aggregation pheromone can be difficult and requires rigorous bioassays demonstrating the behavioural responses of conspecifics to specific compounds [16]. Thus, in cases where there was not sufficient empirical work to determine which designation is correct, we refer to these as aggregation pheromones.

Traits other than chemical signals can also be considered when developing control strategies (Figure 1). Recent advances in understanding the vertical transmission of symbionts and manipulation of transmission offer new possibilities for controlling Pentatomidae [19]. For example, the removal of symbionts can lead to a strong negative effect on insect fitness [10,12,20–24]. Furthermore, several studies have been conducted on vibratory communication (closely associated with stink bug courtship) in order to develop pest control methods that exploit this communication mode [25].

Recent advances in understanding stink bug biology through their symbionts, pheromones, and other traits (e.g., colour vision, flying behaviour) offer new opportunities for the development of integrated pest management tactics. However, their utilisation is still

limited to a few stink bug species, and the complexity and time required to develop commercial applications of these behavioural tools remains a major challenge. Here, we summarise the currently available knowledge of the mating behaviour, alarm and aggregation pheromones, and symbionts within the Pentatomidae, with emphasis on their potential to be exploited for the development of stink bug pest management tactics.

2. Vibratory and Pheromone Signals in Stink Bug Mating

Substrate-borne vibrations are prevalent in insects [26–29]. In the Pentatomidae, these have been described in 36 species [30,31]. Substrate-borne communication has been well studied in the model species *Nezara viridula* (L.), where it is closely associated with their mating behaviour [30,32].

In stink bugs, mating behaviour is mediated by chemical and vibrational signals and involves long-range mate location and close-range courtship behaviour [30–34]. This bimodal communication starts at long distance with the emission of the male pheromone which attracts females towards the chemical signal and can result in aggregations of females on the same host plant or in areas surrounding the calling males [33]. At close range, the male pheromone stimulates the female to start her vibratory calling (e.g., Female Calling Song, FCS). The FCS triggers the male to search for the calling female [35,36]. The male walks on the plant towards the source of vibration and answers with his calling and courtship songs (e.g., Male Calling Song, MSC and Male Courtship Song, MCrS) [37]. The rhythmic FSC repetitions create a vibrational signal which guides the male towards the female, even when complex plant architecture is involved [36]. The vibratory signals of stink bugs are characterised by low frequencies (around 100 Hz) with narrow spectral peaks which are well tuned with the resonance properties of host plants, which act as low-pass filters [38,39]. These characteristics enable short-range communication and facilitate mate location [35,36].

Interference of stink bug communication using substrate-borne vibratory signals has been explored for pest management [25,40]. For example, a few studies have been conducted that explore the use of vibratory signals as a repellent, a mating disruption strategy, and for population monitoring. A vibratory disturbance of 100 Hz can affect communication between males and females of *N. viridula* by decreasing the number of males responding to female calling songs and by reducing the number of males responding to the courtship song [41]. Although disturbance did not affect the production of female calling songs or the location of females by males, females changed the rhythm and the dominant frequency of their songs to avoid overlap with artificial external disturbances. Similar results have also been observed in *Euchistus heros* (F.) [42]. Although the properties of the plant tissues strongly influence vibrational transmission, other factors can also significantly disturb transmission processes. Several studies demonstrated that biotic noise could modify the temporal structure, the frequency, or the amplitude of signals and negatively affect the behavioural responses of conspecifics [41,43,44]. Other abiotic factors such as wind and raindrops have also been suggested as a source of disturbance for stink bug vibratory communication [30].

Substrate-borne vibrations not only affect communication between partners but can also affect the reproductive behaviour of insects [45]. Pure tone vibrations (75–200 Hz) mechanically transmitted to plants significantly (>90%) decreased copulation in *E. heros* by inhibiting male calling and searching for females [46]. In addition, a reduction in fecundity and fertility has also been observed in females exposed to background noise for 24 h, despite the fact that they had been mated. Although this hypothesis remains to be tested, the authors suggested that the background noise could induce stress, resulting in shorter copulation periods and/or a reduction in the efficiency of sperm transfer. A recent study [47] investigated the potential of female rival signals for mating disruption in *E. heros*, *Chinavia ubica* (Rolston) and *Chinavia impicticornis* (Stål). In the presence of rival female signals, fewer males were able to reach females, and a significant reduction in copulating

pairs was observed. These results suggest that female rival signals could be used to disrupt mating of stink bugs.

Indirect effects of vibratory signals, such as the attraction of parasitoids and predators, may also contribute to the regulation of stink bug populations. Predators and parasitoids can use stimuli associated with stink bug prey/hosts to search for and locate stink bugs [26]. For example, the egg parasitoid *Telenomus podisi* (Ashmead) has been shown to be attracted to vibratory signals from *E. heros* [48,49]. Laumann et al. [49] demonstrated that *T. podisi* remained for longer on leaves vibrated by natural or artificial female songs, compared to non-vibrated leaves or leaves vibrated by male songs. These studies suggest that the in situ behavioural response to host female vibratory signals increases the probability of *T. podisi* locating host eggs. Nevertheless, attraction of natural enemies to synthetic vibrational and pheromonal signals does not necessarily imply parasitism. An additional concern is that negative consequences for biological control (i.e., attracting and misleading with no reward) can also be observed [50]. Thus, caution needs to be taken when using these stimuli in pest control.

The detection and monitoring of insect communication with automatic acoustic devices has been investigated in a few species [25,51,52]. For example, algorithms using Gaussian mixture models (GMM), or probabilistic neural networks (PNN) have been used for the identification and detection of Cicadidae [53], as well as the vibratory signals produced by red palm weevils [54]. In both studies, the mathematical models were over 94% accurate. Other methods such as linear prediction cepstral coefficient feature vectors and a multilayer perceptron classifier have also shown potential for vibratory discrimination of leafhoppers [55]. Similarly, the incorporation of an automatic acoustic device has been proposed for stink bug identification and detection [56]. PNN and GMM models were able to identify and discriminate the vibratory signals of *Euschistus servus* (Say) and *N. viridula* species from incidental noise (e.g., locomotion, feeding or tunnelling) with an accuracy, in most cases, higher than 90%. Although this study was conducted under laboratory conditions, thus excluding abiotic noises such as wind and vibrations by other organisms, the results suggest that the method has potential for monitoring stink bugs and improving levels of control realised by pest management programmes.

Sex and aggregation pheromones have been identified in approximately 50 species where the pattern of responses is divided between those that appear to attract only females, and those that attract both sexes, as well as nymphs when this has been tested (Table 1) [16,17,57,58]. The only exception is for *Piezodorus hybneri* (Westwood); its pheromone appears to be bifunctional, acting either as a sex or aggregation pheromone, depending on the time of the year [59,60]. In comparison, sex–aggregation pheromones have only been identified for a few species (Table 1). To date, only male stink bugs have been observed to produce these pheromones. Sex pheromones have been described in various Pentatomidae tribes and consist, in general, of a blend of at least two compounds. For example, among the An-testinii tribe, methyl (E,Z,Z)-2,4,6-decatrienoate was identified as the main sex pheromone compound in *Thyanta perditor* (F.) and *Thyanta pallidovirens* (Stål), along with the sesquiterpenes (+)- α -curcumene, (–)-zingiberene, and (–)- β -sesquiphellandrene [61,62]. Similar methyl esters have been reported to act as sex pheromones in other tribes. In the Carpocorini tribe, methyl 2,6,10-trimethyltridecanoate has been identified as a sex pheromone for *E. heros* [63–65], *Euschistus obscurus* (Palisot) [63,64] and *Agroecus griseus* (Dallas) [66]. In Edessinae, the only known sex pheromone is for *Edessa meditabunda* (Fabricius), where methyl 4,8,12-trimethylpentadecanoate and methyl 4,8,12-trimethyltetradecanoate constitute the major and minor compounds in this species, respectively [67]. Other compounds such as sesquiterpenoids have been reported to act as sex pheromones in Pentatomidae. For example, the sex pheromone of *Tibraca limbativentris* (Stål) is a mixture (7:3) of two isomers of 1,10-bisaboladien-3-ol (i.e., zingiberenol) [68,69], where the main compound alone and the mixture are attractive to females in the field [70]. An isomer of zingiberenol has also been described in the sex pheromone of *Oebalus pœcilus* (Dallas) [71].

Table 1. Defensive compounds and alarm pheromones (D), sex pheromones (S), aggregation pheromones (Ag), and sex–aggregation pheromones (SAg) identified in phytophagous Pentatomidae (list adapted and updated from Borges and Blassioli-Moraes [17] and Weber et al. 2018 [16]. For each of these pheromones, information regarding the life stage and the sex emitting the pheromones is indicated. For the alarm pheromones,  and  indicate adults and nymphs, respectively, whereas the sex, sex–aggregation and aggregation pheromones are emitted by males (“M”). Information regarding the life stage and the sex (males (M); females (F); nymphs (n)) of responder(s) to the alarm, sex, aggregation, or sex–aggregation pheromones are also listed if behavioural studies have been conducted, with red, green, and grey indicating the degree of response (red = does not respond to the pheromone, green = responds to the pheromone, grey = response has not been tested). In terms of compounds listed for each pheromone, only the most abundant compound is listed for the alarm pheromones, except for species in which bioassays have been conducted. For these species, all the compounds (as well as the instar and sex emitter) are listed, and the most abundant compound is underlined. For the sex pheromones, aggregation pheromones and sex–aggregation pheromones all compounds assayed are listed.

Pheromone ¹	Emitter	Responder	Compounds ²	References
Dinidorinae: Dinidorini				
<i>Aspongopus</i> sp.				
D		(E)-2-Hexenal		[72]
<i>Coridus janus</i>				
D		(E)-2-Hexenal		[73]
<i>Cyclopelta siccifolia</i>				
D		(E)-2-Hexenal		[74]
Discocephalinae: Discocephalini				
<i>Antiteuchus innocens</i>				
D		Undecane (M, F)		[75]
D		Undecane (1st–5th instar)		[75]
Discocephalinae: Ochlerini				
<i>Lincus malevolus</i>				
D		Undecane (M, F); (E)-4-oxo-2-decenal (F)		[76]
<i>Lincus spureus</i>				
D		Undecane (M, F)		[76]
D		Undecane (5th instar)		[77]
Edessinae				
<i>Edessa meditabunda</i>				
S	M	M, F, n	Methyl 4,8,12-trimethylpentadecanoate	[67,78]
D			Undecane (1st–2nd instar)	[79]
<i>Edessa rufomarginata</i>				
D			Undecane	[80]
Pentatominae: Aeliini				
<i>Aelia fieberi</i>				
D			(E)-2-Decenal (M, F)	[81]
Pentatominae: Antestiini				
<i>Plautia stali</i>				
Ag	M	M, F, n	Methyl (2E,4E,6Z)-2,4,6-decatrienoate	[82]
<i>Thyanta custator accera</i>				
?	M		Methyl (2E,4Z,6Z)-2,4,6-decatrienoate; (+)- α -curcumene; (–)-zingiberene; (–)- β -sesquiphellandrene	[61]

Table 1. Cont.

Pheromone ¹	Emitter	Responder	Compounds ²	References
<i>Thyanta pallidovirens</i>				
S	M	M, F, n	Methyl (2E,4Z,6Z)-2,4,6-decatrienoate; (+)- α -curcumene; (–)-zingiberene; (–)- β -sesquiphellandrene	[61,83]
D			Tridecane (M, F)	[61]
D			Tridecane (1st–2nd instar)	[84]
<i>Thyanta perditor</i>				
S	M	M, F, n	Methyl (2E,4E,6Z)-2,4,6-decatrienoate	[62]
D			Tridecane (M, F)	[62]
D			Tridecane (1st–2nd instar)	[79]
Pentatominae: Bathycoliini				
<i>Bathycolia distincta</i>				
D		M, F, n	(E)-2-Hexenal; 4-oxo-(E)-2-hexenal; (E)-2-decenal; (E)-2-decenyl acetate; tridecane; dodecane	[85]
Pentatominae: Cappaeini				
<i>Caura rufiventris</i>				
D			Tridecane	[72]
<i>Halyomorpha halys</i>				
Ag	M	M, F, n	Isomers of murgantiol (10,11-Epoxy-1- bisabolen-3-ol)	[86,87]
D			Tridecane (M, F)	[86,88–91]
D			Tridecane (3rd–5th instar)	[86,88]
D	M, F, n	M, F, n	(E)-2-Decenal	[92]
<i>Veterna patula</i>				
D			Tridecane	[72]
Pentatominae: Carpocorini				
<i>Agroecus griseus</i>				
S	M	M, F, n	Methyl 2,6,10 trimethyltridecanoate	[66]
D			Tridecane	[66,93]
D			Tridecane (1st–5th instar)	[66]
<i>Carpocoris fuscispinus</i>				
D			Tridecane (M, F)	[94]
<i>Cosmopepla bimaculate</i>				
D			Tridecane (M, F)	[95]
<i>Euschistus biformis</i>				
D			Tridecane	[96]
<i>Euschistus conspersus</i>				
Ag	M	M, F, n	Methyl (2E,4Z)-2,4-decadienoate; methyl (2E,4E)-decadienoate; geranylacetone	[97]
D			Tridecane (1st–2nd instar)	[84]
<i>Euschistus heros</i>				
S	M	M, F, n	Methy 2,6,10 trimethyltridecanoate; methyl (2E,4Z)-2,4-decadienoate; methyl 2,6,10-trimethyldodecanoate	[63,65,98]
D		M, F, n	(E)-2-Hexenal; 4-oxo-(E)-2-hexenal; hexenoic acid; (E)-2-hexenyl acetate; 2-octenal; (E)-2-octen-1-ol; nonanal; (E)-2-heptenyl acetate; (E)-2-decenal; (E)-2-octenyl acetate; tridecane; tetradecane; (E)-2-decenyl acetate; tridecanal; tetradecanal; pentadecane	[99–101]
D			Tridecane (1st–4th instar); tetradecanal (5th instar)	[79,96,99]

Table 1. Cont.

Pheromone ¹	Emitter	Responder	Compounds ²	References
?	M		<i>Euschistus ictericus</i> Methyl (2E,4Z)-2,4-decadienoate; methyl (2E,4E)-decadienoate	[97]
			<i>Euschistus obscurus</i> Methyl (2E,4Z)-2,4-decadienoate; methyl 2,6,10-trimethyltridecanoate; methyl 2,6,10-trimethyldodecanoate; methyl (2E,4E)-decadienoate	[63,64]
Ag	M	M, F, n	<i>Euschistus politus</i> Methyl (2E,4Z)-2,4-decadienoate; methyl (2E,4E)-decadienoate	[97]
Ag	M	M, F, n	<i>Euschistus servus</i> Methyl (2E,4Z)-2,4-decadienoate; methyl (2E,4E)-decadienoate; geranylacetone	[97]
D			(E)-2-Hexenal	[102]
Ag	M	M, F, n	<i>Euschistus tristigmus</i> Methyl (2E,4Z)-2,4-decadienoate; geranylacetone; decanoic acid	[97]
D			Hexanal	[102]
D			Tridecane (1st–5th instar)	[79]
D			<i>Dichelops melacanthus</i> Tridecane (M, F); 4-oxo-(E)-2-hexenal (M, F)	[99,100,103]
D			4-Oxo-(E)-2-hexenal (5th instar)	[99]
Ag	M	M, F, n	<i>Dolycoris baccarum</i> α -Bisabolol *; trans- α -bergamotene *; (S)- β -bisabolene *	[104]
D			Tridecane (M, F); hexenal	[105,106]
Ag	M	M, F, n	<i>Mormidea v-luteum</i> Isomers of zingiberenol (cis-(1S,4R,1'S)-zingiberenol; trans-(1R,4R,1'S)-zingiberenol); murgantiol *; sesquiperitol	[107]
S	M	M, F, n	<i>Oebalus poecilus</i> Isomer of zingiberenol ((1S,4R,1'S)-4-(1',5'-dimethylhex-4'-enyl)-1-methylcyclohex-2-en-1-ol)	[71]
D			<i>Oebalus pugnax</i> Tridecane	[108]
S	M	M, F, n	<i>Tibraca limbativentris</i> Isomers of zingiberenol ((3S,6S,7R)-1,10-bisaboladien-3-ol; (3R,6S,7R)-1,10-bisaboladien-3-ol); sesquiperitol	[68,69]
Pentatominae: Eysarcorini				
Ag	M	M ⁽³⁾ , F, n	<i>Eysarcoris lewisi</i> Sesquisabinen-1-ol	[109,110]
?	M		<i>Eysarcoris parvus</i> (Z)-exo- α -Bergamotene	[111]
Pentatominae: Halyini				
D			<i>Apodiphus amygdali</i> 4-Oxo-(E)-2-hexenal	[112]
D			4-Oxo-(E)-2-hexenal (1st–5th instar)	[112]
D			<i>Brochymena quadripustulata</i> (E)-2-Hexenal	[113]
D			<i>Erthesina fullo</i> Tridecane (M, F)	[114]
D			<i>Poecilometris strigatus</i> (E)-2-Hexenal; (E)-2-octenal	[115]

Table 1. Cont.

Pheromone ¹	Emitter	Responder	Compounds ²	References
Pentatominae: Myrocheini <i>Delegorguella lautus</i>				
D		Tridecane		[72]
Pentatominae: Nezarini				
<i>Chinavia aseada</i>				
?	M		<i>cis</i> -(Z)-Bisabolene epoxide; <i>trans</i> -(Z)-bisabolene epoxide; (Z)- α -bisabolene	[116]
D			(Z)-4-Nonenal	[102]
D			Tridecane (1st–2nd instar)	[79]
<i>Chinavia hilaris</i>				
S	M	M, F, n	<i>cis</i> -(Z)-Bisabolene epoxide; <i>trans</i> -(Z)-bisabolene epoxide	[117]
D			(E)-2-Hexenal	[102]
D			Tridecane (1st–2nd instar)	[84]
<i>Chinavia impicticornis</i>				
S	M	M, F, n	<i>trans</i> -(Z)-Bisabolene epoxide; <i>cis</i> -(Z)-bisabolene epoxide	[118]
D			Tridecane (M); 4-oxo-(E)-2-hexenal (F)	[99,118]
D			4-Oxo-(E)-2-hexenal (5th instar)	[99]
<i>Chinavia marginata</i>				
?	M		<i>trans</i> -(Z)-Bisabolene epoxide; <i>cis</i> -(Z)-bisabolene epoxide	[119]
<i>Chinavia ubica</i>				
S	M	M, F, n	<i>trans</i> -(Z)-Bisabolene epoxide; <i>cis</i> -(Z)-bisabolene epoxide	[118]
D			Tridecane (M, F)	[99,100,118]
D			4-Oxo-(E)-2-hexenal (5th instar)	[99]
<i>Chlorochroa ligata</i>				
S	M	M ⁽³⁾ , F, n	Methyl (E)-6,2,3-dihydrofarnesoate; methyl farnesoate; methyl (E)-5-2,6,10-trimethyl-5,9-undecadienoate	[120,121]
D			Tridecane (M, F)	[122]
D			Tridecane (5th instar)	[122]
<i>Chlorochroa sayi</i>				
S Ag	M	M, F, n	Methyl geranate; methyl citronellate; methyl farnesoate	[120,123]
D			Tridecane (M, F)	[122]
D			4-Oxo-(E)-2-hexenal (5th instar)	[122]
<i>Chlorochroa uhleri</i>				
Ag	M	M, F, n	Methyl geranate; methyl citronellate; methyl farnesoate	[120,121]
D			Tridecane (M, F)	[122]
<i>Nezara antennata</i>				
?	M		(Z)- α -Bisabolene; <i>trans</i> -(Z)-bisabolene epoxide; <i>cis</i> -(Z)-bisabolene epoxide; (E)-nerolidol	[116]
D			(E)-2-Decenal (M, F)	[81]
<i>Nezara viridula</i>				
Ag	M	M, F, n	<i>trans</i> -(Z)-Bisabolene epoxide; <i>cis</i> -(Z)-bisabolene epoxide; (Z)- α -bisabolene; (E)-nerolidol	[98,116,124–128]
D		M, F, n	Tridecane; (E)-2-decenal; (E)-2-decenyl acetate; (E)-2-hexenal; (E)-2-hexenyl acetate; dodecane	[124,129–131]
D		n	4-Oxo-(E)-2-decenal (1st instar); tetradecane (2nd instar); tridecane (2nd–5th instar); (E)-2-hexenal	[79,84,132,133]

Table 1. Cont.

Pheromone ¹	Emitter	Responder	Compounds ²	References
<i>Palomena viridissima</i>				
D			Unknown carbonyl compound	[106]
Pentatominae: Pentatomini				
<i>Pallantia macunaima</i>				
S	M	M, F, n	(6R,10S)-Pallantione	[134]
D			Tridecane (M, F)	[93,135]
D			(E)-4-Oxo-2-hexenal (1st instar) Tridecane (2nd–5th instar)	[135]
<i>Pellaea stictica</i>				
P	M	M, F, n	Isomers of 2,4,8,13-tetramethyltetradecan-1-ol	[136,137]
D			(Z)-4-tridecene	[93]
<i>Rhaphigaster nebulosa</i>				
D			Tridecane (M, F)	[138]
Pentatominae: Piezodorini				
<i>Piezodorus guildinii</i>				
S	M	M, F, n	(7R)-(+)- β -sesquiphellandrene	[139]
D			(E)-4-oxo-2-hexenal (M, F)	[99,140]
D			Unknown (5th instar)	[99]
<i>Piezodorus hybneri</i>				
SAg	M	M, F, n	β -Sesquiphellandrene *; (R)-15-hexadecanolide; methyl (Z)-8-hexadecenoate	[59,60]
<i>Piezodorus teretipes</i>				
D			(E)-2-Hexenal	[141]
Pentatominae: Rhynchocorini				
<i>Biprorulus bibax</i>				
SAg	M	M, F, n	Biprolure ((3R,4S',1'E)-3,4-bis(1'-buteny1)tetrahydro-2-furano; linalool; isomers of farnesol; nerolidol	[142–146]
D			Tridecane	[147]
<i>Vitellus insularis</i>				
D			Tridecane	[148]
Pentatominae: Strachiini				
<i>Bagrada hiliaris</i>				
Ag	M	M, F, n	(E)-2-octenyl acetate	[149,150]
<i>Eurydema oleraceum</i>				
D			(E)-2-Octenal; (E)-2-hexenal	[102,151]
<i>Eurydema pulchrum</i>				
D		n	(E)-2-Hexenal (1st–3rd instar); tridecane	[133]
<i>Eurydema rugosa</i>				
Ag	M	M, F, n	(E)-2-Hexenal	[152]
D		n	(E)-2-Hexenal (2nd–3rd instar); tridecane	[133]
<i>Eurydema ventrale</i>				
D			(2E,6E)-Octadienedial; (E)-2-octenal	[102,151]
<i>Murgantia histrionica</i>				
Ag	M	M, F, n	Isomers of murgantiol ((3S,6S,7R,10S)-10,11-epoxy-1-bisabolen-3-ol; (3S,6S,7R,10R)-10,11-epoxy-1-bisabolen-3-ol)	[153–155]
D			(2E,6E)-Octadienedial	[151]

Table 1. Cont.

Pheromone ¹	Emitter	Responder	Compounds ²	References
			Podopinae: Graphosomatini	
			<i>Graphosoma lineatum</i>	
D			1-phenanthrenecarboxylic acid (M); tridecane (F); (E)-2-decenal (F)	[156,157]
			<i>Graphosoma rubrolineatum</i>	
D			(E)-2-Decenal (M, F)	[81]
			<i>Graphosoma semipunctatum</i>	
D			Tridecane (M, F)	[158]
			Podopinae: Podopini	
			<i>Scotinophara lurida</i>	
D			(E)-2-Decenal (M, F)	[81]

¹ For compounds where a pheromonal functionality has been assigned but no behavioural study has been conducted, the compounds were isolated from a specific structure where no other function has been observed.

² The most abundant compound of the alarm pheromone can vary among studies, and in these cases, each most abundant compound is listed. ³ Males attraction is unclear. Field tests were suggestive, and the data were limited to confirm the attraction. ? Indicates that it is a putative pheromone. No behavioural bioassays have been conducted to confirm the functionality. * Absolute configuration has not been determined.

In stink bug sex pheromones, the ratio of components appears to be important, with ratios that accurately reflect those produced by males being most attractive. In addition, they are species specific, and can vary with age, physiological status, or among populations [159–162]. For example, Endo et al. (2012) [159] showed that the ratios of the three components in pheromone emissions of *P. hybneri* are variable among individuals and over time. Variation in the pheromone blend has also been observed among *N. viridula* individuals from different geographic populations [116] and within a single population in Australia [162]. Thus, determining the ratio as well as the absolute configuration of the pheromones must be considered when optimizing attractants for monitoring and management purposes.

Field studies have shown that the number of stink bugs recovered in traps baited with sex pheromone does not always correlate with the density of the field population [163]. This is possibly because of sub-optimal trap design. Studies have shown that more pentatomids are observed in the vicinity of traps than inside the traps [97,143], a possible indication that stink bugs require vibrational signals to locate and enter the trap. A trap incorporating olfactory and acoustic stimuli was recently tested in the field for *Halyomorpha halys* (Stal) and demonstrated that the use of vibrations and pheromones can increase the efficacy of pheromone traps [164,165].

3. Alarm Pheromones

Stink bugs are well known for producing odoriferous compounds that play a role in chemical communication and defence [16,17,57,58,96,166]. These volatile compounds are produced and stored in specific glands (DAGs—dorsal abdominal scent glands and MTGs—metathoracic scent glands) according to their life stage (Figure 1) [58,122,127,132,135,166–169]. These glands secrete volatiles through the external efferent thoracic system when insects are disturbed [101,170] and can act as allomones and serve as a defence against natural enemies and microorganisms or mediate alarm or aggregation behaviours among conspecifics [16,96,100]. These compounds are highly conserved across species and are mostly represented by short-chain aliphatic aldehydes, alcohols, esters, alkanes, terpenes, and phenolics (Table 1) [16,57,58,96,99,166].

The use of semiochemicals for behavioural manipulation of natural enemies has been proposed as a tool to improve biological control [171,172]. Natural enemies can exploit the alarm compounds produced by pentatomids as kairomones to locate their hosts [91,173–175]. For example, the egg parasitoids *T. podisi*, *Trissolcus basal* (Wollaston) and *Trissolcus japonicus*

(Ashmead) are attracted to (*E*)-2-hexenal, (*E*)-2-decenal, and tridecane, defensive compounds released by their preferred hosts, *E. heros* [175], *N. viridula* [176], and *H. halys* [91], respectively. Each species seems to be able to recognise the compounds produced by their preferred hosts [174]. *Trissolcus basalis* responded preferentially to (*E*)-2-decenal and 4-oxo-(*E*)-2-hexenal, two compounds in *N. viridula* secretions, whereas *T. podisi* preferred (*E*)-2-hexenal and 4-oxo-(*E*)-2-hexenal compounds in the secretions of *E. heros*. Similarly, in field experiments, traps baited with alarm pheromone compounds such as (*E*)-2-hexenal + α -terpineol and 4-oxo-(*E*)-2-hexenal + linalool + tridecane captured the tachinid flies *Euclytia flava* (Townsend) and *Hemyda aurata* (Robineau-Desvoid) [166,177]. Other predators such as chloropid and milichiid flies, as well as spiders, have also been reported to be attracted to alarm pheromone compounds of Pentatomidae. Aldrich and Barros [178] showed the attraction of male crab spiders *Xysticus ferox* (Hentz) to (*E*)-2-octenal and (*E*)-2-decenal and Eisner et al. [179] demonstrated the attraction of chloropid and milichiid flies to chemicals released by crushed Pentatomidae. These flies are also attracted to defensive compounds such as (*E*)-2-hexenal, (*E*)-2-octenal, (*E*)-2-decenal, and hexanal [178,179]. These results suggest that stink bug natural enemies use scent gland secretions to locate their preferred hosts. However, to date, only one study has been conducted on Pentatomidae with the aim of attracting parasitoids with synthetic alarm pheromone to enhance parasitism of stink bug eggs [175]. Areas treated with (*E*)-2-hexenal showed an increase in the abundance of Telenominae with greater intensity of parasitism and an increase in egg predation. The utilisation of alarm pheromone compounds to improve the action of natural enemies has potential, but it needs further study to develop release devices that release effective doses. In addition, effects on natural enemy foraging and host location may be dose dependent, with some doses positively affecting associated behaviours and others negatively. Further research is needed to determine if release devices and dosages can be developed that enhance positive effects without negative effects. The combination with other semiochemicals or with trap crops could also help. In addition to the volatiles emitted from the scent glands, parasitoids of the Pentatomidae can use other cues when searching for eggs, such as volatiles from plants [180,181], volatiles from eggs [182], sex pheromones [177,183], chemicals left by stink bug footprints [184,185], vibratory signals [49], and visual cues [186]. Thus, specific knowledge about the stimuli that mediate interactions between natural enemies and stink bugs is required to improve the effectiveness of applications of semiochemicals in integrated pest control. Strategies such as attract and reward [187,188] or push–pull tactics [189–191], which combine alarm pheromone compounds with habitat manipulation, could improve the action of natural enemies of Pentatomidae.

4. Aggregation Behaviour and Pheromones in Pentatomidae

Aggregation behaviour is a common phenomenon in Heteroptera [192]. In Pentatomidae, aggregations are first observed when stink bug eggs hatch, but also later by adults (Figure 1). These two separate phenomena are likely mediated by entirely different cues and signals. The maintenance of aggregations of first instar pentatomids is mediated by a combination of tactile cues and chemical compounds, which are normally considered as defensive compounds in later instars [79,84,152]. It has been shown that these defensive compounds can also mediate heterospecific aggregation behaviour of first instars [152], suggesting that compounds which mediate aggregation of first instar stink bugs may be conserved among species. In adults, aggregations can be observed at different times of the year. For example, *H. halys* forms aggregations in both summer and winter during diapause. In the summer, aggregations are mediated by aggregation pheromones which attract both sexes and nymphs [87]. The identity of the stimuli that mediate adult overwintering aggregations remains unknown. Tomaya et al. [193] suggested that tactile and olfactory stimuli might be involved, whereas Bedoya et al. [194] demonstrated that the overwintering aggregations are not mediated via vibration signals.

Aggregation pheromones produced by stink bugs are complex and include saturated hydrocarbons, methyl esters, terpenes, and terpenoids [16,17,57,195]. Although these pheromones

are species specific, some similarities among Pentatomidae occur (Table 1) [16,17]. For example, within the Carpocorini tribe, methyl (*E,Z*)-2,4-decadienoate (MDD) has been identified as an aggregation pheromone in five *Euschistus* species including *E. servus*, *Euschistus tristigmus* (Say), *Euschistus politus* (Uhler), *Euschistus conspersus* (Uhler), and *Euschistus ictericus* (L.) [97]. Other methyl esters have also been reported as aggregation pheromones, such as methyl (*E,E,Z*)-2,4,6-decatrienoate (MDT), methyl (*E*)-5-2,6,10-trimethyl-5,9-undecadienoate, and methyl (8*Z*)-8-hexadecenoate produced by *Plautia stali* Scott [82], *Chlorochroa* sp. [121] and *P. hybneri* [59], respectively. Another example is the presence of sesquiterpenoids such as murgantiol (10,11-Epoxy-1-bisabolen-3-ol), where isomers have been identified in *Murgantia histrionica* (Hahn) [153,154], *Halyomorpha halys* (Say) [87], and *Mormidea v-luteum* (Lichtenstein) [107]. Other similar sesquiterpenes and sesquiterpenoids (α -bisabolol, *trans*- α -bergamotene, β -bisabolene and zingiberenol) have also been detected as sex and aggregation pheromones in various Pentatomidae species [68,69,71,104,124]. A recent study revealed that these similarities of sesquiterpene compounds observed in Pentatomidae are linked to a conserved gene function in terpene biosynthesis [196]. Furthermore, attraction to heterospecific pheromones has been observed in Pentatomidae [16]. For example, the pheromone of the Asian species *P. stali* (MDT) attracts other pentatomids such as *Glaucias subpunctatus* (Walker), *H. halys*, and the North American species *C. hilaris*, despite the fact that *H. halys* and *C. hilaris* do not produce this compound. In fact, trap captures of *H. halys* can be synergised by the addition of MDT [197] and this has been observed in different populations globally [198]. Other examples of cross attraction can, however, be attributed to some overlap in pheromone chemistry, as is the case between *Nezara antennata* (Scott) and *N. viridula*, which share their two compounds [116].

Aggregation pheromones have been used in stink bug management to monitor the phenology and abundance of populations. For *H. halys*, the discovery of its aggregation pheromone has facilitated the development of an extensive monitoring program across the United States to define its infestation range and damage to crops [199]. Pyramid traps baited with MDD lures have been used to monitor populations of *E. servus* and *E. tristigmus* in apple and peach orchards [200,201] and for *E. conspersus* on tomatoes [202]. Nevertheless, studies have shown that although pheromone-baited traps can attract stink bugs to their vicinity, the number of captures often does not correlate with populations in the field [159]. For example, in apple and peach orchards, only 50% of *E. servus* attracted within 10 m of traps were captured [201], whereas traps placed on the border of tomato plantations attracted only *E. conspersus* females [159].

The use of stink bug aggregation pheromones in combination with host plants as trap crops has also been examined. A study with *E. conspersus* has shown that if MDD lures are deployed directly on a host plant, such as mullein plants, higher numbers of bugs are attracted and retained on these baited plants [203], demonstrating the potential of using trap crops for stink bug control. This concept of combining aggregation pheromone with host plants was later used on *P. stali*, where eggplants were baited with pheromones and treated with a high rate of systemic insecticide to kill attracted bugs [204]. However, in this study, significant damage to adjacent trees of the main persimmon crop was observed due to a spillover of attracted stink bugs. More recently, this concept was also applied in commercial apple orchards to control *H. halys* [205–207]. Morrison et al. [206] observed better retention of attracted bugs in the area surrounding lures when they were deployed in host plants, suggesting that deployment protocol can limit spillover and damage to untreated plants. Although this approach looks promising for managing *H. halys* and may be a viable option for other stink bug species [207], important economic considerations still exist. In fact, Morrison et al. [207] compared the cost of the attract and kill strategy and grower standard treatment for insect management. Although yield generated by the attract and kill treatments were higher than the standard treatments, the cost of the attract and kill treatments was seven to ten times higher, and as such remains the biggest limitation of this type of management approach.

5. Symbiotic Relationships and Vertical Transmission of Bacteria in Pentatomidae

Symbiotic interactions between bacteria and insects are ubiquitous [208,209] and can be crucial to insect fitness [210,211]. They play an important role in the digestion and detoxification of food, and provide essential nutrients for development, such as amino acids and vitamins often lacking in xylem/phloem sap [210–212]. Gut symbionts also provide protective functions to their host against pathogens and parasites [210,211].

Various bacterial taxa have been identified as Heteroptera symbionts, including Actinobacteria, Alphaproteobacteria, Bacteroidetes, Betaproteobacteria, Firmicutes and Gammaproteobacteria [213], with Gammaproteobacteria representing the most dominant taxa within the Heteroptera suborder [20,214,215]. These symbionts are essential to the growth, development, and survival of the host [215]. The bacteria *Wolbachia* has also been recorded in several pentatomid species; nevertheless, its role in host biology and, as such, its symbiotic impact has been poorly investigated [216]. The microbial gut symbionts among the Pentatomidae have been well described [10,15,19]. The Pentatomidae primarily harbour bacteria related to the *Pantoea* genus (Enterobacteriaceae family) in their midgut [217]. Pentatomidae are sap feeders and have a stylet (i.e., mouthparts modified from mandibles and maxillae) which allows them to pierce into plant tissue to suck xylem/phloem, and these symbionts provide their hosts with nutrients missing in their diets [218,219] and help to detoxify and deactivate plant chemical defences [220]. Reduced genome size (<1 Mb), high AT content and a fast evolutionary rate have been generally observed in bacterial symbionts of stink bugs [218,219,221,222].

Symbionts in the Pentatomidae are vertically transmitted by females to offspring during or after oviposition via physical deposition upon egg masses (e.g., egg smearing) [9,11,223,224]. After hatching, the first instars aggregate on the egg surface and ingest the bacteria by probing the egg chorions [224–226]. The symbionts of Pentatomidae are located extracellularly in tubular outgrowths called crypts or caeca in the posterior region of the midgut (e.g., M4 or V4 section) [11,15,20,224,227]. The establishment of gut bacteria in these crypts occurs directly from the first instar after ingestion of the symbiont [214,224]. Although these crypts can be found in both males and females, they are morphologically enlarged in females, suggesting that these enlarged crypt structures represent a female-specific specialised morphological trait for vertical symbiont transmission in Pentatomidae [227].

Symbiont acquisition failure can impact nymph survival [20,23,224]. Disruption of symbiont acquisition may thus offer pest control opportunities. Methods for disruption of vertical symbiont transmission in the Pentatomidae have been reviewed recently [19]. Different approaches to egg sterilisation have been tested in the laboratory, such as the utilisation of bleach [12,23] or bleach combined with ethanol [10,20,21], or with ethanol and formaldehyde [22,228,229]. Increased mortality, development time and sterility, reduced fecundity, and morphological abnormalities have been observed in pentatomid adults and nymphs, where symbiont acquisition was disrupted by these sterilisation methods [19]. More recently, egg sterilisation has also been successfully used under laboratory and in field conditions to control *H. halys* populations via the suppression of its primary symbiont *Pantoea carbekii* using antimicrobial treatments [24,230,231]. Since some of these products (i.e., Dentamet®, Keos® and Bio-D®) are commercially available in Europe for organic farming as micronutrient fertilisers [230], this demonstrates the potential of targeting eggs to reduce population levels in the field.

6. Other Important Traits to Consider for Pest Management

Compared with olfactory stimuli, considerably less is known about the visual ecology of Pentatomidae. A few studies have looked at the effect of trap colour and demonstrated that trap colour influences the number of bugs captured, and this increase might result in better management and improved pest control. It has been shown that large numbers of phytophagous insects respond to yellow [232]. A study in the US observed that unbaited yellow traps captured more native stink bug species than traps painted light and dark green, black, or covered with aluminium foil [233]. However, intermediate results for

Euschistus spp. with yellow traps have been observed in comparison to white and green pyramid traps [200]. For *H. halys*, adults and nymphs seem to be more attracted to black pyramid traps than to yellow, white, green, and clear ones [234], for both plastic and wood traps [205]. Similar results have been obtained with black traps for *Bagrada hilaris* (Burmeister) [235]. Other coloured traps such as purple, white, or red also captured more adult *B. hilaris* than yellow traps, while no effect of trap colour was observed for nymphs. Laboratory and field work on the harlequin bug showed that this species is more attracted to green and black than white, yellow, red, or purple [236].

Artificial light can influence insect phototaxis, with both attraction [237] or repellence [238] being observed. Various studies have been conducted on the incorporation of lights into trapping devices or pest management programs [239,240]. The monitoring of Pentatomidae can often be difficult, but the incorporation of UV light traps can improve the performance of surveillance traps significantly [241]. Additional factors that can influence insect behavioural responses to light include light intensity and wavelengths [239,240,242–244]. In this regard, laboratory and field trials on *H. halys* have been conducted in order to determine optimal wavelengths and light intensities for the use of surveillance [245,246]. Leskey et al. [246] observed that this species was attracted to white (full spectrum of the visible light, 400–700 nm wavelength), blue (approximately 410 nm wavelength), and black (ultraviolet, <400 nm wavelength) fluorescent bulbs. However, in the field, blue and “white” sources were more attractive. Cambridge et al. [245] demonstrated recently that *H. halys* was attracted to “white” light (400–700 nm wavelength) at 75 lx of intensity.

Understanding the dispersal of stink bugs is crucial to the improvement of integrated pest management strategies targeting Pentatomidae pests. Although the Pentatomidae are regarded as heavy insects that have a small wing area in relation to their body weight in comparison to other insects [247], studies on *H. halys* suggest they are active fliers with good flight performance [248,249]. The establishment of stink bug flight potential could inform the development of management strategies which are influenced by stink bug movement, such as the attract and kill strategy [207]. A few laboratory studies have examined stink bug flight capacity using flight mills. A recent study assessed the baseline flight potential of *Euschistus servus* collected over 2 years from different host plants with the longest distance flown of 16 km [248]. Previous work with *H. halys* demonstrated that 85% of individuals could fly up to 5 km in 24 h, with the longest distance of 117 km flown by an individual [248,249]. The effects of starvation, age, mating status, sex and pre-flight weight on flight parameters have been examined on laboratory-reared *H. halys* [249]. This work suggested that only starvation impacted the number of flight bouts and velocity, suggesting that this species has a robust dispersal capacity which may contribute to its success as an invasive species. Multiple parameters can impact flight mill results, such as handling of insects, lack of natural stimuli, increased weight load, and reduced energy expenditure [250,251]; thus, caution needs to be taken when interpreting flight mill data. For example, distances flown by laboratory-reared stink bugs in a study by Aita et al. [252] were lower than the distances flown by stink bugs collected from the field [248,249]. Furthermore, flight response can vary among stink bug species and over the season. For example, *H. halys* exhibits a high flight potential toward the end of the season and before entering diapause, compared to the mid-season [249], whereas *E. servus* has a lower mean flight potential during the mid-season and toward the end of the season [253]. Determining when and how long-distance and short-distance movements of stink bugs occur throughout the growing season could help growers to predict the area of infestation and to implement time- and location-appropriate control strategies.

7. Conclusions

Current management programs for stink bug pests rely on the use of chemical insecticides, which often have hidden costs associated with environmental impacts, effects on human health [254,255], and the selection of resistant pest populations [6]. Additionally, the intensive use of chemicals is often incompatible with biological control and establishment

of natural enemies [256]. Pesticide exposure can negatively impact natural enemies and reduce their biological control services, which can lead to pest resurgence and secondary pest outbreaks [257,258]. Alternative management options are needed [259], but the successful development and implementation of these alternatives usually requires considerable knowledge of pest biology. The development of alternative tactics is particularly difficult for new pests or invasive species due to the limited knowledge we often have of the pest life cycle in these situations.

Pentatomidae have specific traits which are promising targets for pest management. The presence of sex and aggregation pheromones is one example. Despite the fact that these pheromones have been identified for several species, their use in pest management is still restricted to monitoring the phenology and abundance of a few major stink bug species. Nevertheless, management tactics such as mass trapping and trap cropping have been improved with the commercialisation of various aggregation pheromone lures. Preliminary results suggest that the attract and kill tactic has potential and warrants additional research [206,207]. Although this tactic is promising, the costs linked with the synthesis and production of compounds, along with the deployment of pheromone devices, are still an obstacle limiting the commercial implementation of this method.

Recent advances in genomics and transcriptomics of stink bugs [260–263], along with new technologies such as reverse chemical ecology [264] or CRISPR-Cas9 [265,266], should facilitate the identification and functional characterisation of semiochemicals and their biosynthetic pathways. This could, in turn, lead to alternative options for synthesis of pheromone compounds in the future. CRISPR-Cas9, as a tool for genome editing, targeting for example pheromone and odour-related genes, genes involved in sexual reproduction or insecticide resistance, could also become a viable option for pest management in the future. In terms of this, research should focus on the development of these gene knockout systems in non-model organisms, similar to Cagliari et al. 2020 [266] who developed this for *E. heros*. Biosafety concerns in terms of the release of genetically transformed insects, which range from the risk of non-target effects if hybridisation (gene flow) can occur between target and non-target species, to the ecological impact on a niche if the target is removed from the environment [267], also exist and represent major challenges to the use of these technologies.

In parallel, precision agriculture methods for detection of pests or crop injury have also evolved, and electronic nose (E-nose) technology using semiochemicals has been developed. Various E-noses have been tested to detect stink bug damage on cotton [56,268,269], and more recently to detect *E. heros* sex pheromone [270]. This new technology may well offer a cheaper and new alternative method for stink bug detection in the future. Finally, it is important to also underline the potential of plant volatiles in combination with pheromones to improve trapping and trap cropping methods in pest management [271]. The use of aggregation pheromones in Pentatomidae offers this opportunity, and this has been recently investigated with *H. halys* [207] and *M. histrionica* [272]. Further research should therefore focus on this underexplored topic for stink bug pest management.

Disruption of symbiont acquisition is another interesting target. Some active substances are already commercially available as antimicrobials or fertilisers, and their utilisation is allowed in organic farming as a sustainable option [230,231]. This method therefore seems promising, although practical aspects still need to be addressed in terms of application, rate, and cost. In addition, questions regarding its applicability to other cropping systems as well as other stink bug species, and the long-term effects of these fertilisers on the cropping system, also need further attention before commercial implementation.

Other traits of the Pentatomidae that can be targeted for pest management are the utilisation of alarm pheromones for enhancing natural enemies [175], and the use of substrate-borne vibration for mating disruption [45,47] and surveillance [164,165]. To date, there is no study which shows that the utilisation of alarm pheromone for enhancing natural enemies can suppress the utilisation of insecticides, but these volatiles might be valuable as complementary tools to minimise insecticide spraying. Precautions may also need to be taken, as some of these volatiles have antifungal effects and their utilisation

might interfere with the use of entomopathogenic fungi as biological control agents [100]. Novel methods such as attract and reward [186] have not been tested in Pentatomidae, and future studies should focus on this aspect, whereas the use of vibrational signals and pheromones in a bimodal trap can increase trap captures and might have utility for mass trapping of stink bugs. Finally, the visual ecology and flight behaviour of Pentatomidae are still poorly studied. Colour preferences may improve the accuracy of mass-trapping and surveillance activities. In addition, advances in stink bug genomics and transcriptomics can help to determine which visual genes occur among Pentatomidae and which colours stink bugs can see. Currently, opsin genes have only been described in one Pentatomidae species [273,274], illustrating that we are far from truly understanding their visual ecology. Finally, knowledge regarding dispersal capacity is another important component for pest monitoring, especially for control tactics such as attract and kill or push and pull methods which rely on pest movement.

While many of these tools are still in the early stages of development, they offer valuable targets for improved and more sustainable Integrated Pest Management systems that should receive more focused attention.

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1.10 Project aim and objectives

The following four data chapters of this PhD dissertation will investigate:

1. The genetic diversity of *B. distincta* in South Africa
2. The alarm pheromone of *B. distincta*
3. The lethal and sublethal effects of insecticides on *B. distincta*
4. Commercial lures for capturing stink bug species associated with macadamia orchards in South Africa

Chapter 2

Genetic and diversity of the two-spotted stink bug *Bathycoelia distincta* (Pentatomidae) associated with macadamia orchards in South Africa

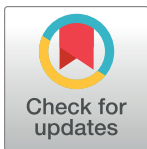
RESEARCH ARTICLE

Genetic diversity of the two-spotted stink bug *Bathycoelia distincta* (Pentatomidae) associated with macadamia orchards in South Africa

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Data Availability Statement: Sequences of COI (accession numbers: OM263477-OM263633), and Cytb (accession numbers: OM219650-OM219806)

Abstract

The South African macadamia industry is severely affected by a complex of stink bugs, dominated by the two-spotted stink bug, *Bathycoelia distincta* Distant (Pentatomidae). This species was first discovered during the spring of 1984 in the Limpopo province. Although considerable effort has been spent trying to manage this pest, it continues to be a pest of concern for the macadamia industry. Information on the genetic diversity of this species is lacking, despite the potential relevance of such information for management strategies. The present study aimed to characterise the genetic diversity of *B. distincta* populations in South Africa. The Cytochrome c Oxidase Subunit 1 (COI) and cytochrome b (Cytb) gene regions were sequenced from individuals collected from the three main regions of macadamia production over three different seasons (2018–2020). An overall high haplotype diversity (COI = 0.744, Cytb = 0.549 and COI+Cytb = 0.875) was observed. Pairwise mean genetic distance between populations from each region varied from 0.2–0.4% in both datasets, which suggests the absence of cryptic species. The median joining network for both datasets consisted of one or two central haplotypes shared between the regions in addition to unique haplotypes observed in each region. Finally, low genetic differentiation ($F_{ST} < 0.1$), high gene flow ($N_m > 1$) and the absence of a correlation between genetic and geographic distance were estimated among populations. Overall, these results suggest that the *B. distincta* populations are not structured among the areas of macadamia production in South Africa. This might be due to its ability to feed and reproduce on various plants and its high dispersal (airborne) between the different growing regions of the country along with the rapid expansion of macadamia plantations in South Africa.

of *B. distincta* were deposited in NCBI GenBank (www.ncbi.nlm.nih.gov). Sequences of COI were also deposited to BOLD (<http://boldsystems.org>: PMSL01-66 sequences from Limpopo; PMSM01-45 sequences from Mpumalanga; PMSK01-46 sequences from KwaZulu-Natal).

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Competing interests: The authors have declared that no competing interests exist.

Introduction

The Pentatomidae is one of the largest families within the Heteroptera with more than 4,700 species distributed worldwide [1]. Phytophagous and polyphagous, Pentatomidae are a major concern to agricultural production around the world, including nut crops [2, 3]. In nut crops, stink bugs can cause direct feeding damage (e.g., nut abortion, discolouration, fruit drop) by insertion of their mouthparts into developing nuts inducing losses in yield and kernel nut quality [4–6]. Stink bugs can also cause indirect feeding damage by transmission of pathogens. This has been demonstrated especially in *Nezara viridula* (L.) with the transmission of *Pantoea agglomerans* into cotton bolls [7, 8].

In South Africa, several nuts are produced and exported worldwide, including macadamia nuts. Considered as the current world's largest producer of macadamia nuts, the industry is continuously growing with about 5000 ha of trees planted annually [9]. However, this production is severely affected by various Heteroptera from the families Coreidae and Pentatomidae [10–12], causing approximately 15.23 million USD in losses due to nut damage annually [12, 13]. Among the different stink bug species occurring in macadamia orchards, *Bathypoelia distincta* Distant (Heteroptera: Pentatomidae) is the most abundant [10, 14, 15] and one of the most damaging pests for the industry due to its long proboscis which can cause kernel damage throughout the entire season [11, 16]. *Bathypoelia distincta* was discovered during the spring of 1984 in the Limpopo province and is now present in plantations in all macadamia production regions. Nymphs and adults have been recovered in the orchards even when no or few nuts are available on trees [10, 14], suggesting that this pest is breeding in the orchards and well established in all production areas in the country.

Control of *B. distincta* and other stink bug species has relied mainly on chemical insecticides. However, the prevalence of insecticide resistance and outbreaks of secondary pests coupled with increased environmental concerns have led to more integrated pest control approaches [17–19]. Some studies have been conducted to understand the seasonal occurrence [14] and the distribution patterns of *B. distincta* [20] to help with the implementation of IPM methods in South Africa [21]. Nevertheless, a comprehensive understanding of *B. distincta* population dynamics is lacking and required for the design of effective management strategies.

Population genetic studies can provide knowledge about the origin, migration patterns, genetic structure, and dynamics of pest populations. Exploring the genetic variability of a species among regional populations is also crucial to detect the existence of cryptic species complexes that could directly affect the efficiency of pest control efforts [22, 23]. Indeed, the utilisation of pheromones is often species-specific and may not work if subpopulations or cryptic species occur in the same geographical area, as has been demonstrated in various lepidopteran species [24–26]. Similarly, successful biological control depends on the use of species-specific parasitoid wasps. For example, a recent study discovered that cryptic species of the parasitoid *Ganaspis brasiliensis* have different affinities towards their hosts regardless of their food source, and as such impact the biological control of *Drosophila suzukii* (Matsumura) [27]. Another study on generalist parasitoids from the subfamily Aphidiinae revealed the presence of multiple cryptic species which are each in fact associated with a different host species [28]. Host preferences also exist in parasitoids of the Pentatomidae [29]. For example, *Trissolcus utahensis* (Ashmead) parasitizes more eggs of *Chlorochroa uhleri* (Stål) than *Chlorochroa sayi* (Stål) [30].

Population genetic studies have also highlighted genetic differentiation which may occur between insecticide resistant and susceptible populations. This may be a result of insecticide selection [31–33], management practices [34, 35], or other factors such as species isolation [36, 37]. Insecticide resistance has been reported for several Pentatomidae species [38]. For

example, *Euschistus heros* (Fabricius) populations in Brazil have shown reduced susceptibility to various insecticides such as organophosphates and endosulfan [38, 39]. Concern regarding the reduced susceptibility of *E. heros* led to a large geographical survey and geostatistical analysis of bioassays with various insecticide formulations in order to map and identify areas with high risk of insecticide control failure [40].

The utilisation of mitochondrial DNA (mtDNA) has become an effective method to detect genetic variation among regional populations [41–44] due to its central role in metabolism and its high conservation rate across species in some sites, but high mutation rate in other sites to allow for species delimitation [41–43]. Although this method has been criticized [45–48], population genetic studies have been widely used among insect taxa in a variety of disciplines, including general ecology and evolution [49], to inform and improve management strategies. In addition, the mitochondrial Cytochrome c Oxidase Subunit 1 (COI) gene region has been used to determine intraspecific divergence rates. To this end intraspecific divergence of 3 to 5% in Heteroptera has been found to delineate cryptic species within populations [50–53] in comparison to 2% intraspecific divergence rates for some other insect groups [42].

Genetic diversity of Pentatomidae species has mostly been studied using the COI gene region [50–52, 54, 55], although additional genes encoded by the mitochondria such as the cytochrome b (Cytb) are also commonly used [49, 56]. The occurrence and genetic diversity of *Halyomorpha halys* (Stål) has been widely studied in several countries [57–62] and such information is also available for *N. viridula* [63–65]. For example, in Brazil, various studies examined population structure and variation among populations of *N. viridula* [66, 67], *E. heros* [68, 69] and *Loxa spp.* [70] from different geographic regions. The genetic variability of *China-via hilaris* (Say), *C. uhleri*, *C. sayi*, and *Thyanta pallidovirens* (Stål) has also been examined to determine the presence of cryptic species in order to assist with pest management in pistachio orchards [53].

The present study aimed to investigate the genetic diversity of *Bathypoelia distincta* in South Africa. We examined the COI and Cytb gene regions from individuals collected from different regions where macadamia is planted commercially. We determined the genetic variability of *B. distincta* populations within and between the three main macadamia production areas of South Africa. We also consider whether there is any evidence of cryptic species.

Materials and methods

Ethics statement

No endangered or protected species were involved in this study. No national permissions were required for this study. All work on this project was conducted with permission from landowners.

Sample collection

Stink bugs were collected from different macadamia farms in Limpopo, Mpumalanga, and Kwazulu-Natal from October 2017 to March 2020 (Tables 1 and 2). Insects were collected as adults, from macadamia trees after insecticide application using a beating cloth under the trees. Samples were preserved in ethanol (> 95%) at -20°C until molecular analysis.

The two-spotted stink bug specimens were identified based on external morphological features described in literature [71] and also confirmed morphologically by an entomologist at the Agricultural Research Council—Plant Health and Protection (Pretoria, South Africa). Insects used for this study are conserved in our collection located at University of Pretoria. Vouchered specimens were pinned, accession number assigned (PENT00026–PENT00030)

Table 1. Sampling locations where *B. distincta* was collected between October 2017 to March 2020 in South Africa provinces.

Location	n ¹	Latitude (S)	Longitude (E)
Farm L1	38	23°04'41.1"	30°08'31.1"
Farm L2	28	23°05'34.6"	30°14'23.3"
Farm M1	45	25°04'50.4"	31°01'08.4"
Farm K1	46	31°01'41.2"	30°13'13.1"

¹ Number of specimens collected and used in this study to determine genetic diversity.

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Table 2. Distance in straight line (Km) among the different sampling sites of *Bathycoelia distincta* population.

Distance (Km) in straight line	Farm L1	Farm L2	Farm M1	Farm K1
Farm L1	-	-	-	-
Farm L2	10	-	-	-
Farm M1	242	234	-	-
Farm K1	885	881	667	-

<https://doi.org/10.1371/journal.pone.0269373.t002>

and deposited in the National Collection of Insects located at the Agricultural Research Council—Plant Health and Protection (Pretoria, South Africa).

DNA extraction, PCR amplification and sequencing

The genetic diversity of *B. distincta* was determined by analysing sequence data from sections of the mitochondrial COI and Cytb genes. DNA was extracted from leg tissue of 157 adult specimens (Table 1) using the NucleoSpin[®] DNA insect (Macherey-Nagel GmbH & Co. KG, Düren, Germany) kit, following the manufacturer's protocol for tissue extraction. A negative control was also carried out with all the kit solutions but without insect tissue to check for contamination. The DNA quantity was measured using the Thermo Scientific NanoDrop[®] ND-1000 spectrophotometer (Wilmington, DE, USA). The quantity of DNA in samples ranged from 20–40 ng/μl.

The COI gene region was amplified using the universal forward LCO1490 (5'-GGTCAA-CAAA TCATAAAGATATTGG-3') and reverse HCO2198 (5'-TAAACTTCAGGGTGACCA AAAAATCA-3') primer [72], and the Cytb gene region was amplified using the forward (5'-GGATATGTTTTACCTTGAGGACA-3') and the reverse (5'-GGAATTGATCGTAA-GATTGCGTA-3') primer [66, 73]. Polymerase Chain Reactions (PCR) were performed in a total volume of 25 μL. For amplification of the COI gene, each PCR contained 5 μL of 5X PCR Buffer (dNTPs and MgCl₂ included), 0.5 μL Taq DNA Polymerase (Bioline, South Africa), 0.5 μL of each primer (10 mM), 16.5 μL of distilled water and 2 μL of 50–100 ng DNA. PCR cycling conditions consisted of denaturation at 94°C for 1 min, followed by five cycles with denaturation at 94°C for 1 min, annealing at 45°C for 90 sec, and extension at 72°C for 90 sec, followed by another 30 cycles with denaturation at 94°C for 1 min, annealing at 50°C for 90 sec, extension at 72°C for 1.30 min, and final extension at 72°C for 5 min. The amplification of the Cytb gene was performed with 50–100 ng of DNA, 1.5 μL of MgCl₂, 1 μL of dNTPs (10 μM), 1 μL of each primer (10 mM), 2.5 μL of 10X PCR buffer, and 0.5 μL of FastStart Taq DNA Polymerase (Roche, Molecular Biochemicals, Mannheim, Germany). The PCR cycles consisted of initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 50°C for 30 sec, extension at 72°C for 2 min and final extension at

72°C for 10 min. With each run, negative controls without DNA in the PCR reaction were performed for PCR validation.

The PCR products were verified on agarose gel (1.5% w/v) with BioRad Gel Doc™ Ez Imager and then purified using the ExoSAP-IT™ (Applied Biosystems, Foster City, CA) PCR Product clean-up kit. Forward and reverse sequence reaction was prepared using the BigDye® Terminator Kit v3.1 (Applied Biosystems, Foster City, CA). Sequencing products were cleaned and precipitated using ethanol and NaAC and sequenced using an ABI Prism™ 3100 Automated Capillary DNA sequencer (Applied Biosystems) at the Bioinformatics Sequencing facility of the University of Pretoria (South Africa).

Population genetic analyses

Electropherograms for all sequences were visualised and a consensus sequence generated using the Biological Sequence Alignment Editor (BioEdit) software (version 7.0.5) [74] and aligned using the online software Multiple Alignment using Fast Fourier Transform (MAFFT) v.7 [75]. To account for different sequence lengths, sequences were trimmed at 642 bp for the COI gene and 443 bp for the Cytb gene. Sequences of both COI and Cytb were concatenated to yield a total length of 1085 bp. Sequences of COI (accession numbers: OM263477-OM263633), and Cytb (accession numbers: OM219650-OM219806) of *B. distincta* were deposited in NCBI GenBank (www.ncbi.nlm.nih.gov). All the COI sequences obtained in this study were also submitted in the BOLD database (<http://boldsystems.org/>) under the project “PBDSA-*Bathypoelia distincta* Pentatomidae South Africa” (ID numbers: PMSL01-66 sequences from Limpopo; PMSM01-45 sequences from Mpumalanga; PMSK01-46 sequences from Kwazulu-Natal).

The COI and Cytb haplotype networks were constructed using Population Analysis with Reticulate Trees (PopART) version 1.7 [76]. Uncorrected (p) pairwise mean genetic distances between populations were calculated using the Kimura 2-parameter substitution model with 1000 bootstraps replicated in MEGA version 7.0.21 [77]. Genetic diversity parameters were determined using DnaSP 5.10.01 [78] and included the number of haplotypes (h), haplotype diversity (Hd), nucleotide diversity (Pi), genetic differentiation (Fst) and gene flow (Nm) values. The levels of genetic differentiation can be categorized as $F_{ST} > 0.25$ (high differentiation), 0.15 to 0.25 (moderate differentiation), and $F_{ST} < 0.05$ (negligible differentiation) [79]. The levels of gene flow can be categorized as $Nm > 1$ (high gene flow), 0.25 to 0.99 (intermediate gene flow), and $Nm < 0.25$ (low gene flow). Tajima’s D [80] and Fu’s Fs [81] values were estimated to test for changes in population size of *B. distincta* using DnaSP (v5.10.01). Significant negative values generally suggest population expansion. One thousand simulations under a model of selective neutrality were used to generate Tajima’s D and Fu’s Fs values. To determine the occurrence of isolation by distance (IBD), Mantel tests between the genetic and geographic distances between each population and marker were conducted using GenAlEx 6.5 with 9999 permutations [82].

Results

Sequence variation

The final sequence aligned matrix of the COI gene from 157 individuals was 642 bp in length. Genetic diversity indices for the COI gene are shown in Table 3. A total of 40 polymorphic nucleotides were observed. Thirty-five haplotypes were identified amongst COI sequences. The farms L1 and L2 showed 14 and 13 haplotypes from a total of 38 and 28 samples, respectively. Only 11 and 8 haplotypes were obtained from 45 and 46 samples of the farms M1 and K1, respectively. The haplotype diversity was the highest in Limpopo (farm L2, Hd = 0.754) and the lowest in the Kwazulu-Natal (farm K1, Hd = 0.571). The estimated nucleotide diversity

Table 3. Summary of molecular diversity indices and population expansion test statistics of COI, Cytb and COI+Cytb genes in *Bathycocelia distincta* populations.

Gene		n	h	S	k	Hd	Pi	D	F _s
COI	Population								
	Farm L1	38	14	15	0.989	0.606	0.00154	-2.32655**	-13.391**
	Farm L2	28	13	18	1.677	0.754	0.00261	-2.22585**	-8.615*
	Farm M1	45	11	13	1.285	0.725	0.00200	-1.73408	-5.511*
	Farm K1	46	8	11	0.899	0.571	0.00140	-1.89467*	-3.600**
All samples		157	35	40	1.316	0.739	0.00206	-2.46755**	-43.196**
Cytb	Population								
	Farm L1	38	14	13	2.004	0.782	0.00452	-1.10959	-7.080
	Farm L2	28	11	8	1.587	0.746	0.00358	-0.99040	-5.860
	Farm M1	45	10	12	0.786	0.431	0.00177	-2.15056*	-7.174*
	Farm K1	46	7	9	0.391	0.246	0.00088	-2.30002**	-5.923**
All samples		157	34	30	1.174	0.549	0.00265	-2.31555**	-33.271**
COI+Cytb	Population								
	Farm L1	38	23	28	2.993	0.936	0.00276	-1.90073*	-18.195*
	Farm L2	28	21	26	3.265	0.955	0.00301	-1.92193*	-17.779*
	Farm M1	45	19	25	2.071	0.831	0.00191	-2.11947*	-13.579*
	Farm K1	46	12	20	1.290	0.643	0.00119	-2.31566**	-6.770**
All samples		157	62	70	2.490	0.875	0.00229	-2.52468***	-86.981**

For each population and gene marker, the number of specimens (n), number of haplotype (h), number of polymorphic (segregation) sites (S), average number of nucleotide differences (k), haplotype diversity (Hd), nucleotide diversity (pi) and Tajima's D (D) and Fu's F_s (F_s) tests statistics are given. Values are significant at

* $P \leq 0.05$;

** $P \leq 0.01$;

*** $P \leq 0.001$.

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(*Pi*) was low overall, ranging from 0.00140 to 0.00261. When all samples were included, the total diversity was Hd = 0.739, but the nucleotide diversity was quite low *Pi* = 0.00206.

Genetic diversity indices for the Cytb gene are shown in Table 3. The final aligned sequence was composed of 443 nucleotides for the Cytb with a total of 30 polymorphic nucleotides observed. The total number of haplotypes was 34. The farm L1 revealed the highest number of haplotypes (14 haplotypes for 38 samples), while the farm K1 showed the lowest number of haplotypes (only 7 haplotypes for 46 samples). The total haplotype diversity was Hd = 0.549, ranging from 0.246 to 0.782. Low nucleotide diversity (*Pi*) was observed (*Pi* < 0.01). It was the highest in Limpopo (farm L1, *Pi* = 0.00452) and lowest in Kwazulu-Natal (farm K1, *Pi* = 0.00088).

The results of the combined analysis of both COI and Cytb genes are presented in Table 3. The 1085 paired bases analysed revealed 70 polymorphic nucleotides. In total, 62 haplotypes were obtained with haplotype diversity Hd = 0.875 and a nucleotide diversity *Pi* = 0.00229. The highest number of haplotypes was observed in Limpopo at the farm L1 with 23 haplotypes, while the lowest number was observed in Kwazulu-Natal with 12 haplotypes. However, the highest haplotype diversity and nucleotide diversity was observed in Limpopo at the farm L2 (Hd = 0.955, *Pi* = 0.00301). It was the lowest in Kwazulu-Natal (Hd = 0.643 and *Pi* = 0.00119).

The pairwise distance comparison among *B. distincta* populations based on COI, Cytb and both COI+Cytb genes are shown in Table 4. Sequence divergence among the four populations by pairwise comparison ranged from 0.2–0.4%. The highest sequence divergence was found

Table 4. Uncorrected “p” distance matrix between different locations based on COI, Cytb and COI+Cytb DNA sequences of *Bathycocelia distincta* from South Africa.

COI	Farm L1	Farm L2	Farm M1	Farm K1
Farm L1	-			
Farm L2	0.002	-		
Farm M1	0.002	0.003	-	
Farm K1	0.002	0.003	0.002	-
Cytb	Farm L1	Farm L2	Farm M1	Farm K1
Farm L1	-			
Farm L2	0.004	-		
Farm M1	0.003	0.003	-	
Farm K1	0.003	0.003	0.001	-
COI+Cytb	Farm L1	Farm L2	Farm M1	Farm K1
Farm L1	-			
Farm L2	0.003	-		
Farm M1	0.003	0.003	-	
Farm K1	0.003	0.003	0.002	-

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when farm L1-Cytb was compared with farm L2-Cytb. The lowest sequence divergence was observed between the farms M1-Cytb and K1-Cytb.

Haplotype network analysis

To clarify the genetic relationship between *B. distincta* populations collected from various farms over different regions in South Africa, median-joining networks were generated (Figs 1–3). The obtained networks were generated with the genetic indices and neutrality tests calculated previously. The COI network (Fig 1) showed a star-like pattern with the two common haplotypes (Hap_C2 and Hap_C19) (S1 Table). The two common haplotypes included *B. distincta* collected from all the different farms in all three regions. They were separated by a single mutational change. For the Cytb, the generated haplotype network showed a dominant haplotype Hap_B1 where *B. distincta* collected from the four farms in all three regions are represented (Fig 2, S2 Table). Similar to the COI network, two common haplotypes Hap_CB3 and Hap_CB13 were found in the COI+Cytb median-joining network (Fig 3, S3 Table). The median-joining network of COI, Cytb, and COI+Cytb also showed a high number of unique haplotypes (35 for COI, 34 for Cytb, 62 for COI+Cytb), suggesting population expansions.

Neutrality test

The neutrality test was conducted using Tajima’s D and Fu’s F_s statistics (Table 3). The results of the tests indicated significant negative D and F_s values for the total of the populations for each gene (COI: D = -2.467, $P < 0.01$, $F_s = -43.196$, $P < 0.01$; Cytb: D = -2.315, $P < 0.01$, $F_s = -33.271$, $P < 0.01$; COI+Cytb: D = -2.525, $P < 0.001$, $F_s = -86.981$, $P < 0.01$), suggesting population expansion. Considering each population separately, all the farms presented significant negative values for the neutrality tests, indicating that there is an excess of rare mutations in *B. distincta* populations which can imply recent population growth. In concordance, the haplotype networks indicated that the different sequence types observed in South Africa would have derived from a common ancestral haplotype (Hap_2).

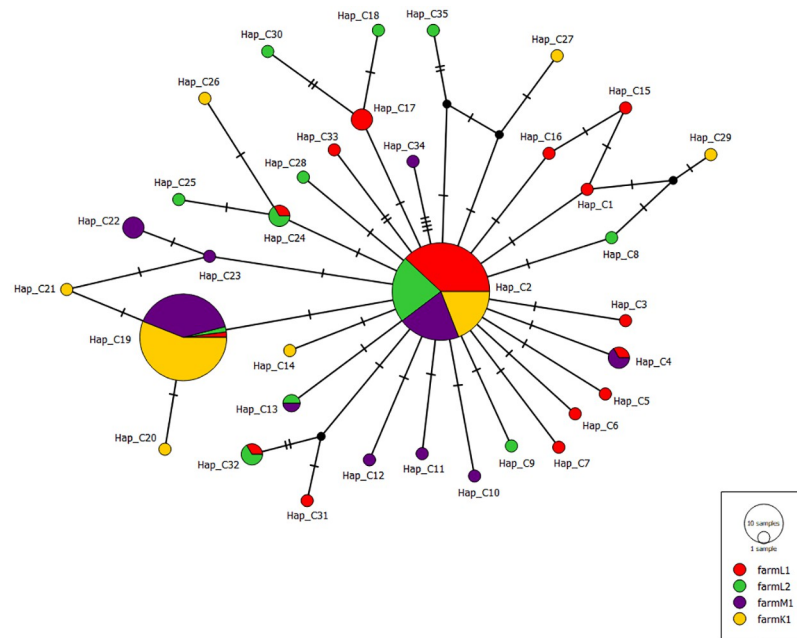


Fig 1. Median-joining haplotype network of *Bathycyelia distincta* for COI gene. Each haplotype is represented by a circle. Relative sizes of the circle indicate haplotype frequency. Color patterns demonstrate samples collected from different farms and regions of South Africa (Limpopo: farm L1 (n = 38), farm L2 (n = 28); Mpumalanga farm M1 (n = 45); Kwazulu-Natal farm K1 (n = 46)). Crossbars indicate one mutational step.

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Genetic structure

Genetic distance (F_{ST}) and migration rates (Nm) of the *B. distincta* populations were calculated (Table 5). Based on COI sequence data, the pairwise F_{ST} among 4 pairs of *B. distincta* populations ranged from -0.005 to 0.291. *Bathycyelia distincta* samples from the Limpopo farms (L1 and L2) exhibited statistically significant genetic differentiation when compared to Mpumalanga (M1) and Kwazulu-Natal (K1) farms. Interestingly, similar results were obtained based on the COI+Cytb data set. Consistent results were also observed based on the Cytb dataset, which showed no significant differences ($P < 0.05$) of pairwise F_{ST} in most population pairs except for the farms L1 and L2 when compared to the farm K1. Regarding the migration rates (Nm) values, all population pairs were greater than one, except for the Limpopo farms (COI, $Nm = -48.96$), or between the Mpumalanga and Kwazulu-Natal farms (Cytb, $Nm = -90.55$). Finally, for each marker, the Mantel test showed no statistically significant IBD, indicating no positive correlation between the geographic and genetic distances among *B. distincta* populations (COI: $r = 0.731$; $P > 0.05$; Cytb: $r = 0.310$, $P > 0.05$; COI+Cytb: $r = 0.669$, $P > 0.05$) (S1–S3 Figs). Our results suggested that more than one stink bug female per generation was estimated to migrate between all pairs of populations, except between Limpopo and Mpumalanga or Kwazulu-Natal.

Discussion

Population genetic studies have played a significant role in the identification of cryptic species and the study of their genetic diversity [42, 51]. In this study, we investigated the population genetics of *B. distincta* for the first time. DNA sequences were analysed employing COI and Cytb markers from specimens collected in the three main macadamia production regions of

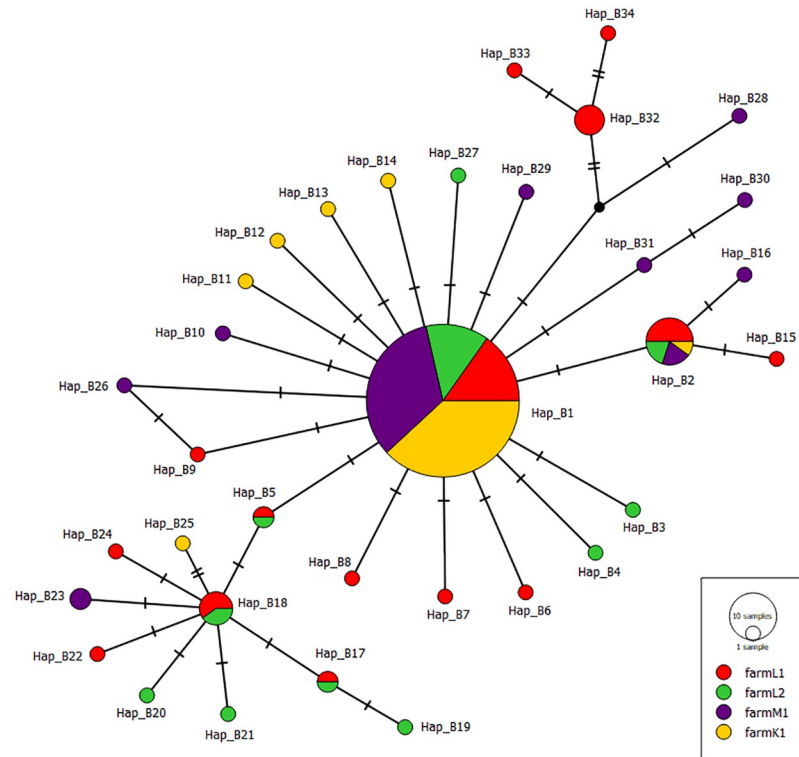


Fig 2. Median-joining haplotype network of *Bathycoelia distincta* for Cytb gene. Each haplotype is represented by a circle. Relative sizes of the circle indicate haplotype frequency. Color patterns demonstrate samples collected from different farms and regions of South Africa (Limpopo: farm L1 (n = 38), farm L2 (n = 28); Mpumalanga farm M1 (n = 45); Kwazulu-Natal farm K1 (n = 46)). Crossbars indicate one mutational step.

<https://doi.org/10.1371/journal.pone.0269373.g002>

South Africa. The highest genetic diversity was observed within the Limpopo province populations, the area from where the species was first discovered. Pairwise mean distance analysis between populations suggested the absence of cryptic species. The median joining network for both datasets consisted of a few central haplotypes shared among populations in South Africa, with several unique haplotypes (35 for COI, 34 for Cytb, 62 for COI+Cytb) among the 157 *B. distincta* individuals examined (Figs 1–3). Considering the direct relationship between haplotype frequency and the ages of haplotypes [83, 84], the existence of a star-like structure (central haplotype and several less frequently derived haplotypes) suggests that most of the haplotypes originated recently, and is indicative of a population expansion during the recent history of the species [85].

Bathycoelia distincta was originally discovered from the Limpopo region [14], and became a serious problem in South African macadamia areas in Limpopo and Mpumalanga in the early 2000s [10, 15, 86] and more recently in Kwazulu-Natal. The rapid and intensive increase in macadamia planted area in the last decade may be related to the genetic diversity observed in this study. Beck and Reese [87] proposed that insect survivorship, fecundity, growth rate and activity can be affected by the quantity and quality of the host. An abundance of hosts in a habitat will increase survival and fecundity and reduce mortality. As macadamia trees are the primary cultivated host for *B. distincta*, the increase in population size of this host would distinctly improve the fecundity and survival of the insect, and thus the extensive commercial

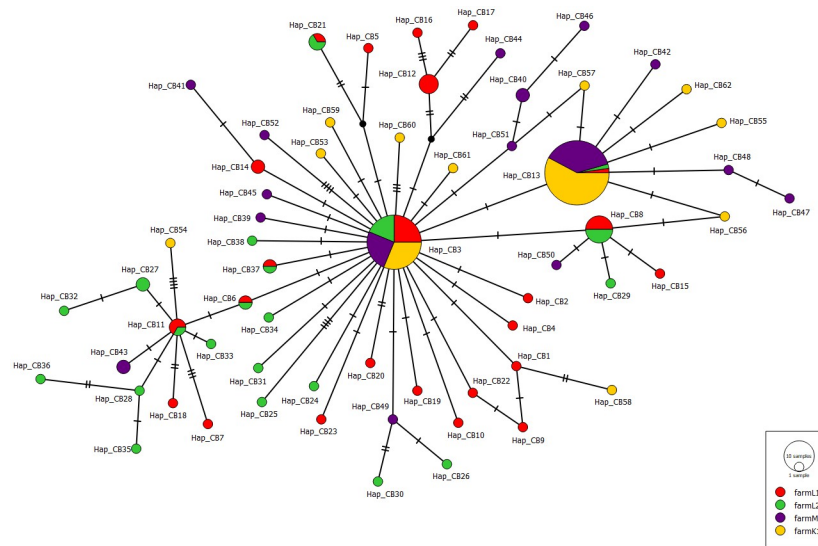


Fig 3. Median-joining haplotype network of *Bathycoelia distincta* for COI+Cytb genes. Each haplotype is represented by a circle. Relative sizes of the circle indicate haplotype frequency. Color patterns demonstrate samples collected from different farms and regions of South Africa (Limpopo: farm L1 (n = 38), farm L2 (n = 28); Mpumalanga farm M1 (n = 45); Kwazulu-Natal farm K1 (n = 46)). Crossbars indicate one mutational step.

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cultivation of macadamia trees in recent years may have contributed to an expansion of *B. distincta*'s range in South Africa. Similar results have been obtained in *E. heros* populations in Brazil after the rapid expansion of the soybean-planted area, leading to an absence of genetic differentiation within populations [68], compared to the results obtained in the same area 14 years earlier [69]. This hypothesis is reinforced by the significant negative values found for the

Table 5. Genetic differentiation (F_{ST}) and gene flow (Nm) based on COI, Cytb and COI+Cytb DNA sequences of *Bathycoelia distincta* from three different regions of South Africa.

COI	Farm L1	Farm L2	Farm M1	Farm K1
Farm L1	-	-48.96	1.58	0.61
Farm L2	-0.00513	-	2.01	0.84
Farm M1	0.13647**	0.11052**	-	7.05
Farm K1	0.29150***	0.23025***	0.03427	-
Cytb	Farm L1	Farm L2	Farm M1	Farm K1
Farm L1	-	5.07	4.44	2.75
Farm L2	0.04703	-	2.21	1.44
Farm M1	0.05335	0.10151	-	-90.55
Farm K1	0.08331*	0.14768*	-0.00277	-
COI+Cytb	Farm L1	Farm L2	Farm M1	Farm K1
Farm L1	-	9.56	2.45	1.08
Farm L2	0.02548	-	2.10	1.02
Farm M1	0.09256*	0.10654	-	11.32
Farm K1	0.18840*	0.19643**	0.02161	-

The data above and below the diagonal correspond to Nm and F_{ST} respectively.

*, ** and *** indicate significant difference at $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively.

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neutrality tests (Tajima's D and Fu's F_s), confirming a recent population expansion of *B. distincta*.

In our study, high gene flow ($Nm > 1$), lack of genetic differentiation ($F_{ST} < 0.1$) and no IBD ($P > 0.05$) were observed among the different populations (Table 5, S1–S3 Figs). Genetic differentiation between insect populations can be influenced by different factors such as the geographic distance between populations [53, 88, 89]. Although population differentiation by distance has been observed previously within Pentatomidae species [53, 57, 69], our results suggest that geographic distances did not affect the genetic structure of *B. distincta*. While limited genetic structure and high levels of population connectivity were expected for the farms located in Limpopo (distance between farms L1–L2 is 10 km), this was not expected for our regional scale investigation as the farms sampled are geographically distant, being up to 800 km apart (Table 2). Few studies have been conducted on the flight capacities of Pentatomidae using flight mills. Babu et al. [90] showed that *Euschistus servus* (Say) can fly a maximum distance of 15.9 km in 22 h, especially after overwintering emergence, whereas most individuals only flew between 0–1 km. For *H. halys*, adults can fly 5–7 km in 24 h and up to 117 km, with longer and faster flights achieved in summer [91–93], while nymphs can easily walk among host plants [94]. Considering that genetic isolation can also be higher in species with a limited capacity for dispersal [95], a high dispersal capacity in *B. distincta* could explain the genetic homogeneity among its populations in South Africa.

Factors other than flight ability might also facilitate movement of *B. distincta*. One such a factor is the distribution of suitable host plants (both wild and cultivated) in South Africa. Stink bugs can feed on several plant-hosts [2, 96] and higher levels of damage are often observed when forests and natural vegetation border crops [97, 98], a feature quite common in the South African landscape. In addition, the two-spotted stink bug population can reach high densities especially in the canopies [20], mainly from November to March when nuts are present but they can remain in the field even after the nuts have been harvested [10, 14]. After harvest *B. distincta* may disperse, looking for shelter to remain in diapause during winter. High levels of gene flow, as determined for *B. distincta* in this study, are determinants of the maintenance of high levels of genetic diversity and low population differentiation [99, 100]. This, in addition to its multivoltinism, may have contributed to high population densities, a wide distribution, and low levels of population differentiation throughout the country. Therefore, these long-distance dispersal events might occur in parallel with some anthropogenic driven dispersal (e.g., transport of seedlings or fruits among regions of South Africa).

Genetic divergence and nucleotide diversity in this study among the different locations was comparable to results for Hemiptera in previous studies. The intraspecific genetic divergence in this study was in the range of 0.2 to 0.4% (Table 4) and a low nucleotide diversity was observed ($P_i < 3\%$) (Table 3). Previous studies observed intraspecific divergence of 4.7% between individuals of *C. hilaris* [53], and $> 2\%$ in *N. viridula* [54]. Nevertheless, an intraspecific genetic divergence value of 4.7% has been suggested to delineate cryptic species within populations of Heteroptera [51]. Considering the previous results obtained and the morphological and ecological similarity of *B. distincta* between the different locations analysed, we can confirm that our study did not show any evidence of cryptic species in its populations in South Africa. Knowledge of the existence of cryptic species is important for IPM because different species can respond differently to pest management strategies [23, 35]. For example, variation in the pheromone blends have been found among different populations of *Diatraea saccharalis* (Fabricius), suggesting that the trapping efficiency could vary among the regions [26]. Similarly, insect populations from different geographic regions can vary in their level of susceptibility to insecticides as it has been recently demonstrated in wireworm populations [101]. Thus,

it would be useful to determine the effectiveness of future pheromone lures and other control methods across the wide geographical range of *B. distincta*.

In conclusion, this study of *B. distincta* collected within the three main macadamia production regions in South Africa revealed a high genotypic diversity and a lack of genetic differentiation among localities. The high genotypic diversity suggests favourable environmental conditions for reproduction and growth of the species in its native range. The high gene flow observed, even across a wide geographic area, appears to be the major ecological force shaping the overall genetic pattern observed. Regional population dynamics of *B. distincta* are likely linked to the rapid expansion of macadamia planted areas. To our knowledge this is the first study on the population genetics of *B. distincta*. This information provides a critical starting point for understanding this species in South Africa and might assist future development of pest management strategies that incorporate pheromones and biological control. Furthermore, future studies using other genetic and genomic tools and including a larger sample size and geographic range, could help understand the movement of on *B. distincta* populations between orchards and native plants, and within regions and over shorter time scales. Such genetic and genomic tools are currently under-utilized as a resource for stink bug management and our study provides a foundation for such further work.

Supporting information

S1 Fig. Isolation by distance of *Bathycocelia distincta* populations for COI marker (Mantel test, $r = 0.731$, $P > 0.05$).

(TIF)

S2 Fig. Isolation by distance of *Bathycocelia distincta* populations for Cytb marker (Mantel test, $r = 0.310$, $P > 0.05$).

(TIF)

S3 Fig. Isolation by distance of *Bathycocelia distincta* populations for COI+Cytb combined marker (Mantel test, $r = 0.669$, $P > 0.05$).

(TIF)

S1 Table. List of the individual for each haplotype generated in the study for the COI marker.

(DOCX)

S2 Table. List of the individual for each haplotype generated in the study for the Cytb marker.

(DOCX)

S3 Table. List of the individual for each haplotype generated in the study for the COI +Cytb combined markers.

(DOCX)

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Writing – original draft: Elisa Pal.

Writing – review & editing: Elisa Pal, Jeremy D. Allison, Brett P. Hurley, Bernard Slippers, Gerda Fourie.

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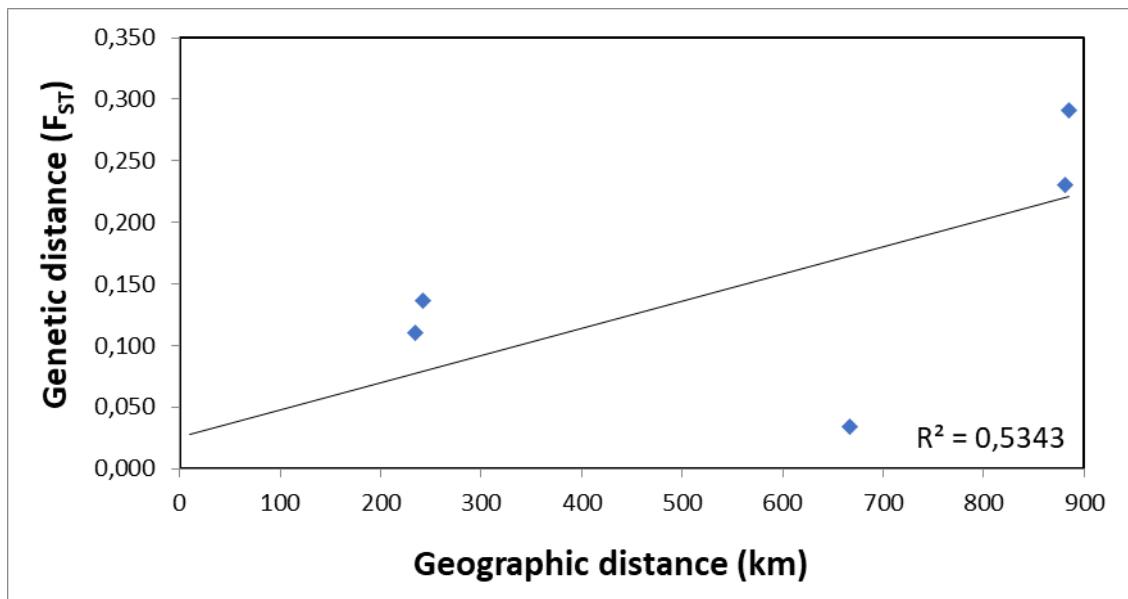
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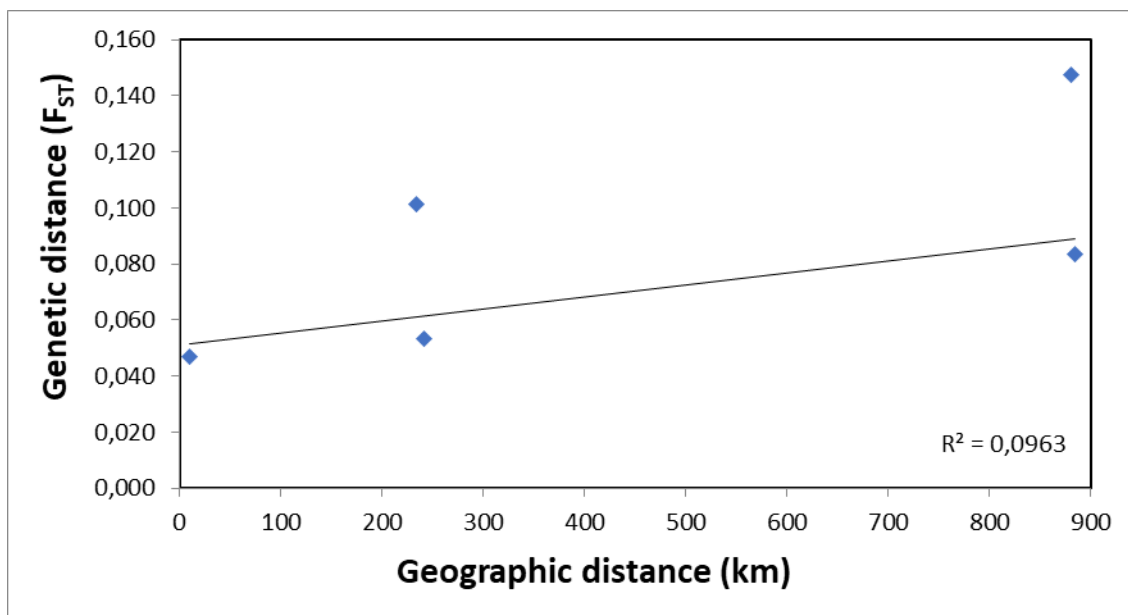
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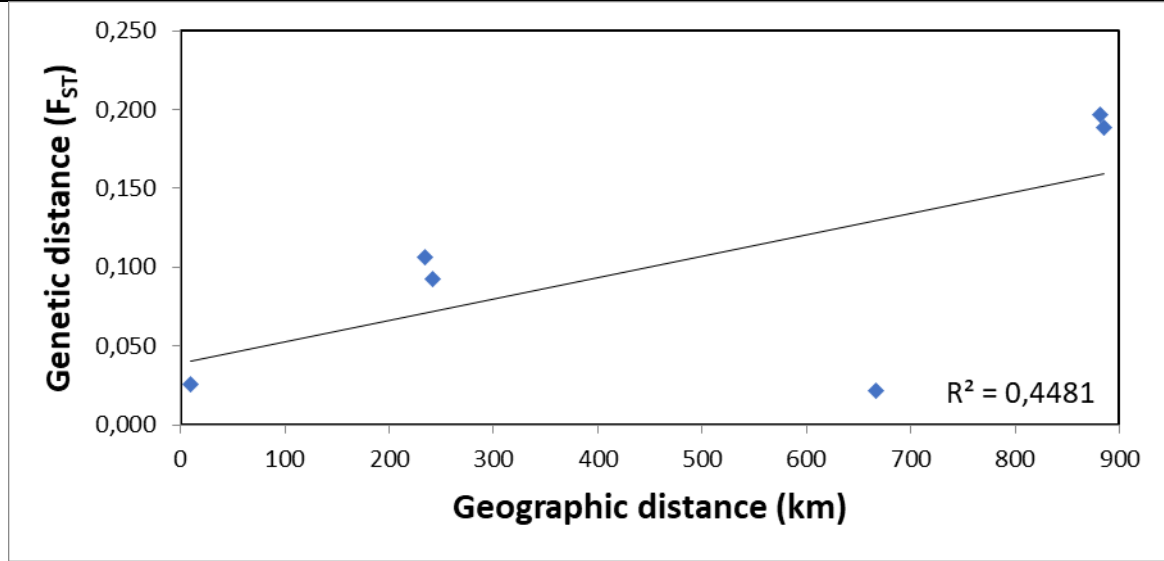
2.7 Supplementary materials



S1 Fig. Isolation by distance of *Bathycoelia distincta* populations for COI marker (Mantel test, $r = 0.731$, $P > 0.05$).



S2 Fig. Isolation by distance of *Bathycoelia distincta* populations for Cytb marker (Mantel test, $r = 0.310$, $P > 0.05$).



S3 Fig. Isolation by distance of *Bathycoelia distincta* populations for COI+Cytb combined marker (Mantel test, $r = 0.669$, $P > 0.05$).

S1 Table. List of the individual for each haplotype generated in the study for the COI marker.

Haplotype	n	Individuals
Hap_C1	1	MSL1
Hap_C2	63	MSL2; MSL3; MSL6; MSL7; MSL9; MSL11; MSL12; MSL13; MSL14; MSL15; MSL16; MSL19; MSL20; MSL21; MSL22; MSL23; MSL25; MSL26; MSL27; MSL29; MSL31; MSL36; MSL37; MSL38; MSL42; MSL43; MSL45; MSL47; MSL49; MSL50; MSL52; MSL54; MSL55; MSL56; MSL61; MSL62; MSL63; MSL66; MSM5; MSM9; MSM11; MSM19; MSM21; MSM23; MSM30; MSM36; MSM39; MSM40; MSM41; MSM45; MSK8; MSK10; MSK21; MSK23; MSK26 ; MSK36 ; MSK38; MSK42; MSK43; MSK44; MSK45; MSK46; MSM28
Hap_C3	1	MSL4
Hap_C4	3	MSL17; MSM10; MSM27
Hap_C5	1	MSL32
Hap_C6	1	MSL35
Hap_C7	1	MSL39
Hap_C8	1	MSL46
Hap_C9	1	MSL51
Hap_C10	1	MSM1
Hap_C11	1	MSM20
Hap_C12	1	MSM22
Hap_C13	2	MSM29; MSL60

Hap_C14	1	MSK27
Hap_C15	1	MSL10
Hap_C16	1	MSL34
Hap_C17	3	MSL24; MSL30; MSM38
Hap_C18	1	MSL41
Hap_C19	50	MSL18; MSL65; MSM2; MSM3; MSM4; MSM7; MSM8; MSM12; MSM13; MSM14; MSM15; MSM16; MSM17; MSM18; MSM24; MSM25; MSM31; MSM32; MSM34; MSM35; MSM37; MSM44; MSK1; MSK2; MSK3; MSK4; MSK5; MSK6; MSK7; MSK9; MSK11; MSK14; MSK15; MSK16; MSK17; MSK19; MSK20; MSK22; MSK25; MSK28; MSK29; MSK30; MSK31; MSK32; MSK33; MSK34; MSK35; MSK37; MSK39; MSK40
Hap_C20	1	MSK13
Hap_C21	1	MSK13
Hap_C22	3	MSM6; MSM26; MSM33
Hap_C23	1	MSM42
Hap_C24	1	MSL28; MSL44; MSL57
Hap_C25	1	MSL59
Hap_C26	1	MSK41
Hap_C27	1	MSK12
Hap_C28	1	MSL64
Hap_C29	1	MSK24
Hap_C30	1	MSL48
Hap_C31	1	MSL5
Hap_C32	3	MSL33; MSL53; MSL58
Hap_C33	1	MSL8
Hap_C34	1	MSM43
Hap_C35	1	MSL40

S2 Table. List of the individual for each haplotype generated in the study for the Cytb marker.

Haplotype	n	Individuals
Hap_B1	105	MSL39; MSL41; MSL43; MSL45; MSL47; MSL48; MSL53; MSL55; MSL58; MSL60; MSL61; MSL62; MSL64; MSL65; MSL14; MSL18; MSL17; MSL20; MSL22; MSL25; MSL33; MSL34; MSL35; MSL36; MSL38; MSL1; MSL3; MSL4; MSL5; MSL7; MSL10; MSK17; MSM18; MSM21; MSM22; MSM23; MSM24; MSM25; MSM27; MSM29; MSM30; MSM31; MSM32; MSM33; MSM37;

		MSM38; MSM39; MSM42; MSM43; MSM44; MSM45; MSM1; MSM2; MSM3; MSM4; MSM5; MSM6; MSM7; MSM8; MSM9; MSM11; MSM12; MSM13; MSM14; MSM15; MSK15; MSK16; MSK17; MSK18; MSK19; MSK20; MSK21; MSK22; MSK23; MSK24; MSK25; MSK26; MSK27; MSK28; MSK29; MSK30; MSK31; MSK32; MSK33; MSK34; MSK35; MSK37; MSK38; MSK39; MSK42; MSK43; MSK44; MSK45; MSK46; MSK10; MSK11; MSK13; MSK6; MSK7; MSK9; MSK1; MSK2; MSK3; MSK4; MSK5
Hap_B2	10	MSL46; MSL52; MSL11; MSL13; MSL19; MSL37; MSL9; MSM36; MSM41; MSK14
Hap_B3	1	MSL49
Hap_B4	1	MSL56
Hap_B5	2	MSL63; MSL6
Hap_B6	1	MSL12
Hap_B7	1	MSL31
Hap_B8	1	MSL32
Hap_B9	1	MSL2
Hap_B10	1	MSM10
Hap_B11	1	MSK41
Hap_B12	1	MSK36
Hap_B13	1	MSK40
Hap_B14	1	MSK8
Hap_B15	1	MSL21
Hap_B16	1	MSM40
Hap_B17	2	MSL42; MSL66
Hap_B18	5	MSL44; MSL51; MSL54; MSL15; MSL23
Hap_B19	1	MSL50
Hap_B20	1	MSL57
Hap_B21	1	MSL59
Hap_B22	1	MSL8
Hap_B23	2	MSM19; MSM28
Hap_B24	1	MSL30
Hap_B25	1	MSK12
Hap_B26	1	MSM16
Hap_B27	1	MSL40
Hap_B28	1	MSM20
Hap_B29	1	MSM26

Hap_B30	1	MSM34
Hap_B31	1	MSM35
Hap_B32	4	MSL26; MSL27; MSL29; MSL16
Hap_B33	1	MSL28
Hap_B34	1	MSL24

S3 Table. List of the individual for each haplotype generated in the study for the COI+Cytb combined markers.

Haplotype	n	Individuals
Hap_CB1	1	MSL1
Hap_CB2	1	MSL2
Hap_CB3	32	MSL3; MSL7; MSL14; MSL20; MSL22; MSL25; MSL36; MSL38; MSL43; MSL45; MSL47; MSL55; MSL61; MSL62; MSM5; MSM9; MSM11; MSM21; MSM23; MSM30; MSM39; MSM45; MSK10; MSK21; MSK23; MSK26; MSK38; MSK42; MSK43; MSK44; MSK45; MSK46
Hap_CB4	1	MSL4
Hap_CB5	1	MSL5
Hap_CB6	2	MSL6; MSL63
Hap_CB7	1	MSL8
Hap_CB8	8	MSL9; MSL11; MSL13; MSL19; MSL37; MSL52; MSM36; MSM41
Hap_CB9	1	MSL10
Hap_CB10	1	MSL12
Hap_CB11	3	MSL15; MSL23; MSL54
Hap_CB12	4	MSL16; MSL26; MSL27; MSL29 MSL18; MSL65; MSM2; MSM3; MSM4; MSM7; MSM8; MSM12; MSM13; MSM14; MSM15; MSM17; MSM18; MSM24; MSM25; MSM31; MSM32; MSM37;
Hap_CB13	45	MSM44; MSK1; MSK2; MSK3; MSK4; MSK5; MSK6; MSK7; MSK9; MSK11; MSK15; MSK16; MSK17; MSK19; MSK20; MSK22; MSK25; MSK28; MSK29; MSK30; MSK31; MSK32; MSK33; MSK34; MSK35; MSK37; MSK39
Hap_CB14	2	MSL17 MSM27
Hap_CB15	1	MSL21
Hap_CB16	1	MSL24
Hap_CB17	1	MSL28
Hap_CB18	1	MSL30
Hap_CB19	1	MSL31

Hap_CB20	1	MSL32
Hap_CB21	3	MSL33; MSL53; MSL58
Hap_CB22	1	MSL34
Hap_CB23	1	MSL35
Hap_CB24	1	MSL39
Hap_CB25	1	MSL40
Hap_CB26	1	MSL41
Hap_CB27	2	MSL42; MSL66
Hap_CB28	1	MSL44
Hap_CB29	1	MSL46
Hap_CB30	1	MSL48
Hap_CB31	1	MSL49
Hap_CB32	1	MSL50
Hap_CB33	1	MSL51
Hap_CB34	1	MSL56
Hap_CB35	1	MSL57
Hap_CB36	1	MSL59
Hap_CB37	2	MSL60; MSM29
Hap_CB38	1	MSL64
Hap_CB39	1	MSM1
Hap_CB40	2	MSM6; MSM33
Hap_CB41	1	MSM10
Hap_CB42	1	MSM16
Hap_CB43	2	MSM19; MSM28
Hap_CB44	1	MSM20
Hap_CB45	1	MSM22
Hap_CB46	1	MSM26
Hap_CB47	1	MSM34
Hap_CB48	1	MSM35
Hap_CB49	1	MSM38
Hap_CB50	1	MSM40
Hap_CB51	1	MSM42
Hap_CB52	1	MSM43
Hap_CB53	1	MSM43
Hap_CB54	1	MSK12
Hap_CB55	1	MSK13

Chapter 2

Genetic diversity of the two-spotted stink bug

Hap_CB56	1	MSK14
Hap_CB57	1	MSK18
Hap_CB58	1	MSK24
Hap_CB59	1	MSK27
Hap_CB60	1	MSK41
Hap_CB61	1	MSK36
Hap_CB62	1	MSK40

Chapter 3

Characterisation of the alarm
pheromone of *Bathycoelia distincta*
(Pentatomidae)



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 a 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*.

Keywords Stink bug · Scent gland · GC-MS · Antennal activity · Behaviour

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 Viličková 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; Fávoro et al. 2011, 2012; Ho and Millar 2001). For example, the secretions of adult *Chinavia impicticornis* (Stål) 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

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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, 2018), but nymphs showed 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).

Bathycorixa distincta (Distant), the two-spotted stink bug, is a major pest on macadamia in South Africa (Schoeman 2013, 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, 2018) and parameters affecting the distribution and dispersal behaviour of stink bugs in South Africa (Schoeman 2014a, b). 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), 14 L : 10D photoperiod) in plastic cages (27 × 15 cm) 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 (1 µl) were immediately analysed after collection by gas chromatography coupled to mass spectrometry (GC/MS) or

stored at $-20\text{ }^{\circ}\text{C}$ until analysis. A blank which consisted of $1\text{ }\mu\text{l}$ 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 ($110\text{ }^{\circ}\text{C}$) 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, 150/75 mg) at 40 ml min^{-1} . After sampling, PorapakQ beads were transferred to GC vials (vial N9 702,293, Macherey-Nigel) and eluted with 1 ml of double distilled *n*-hexane for 1 h . After 1 h the solvent was removed and stored at $4\text{ }^{\circ}\text{C}$ 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 was immediately closed. The stressed bugs, each in a separate glass jar, were aerated for 4 h . 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 h 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 ($30\text{ m} \times 0.320\text{ mm I.D.} \times 0.25\text{ }\mu\text{m}$ film thickness; 7HM-G002-11; Zebron) and ZB-5 ($30\text{ m} \times 0.320\text{ mm I.D.} \times 0.25\text{ }\mu\text{m}$ film thickness; 7HM-G007-11; Zebron) column. The oven temperature was maintained at $50\text{ }^{\circ}\text{C}$ for 2 min , ramped at $10\text{ }^{\circ}\text{C min}^{-1}$, and then maintained for 2 min at $250\text{ }^{\circ}\text{C}$ or $300\text{ }^{\circ}\text{C}$ 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 ($30\text{ m} \times 0.250\text{ mm i.d.}, 0.25\text{ }\mu\text{m}$ film thickness) and a DB-wax ($30\text{ m} \times 0.250\text{ mm i.d.}, 0.25\text{ }\mu\text{m}$ film thickness) column. The oven temperature was maintained at $40\text{ }^{\circ}\text{C}$ for 2 min , ramped at $20\text{ }^{\circ}\text{C min}^{-1}$, and then maintained for 2 min at $300\text{ }^{\circ}\text{C}$ or $250\text{ }^{\circ}\text{C}$ for the HP5-MS-UI or the DB-wax column, respectively. All solutions were injected in splitless-mode at $250\text{ }^{\circ}\text{C}$ at constant column head pressure of 9.8 psi (helium). The mass

spectrometer was operated in scan mode ($m/z\text{ }45\text{--}550$). Electron impact ionization (70 e-V) was used to generate ions. The ion source and MS quadrupole temperature were $230\text{ }^{\circ}\text{C}$ and $150\text{ }^{\circ}\text{C}$, 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 ($1\text{--}500\text{ ng}/\mu\text{l}$ 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 ($1\text{ }\mu\text{l}$) 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 $10\text{ }\mu\text{L}$ 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 $10\text{ }000\text{ ng}$ 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 s 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 centered 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, CaCl₂: 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 s. 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 center. 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 × 100 × 100 cm). The behaviour of each insect was recorded using a Logitech® C922 Pro HD stream webcam positioned approximately 20 cm directly above the center 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

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 $\pm$ std. Compounds in bold have been identified by matches with authentic standards

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

(8 $\pm$ 9% females; 10 $\pm$ 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%.

Bathycelia 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 was not significantly different ($P > 0.05$). Adults of *B. distincta* released tridecane (150 $\pm$ 53 ng females; 183 $\pm$ 52 ng males), (E)-4-oxohe-2-enal (27 $\pm$ 9 ng females; 33 $\pm$ 11 ng males), (E)-2-decenal (22 $\pm$ 4 ng females; 16 $\pm$ 5 ng males), dodecane (19 $\pm$ 6 ng females; 23 $\pm$ 6 ng males), (E)-2-hexenal (11 $\pm$ 3 ng females; 7 $\pm$ 2 ng males) and (E)-2-decenyl acetate (3 $\pm$ 1 ng females; 9 $\pm$ 3 ng males). Analyses of the

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

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

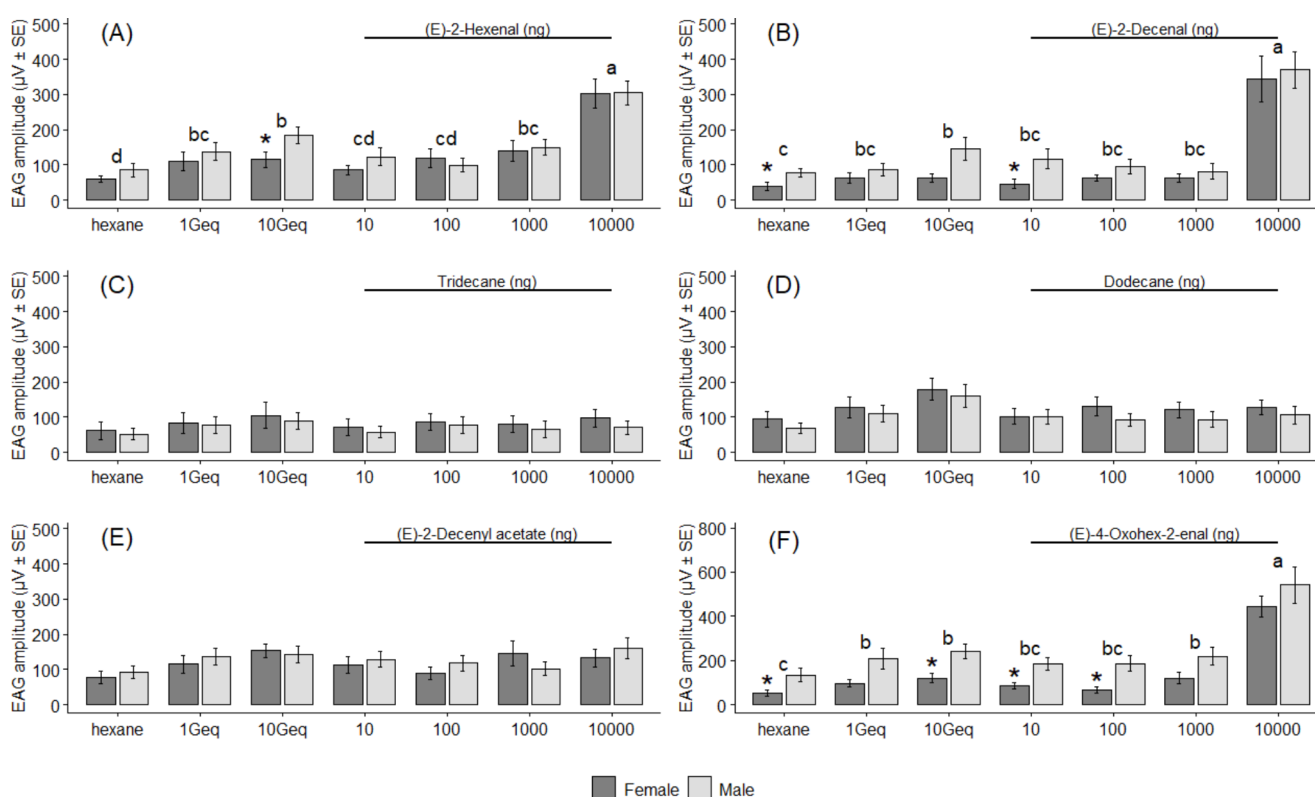


Fig. 1 Electro-antennogram dose-response profiles of major compounds found in MTGs and volatiles of stressed *B. distincta*. EAG response amplitudes (mean μ V $\pm$ SE) of (*E*)-2-hexenal (A), (*E*)-2-decenal (B), tridecane (C), dodecane (D), (*E*)-2-decenyl acetate (E) and (*E*)-4-oxohex-2-enal (F) 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

extracts from the unstressed stink bugs were similar to the blank.

Electrophysiology. No differences were observed between the size of the depolarisation events with 30, 45, and 60 sec between successive odour puffs (Supplementary material Fig. S1, $P=0.79$). Therefore, an interval of 30 sec

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) 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 were 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, 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. 2012, 2015; 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.*

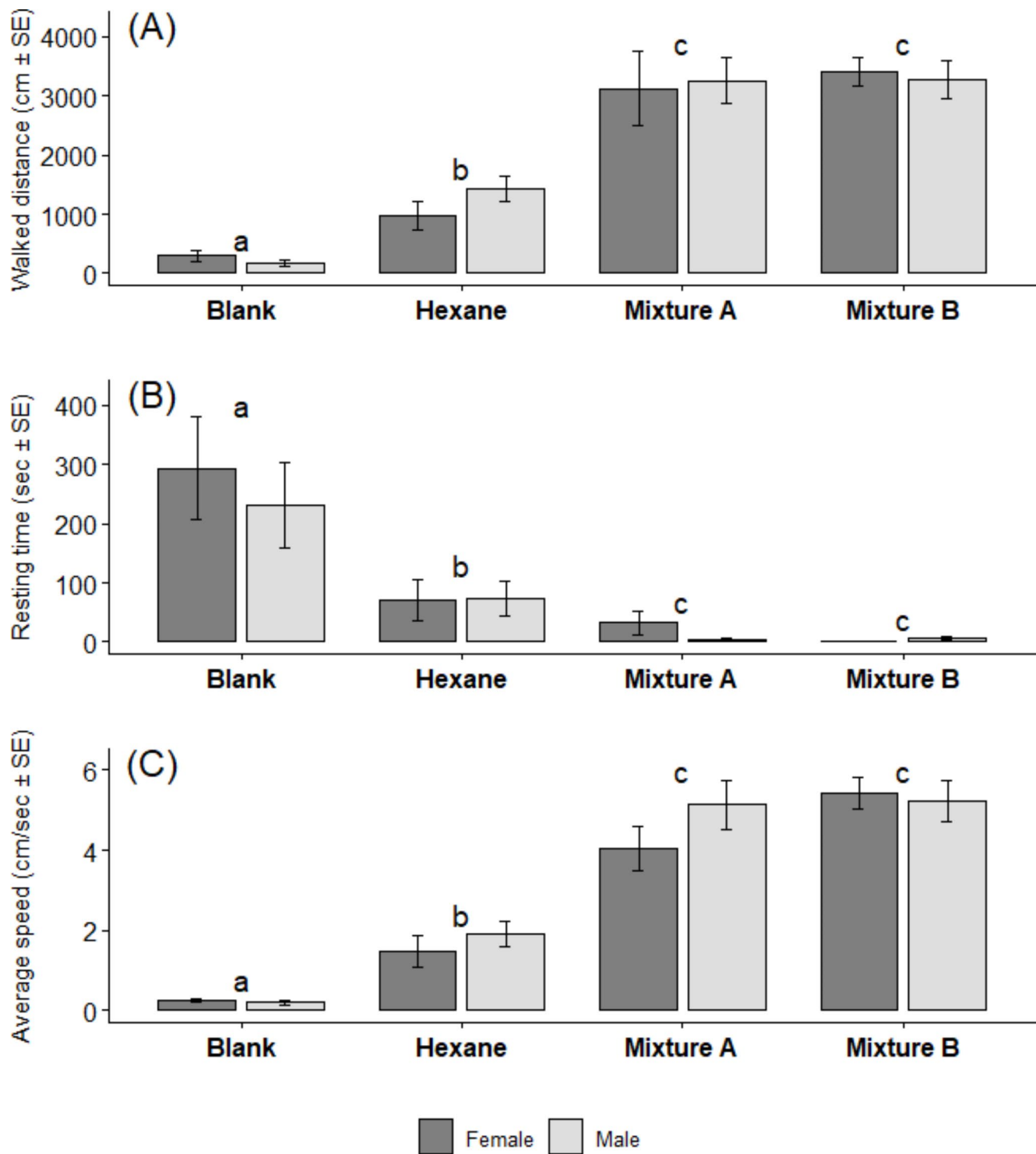


Fig. 2 Behavioural responses of *B. distincta* to putative alarm pheromone compounds. Distance walked (cm ± SE) (A), resting time (sec ± SE) (B) and average of speed (cm / sec ± SE) (C) of male (n = 10) and female (n = 10) insects after 10 min of exposure to mixture A ((*E*)-2-hexenal, (*E*)-2-decenal, 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

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 *Begrada 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. 2017, 2018). (*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 μ V 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) *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 significantly 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, 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) *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.

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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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Declarations

Conflict 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.

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3.7 Supplementary materials

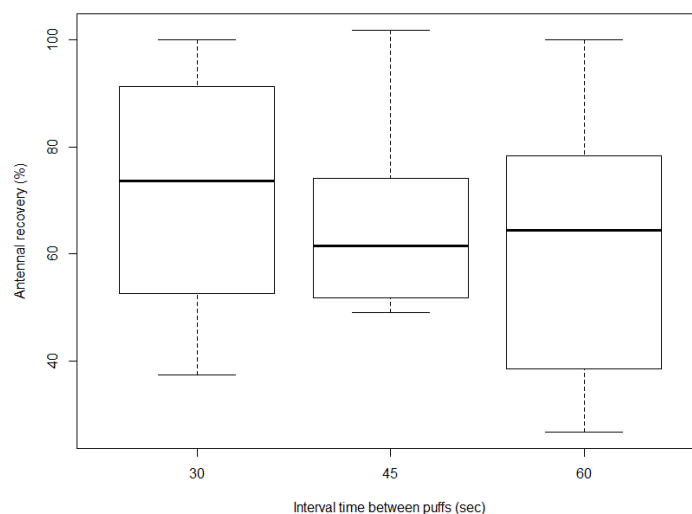


Figure S1. Antennal recovery (in %) 30, 45 and 60 sec after 1000 ng of Verbenol puffed (n = 3 males and 3 females) (ANOVA test, $P = 0.795$).

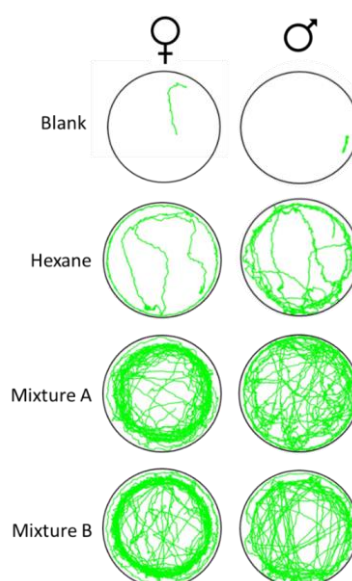


Figure S2. Representative tracks showing the walking activity of *B. distincta* for 10 min period after exposition to different treatments. Filter papers impregnated with to mixture A ((*E*)-2-hexenal, (*E*)-2-decenal, tridecane, dodecane, (*E*)-2-decenyl acetate and 4-oxohex-2-enal diluted in hexane), mixture B ((*E*)-2-hexenal, (*E*)-2-decenal and 4-oxohex-2-enal diluted in *n*-hexane), *n*-hexane or no solvent (blank) were placed at the centre arenas. Green tracks indicate the walking path realized by the female (left) and male (right) stink bugs during 10 min period.

Chapter 4

Lethal and sublethal effects of insecticides on *Bathycoelia distincta* (Heteroptera: Pentatomidae) in South Africa

4.1 Abstract

Bathycoelia distincta is one of the most dominant stink bug pests associated with macadamia orchards in South Africa. Understanding the toxicity and sublethal effects of insecticides on this pest is important for its effective management. In this study, four commercial insecticide formulations were tested, consisting of one organophosphate (acephate) and three pyrethroids (lambda-cyhalothrin, beta-cyfluthrin and tau-fluvalinate). The toxicity of these insecticides and their behavioural effects on mobility were assessed. The sublethal effects of lambda-cyhalothrin on the biological parameters of parent *B. distincta* (F0) and offspring generations (F1) were also determined by treating *B. distincta* adults with sublethal concentrations (LC₁₀ and LC₃₀). In toxicity bioassays, acephate was more toxic to *B. distincta* than lambda-cyhalothrin, beta-cyfluthrin and tau-fluvalinate. Behavioural changes were only observed in bugs exposed to pyrethroids, resulting in an increase in the distance walked and decrease of angular velocity. In the F0 generation, LC₃₀ reduced the fecundity whereas the LC₁₀ and LC₃₀ accelerated development of the F1 generation. These results suggest that pyrethroids may enhance the dispersal of this pest and stimulate the growth of offspring populations. Further experiments should be conducted to confirm these results and understand the mechanism of action of pyrethroids on *B. distincta*.

4.2 Introduction

Stink bugs (Hemiptera: Pentatomidae) are important pests of various fruit and vegetable crops (McPherson, 2018; McPherson & McPherson, 2000). Nymphs and adults primarily feed on the reproductive parts of crops including the flowers, seeds, fruits, although other tissues (e.g., roots, stems, leaves) are also fed on. This feeding results in the stunting of plants and dropping of bolls and fruits and in some cases results in yield losses (McPherson, 2018; McPherson & McPherson, 2000).

Broad-spectrum insecticides such as organophosphates, pyrethroids and neonicotinoids remain the main strategy for controlling populations of stink bugs (Greene et al., 2018; Sosa-Gómez et al., 2020). Repeated and long-term use of insecticides against stink bugs can result in the development of resistance (Sosa-Gómez et al., 2020; Tuelher et al., 2018). For

example, intensive use of pyrethroids and organophosphates on soybean in Brazil reduced the susceptibility of *Euschistus heros* (Fabricius) considerably, which led to increased concentration of active ingredients in the formulation of some acephate products (Sosa-Gómez et al., 2020; Tuelher et al., 2018).

Evaluation of insecticide efficacy should not only measure mortality in pest populations within a given time period at lethal concentrations (Desneux et al., 2007; Stark & Banks, 2003), but should also study sublethal effects. The high mobility of stink bugs can limit their exposure to lethal concentrations, but these insecticides may still have important sublethal impacts (Lee et al., 2014; Morrison et al., 2017). Furthermore, many active components of chemicals can be influenced by factors such as the temperature, rainfall, UV light or plant metabolism (Burrows et al., 2002; Hulbert et al., 2011; Maia et al., 2016), resulting in a degradation of the insecticide into low or sublethal concentrations. Measuring sublethal effects in addition to lethal effects of insecticides is therefore viewed as a more accurate assessment of insecticide efficacy (Desneux et al., 2007; Haynes 1988; Müller, 2018; Stark & Banks, 2003).

Sublethal concentrations of insecticides can affect many physiological parameters such as development, longevity, and fecundity (Lu et al., 2020; Wu et al., 2022; Zhou et al., 2017), as well as mobility (da Silva et al., 2022) and feeding behaviour (Koo et al., 2015; Zeng et al., 2016). These effects can vary according to the species and the insecticides used. For example, sublethal concentrations of dinotefuran can reduce the fecundity of the planthopper *Nilaparvata lugens* (Stal) (Bao et al., 2009) whereas no effect was observed on the mirid bug *Apolygus lucorum* (Meyer-Dur) (Lu et al., 2020). In contrast, other studies have shown that sublethal concentrations of insecticides can stimulate pest population dynamics (e.g., insecticide-induced hormetic responses) by influencing their reproductive output. For example, the insecticides triazophos and deltamethrin stimulate the reproductive systems of male adults of *N. lugens*, leading to increased fertility in females after mating (Wang et al., 2010). A similar effect has been observed with *Laodelphax striatellus* (Fallen) where the fecundity and reproductive rate of females were increased after exposure to a sublethal dose of sulfoxaflor (Xu et al., 2016). On aphids, sublethal concentrations of beta-cypermethrin had no significant effect on *Aulacorthum solani* (Kaltenbach) whereas it can stimulate the oviposition of *Aphis glycines* (Matsumura) (Qu et al., 2020). Acceleration of development

after exposure to sublethal concentrations of insecticides has also been reported in various species such as *Bemisia tabaci* (Gennadius) (Fang et al., 2018), *Aphis gossypii* (Glover) (Koo et al., 2015; Yuan et al., 2017) and *N. lugens* (Xu et al., 2022).

To date, research on the effects of insecticides on stink bugs has mainly focused on direct mortality (Sosa-Gómez et al., 2020) with few studies characterizing sublethal effects. Cira et al. (2017) measured the sublethal effects of various insecticides on development and feeding of *Halyomorpha halys* (Stal), whereas Morrison et al. (2017) investigated the consequence of brief exposure on the survivorship and mobility of this pest. Hormetic effects have also been reported in *Euschistus heros* (F.) after exposure to sublethal concentrations of imidacloprid (Haddi et al., 2016; Santos et al., 2016).

The two-spotted stink bug, *Bathycoelia distincta* (Distant) is one of the major pests of macadamia in South Africa (Schoeman, 2013, 2018; Sonnekus et al., 2022). The damage from *B. distincta* is caused by the insertion of its stylet into the nuts for feeding which can result in pre-mature nut abortion and yield loss (Bruwer et al., 2021; Schoeman, 2020). Stink bug feeding can also result in the deformation of nuts and development of lesions on the kernel, which decrease the overall quality of the nuts (Bruwer et al., 2021; Schoeman, 2020). The use of pyrethroids remains the main strategy for controlling *B. distincta* despite considerable effort in the implementation of integrated pest management methods in macadamia orchards in South Africa (Schoeman, 2014a). Furthermore, macadamia trees can be very tall with dense branches which reduces the efficacy of insecticide applications (Schoeman, 2014b).

To date, no experimental study has been conducted on the lethal and sublethal effects of insecticides on *B. distincta*. Here, we first evaluated the toxicity and effects on the mobility of *B. distincta* with four commercial products frequently used in macadamia orchards, the insecticides acephate, lambda-cyhalothrin, beta-cyfluthrin and tau-fluvalinate. We then focused on lambda-cyhalothrin, the most commonly applied insecticide in macadamia orchards, and assessed its acute toxicity on adult *B. distincta*. Finally, the sublethal effects of lambda-cyhalothrin on biological parameters of the parent *B. distincta* (F0) and offspring generation (F1) were determined with the LC₁₀ and LC₃₀. Our objective was to determine whether the population-level performance and behavioural traits of *B. distincta* were

influenced by insecticide exposure. The results from this study will be useful as a baseline reference point for future studies and may lead to improved stink bug management strategies for macadamia growers in South Africa.

4.3 Materials and methods

Insects

To test the various commercial products on *B. distincta*, egg masses of *B. distincta* were collected from a macadamia plantation in Limpopo (23°05'34.6"S, 30°14'23.3"E; South Africa), from February to April 2022 to start a laboratory colony at the Forestry and Agricultural Biotechnology Institute (FABI), Biological Control Centre of the University of Pretoria. Eggs were maintained in small containers until hatching and nymphs were subsequently transferred to a larger plastic container (27 x 15 cm) with a mesh lid for ventilation (approximately 25 nymphs per container). The stink bugs used to assess the sublethal effects of the pyrethroid lambda-cyhalothrin were initially collected in 2018 from a macadamia orchard in Limpopo (23°03'13''S, 30°14'02''E). The colony was maintained without any insecticide exposure since collection. All the insects used in this experiment were maintained in a climatic chamber at standard environmental conditions (25 ± 2°C, 20 ± 5% RH, 14L : 10D) and reared on green beans and corn *ad libitum*.

Time-mortality bioassays under insecticide field rates

Acute (lethal) toxicity was evaluated by direct contact of insecticides to the insect body of *B. distincta* via a glass-vial method (De Castro et al., 2018; Snodgrass et al., 2005). The bioassay was conducted in 20 mL glass vials treated with 0.5 mL solution of each insecticide. All the insecticides used are registered for controlling *B. distincta* in macadamia orchards (SAMAC, 2022). The insecticides used and their respective commercial formulations were: the organophosphate acephate (Ace[®] 750SP; 750 g a.i.kg⁻¹; Nulandis), and the pyrethroids lambda-cyhalothrin (Karate Zeon[®] 10 CS; 100 g a.i.L⁻¹; Syngenta South Africa Ltd), beta-cyfluthrin (Buldock[®] Beta 125 SC; 125 g a.i.L⁻¹; Bayer (Pty) Ltd) and tau-fluvalinate (Klartan[®] 240 EW; 240 g a.i.L⁻¹; Adama South Africa (Pty) Ltd). The commercial formulations were diluted in 1 L of distilled water achieving the following concentration for the bioassays: 75 mg/L (acephate), 5 mg/L (lambda-cyhalothrin), 7.5 mg/L (beta-cyfluthrin) and 72 mg/L (tau-fluvalinate). The vials were rolled on a hotdog roller with the heat unit

disconnected in a fume hood until the commercial formulations diluted with water evaporated and the insecticide covered the inner vial surface. For the control treatment vials were treated with distilled water only. Insects were placed individually into treated vials plugged with cotton to prevent escape with no food. Preliminary tests confirmed that *B. distincta* can survive without food or water for time periods up to 48h in the glass vials.

Twenty *B. distincta* adults (sex ratio 1:1) were evaluated individually for response to each insecticide treatment. The insects were kept in a climate-controlled chamber ($25 \pm 2^\circ\text{C}$, $20 \pm 5\%$ RH, L14: D10) and exposure was replicated three times. Insect mortality was observed every two hours during the initial 12h exposure and at 6h intervals afterwards until death. Adults were recorded as dead if they did not move when probed with a fine hairbrush.

Concentration-mortality bioassays

Lambda-cyhalothrin toxicity to *B. distincta* was determined via the same method described above. A serial dilution of stock of lambda-cyhalothrin (99% purity, Sigma-Aldrich, Johannesburg, South Africa) was prepared and diluted in acetone (Sigma-Aldrich, Johannesburg, South Africa). Insects were placed individually into treated vials plugged with cotton to prevent escape with no food.

Three replicates of seven concentrations (10 µg/L, 30 µg/L, 75 µg/L, 100 µg/L, 300 µg/L, 750 µg/L, 1000 µg/L), and a control without insecticide were tested, and ten stink bugs per concentration were used (sex ratio 1:1, $n = 240$). The concentrations used were established through preliminary bioassays to facilitate identification of the concentration range leading to a range in mortality between 0% and 100%. All the vials were maintained in a climate-controlled chamber ($25 \pm 2^\circ\text{C}$, $20 \pm 5\%$ RH, L14: D10). Mortality was assessed after 24 and 48h of exposure. Insects were considered dead if they did not move when probed with a fine hairbrush.

Behavioural bioassays

Horizontal movement

Bathycoelia distincta movement was analysed using a Petri-dish method (Leskey et al., 2012; Morrison et al., 2017). Adults were briefly exposed to commercial formulation of insecticides in glass-treated vials for 10 min and subsequently transferred into an untreated

glass Petri dish arena (Ø 90 mm x 15 mm high) with lids to prevent escape. Ten insects (sex ratio 1:1) per insecticide were tested per treatment in a complete randomized experimental design. Petri dishes were washed after each use with soap and water and dried in an oven overnight at 110°C.

The horizontal movement of adults was tracked for 10 min immediately after insecticide exposure with a Logitech® C922 Pro HD stream webcam suspended approximately 20 cm directly above the centre of the arena. All bioassays were conducted in a custom-made white box (90 x 100 x 100 cm) in a climate-controlled chamber maintained at $25 \pm 2^\circ\text{C}$ and $20 \pm 5\%$ relative humidity. The software EthoVision® XT (version 16) was used to analyse the videos and to measure the distance travelled (cm) and angular velocity (degrees/sec) of the insects in the arena.

Sublethal effects of lambda-cyhalothrin on life-history traits of *B. distincta*

Fifth instar stink bugs were placed on green beans and corn until they moulted to adults. These adults were then used in the study as the F0 generation individuals to ensure that all stink bugs were similar in age prior to exposure to insecticide. The LC_{10} and LC_{30} obtained from the experiment above were used to evaluate the sublethal effect of lambda-cyhalothrin on *B. distincta*. The LC_{10} and LC_{30} were chosen to mimic lower concentrations of lambda-cyhalothrin that may occur in the field following initial insecticide application owing to its degradation by various factors. The two concentrations were prepared in acetone following the method described above, and acetone was used as a control. Adults were exposed via treated glass-vials. After 48h, male and female survivors of each treatment were paired (1 male and 1 female, 6 pairs per treatment) and transferred to small plastic containers (11 x 11 x 15 cm) with food. Every two days, adult mortality and fecundity were recorded. The number of eggs were counted until female adult death. The hatched eggs were recorded, and the nymphs were transferred into small plastic containers. Nymphal stage and survival were checked and recorded every two days. Finally, when the nymphs reached the adult stage, the sex ratio was assessed for each treatment. All the experiments were conducted in a climate-controlled chamber ($25 \pm 2^\circ\text{C}$, $20 \pm 5\%$ RH, L14: D10) and insects were reared on green beans and corn *ad libitum*.

Statistical analyses

The time-mortality data were subjected to survival analysis using the packages *survival* and *survminer* (Kassambara and Kosinski, 2018; Therneau, 2015), and the Kaplan-Meier method was used followed by a post hoc test for comparison of survival between treatments. Concentration-mortality bioassays were subjected to Probit analysis (Finney, 1971; Robertson et al., 2007; Wheeler et al., 2006) using the *ecotox* package in R (Hlina, 2020). Concentration was log-transformed to calculate the lethal concentration for 10%, 30% and 50% (LC₁₀, LC₃₀ and LC₅₀) of the population after 48h, as well as their 95% confident limits (CI). No stink bug mortality occurred in the control treatment in the toxicity bioassays, so adjustment of treatment mortalities was not required (Abbott, 1925). The data from the behavioural bioassays (distance walked, angular velocity) and the sublethal bioassays were tested for normality of errors and homogeneity of variance using a Kolmogorov–Smirnov and Bartlett’s test, respectively. When these assumptions were met (inspection of residuals and by Bartlett’s test), one-way ANOVA was performed, and the means were compared using Tukey’s honestly significant difference (HSD) test. If the assumptions were violated a Kruskal-Wallis test was performed followed by a Dunn post hoc test to compare the differences between treatments using the package *FSA* (Ogle et al. 2023). Only the adult longevity data were analysed with a two-way ANOVA (factors: sex and treatment). All data were analysed using R (version 4.1.2) and significance was accepted at $\alpha = 0.05$.

4.4 Results

Time-mortality bioassays

The survival analysis of *B. distincta* exposed to dried insecticide residues indicated significant differences among treatments (Log-rank test, $\chi^2 = 117$, $df = 4$, $P < 0.001$) (Figure 1). No mortality of *B. distincta* was observed in the control (without insecticide exposure) after 72h of exposure, while 100% mortality was observed for the insecticides acephate, beta-cyfluthrin, lambda-cyhalothrin and tau-fluvalinate after 36, 48, 72 and 72h, respectively. Such differences were reflected in the mean survival time (LT₅₀) observed for each insecticide. The LT₅₀ were 8h and 30h for the insecticide acephate and beta-cyfluthrin, and 36h for the insecticide lambda-cyhalothrin and tau-fluvalinate. The mean survival time was not estimated for stink bugs without exposure (control) because no mortality was observed.

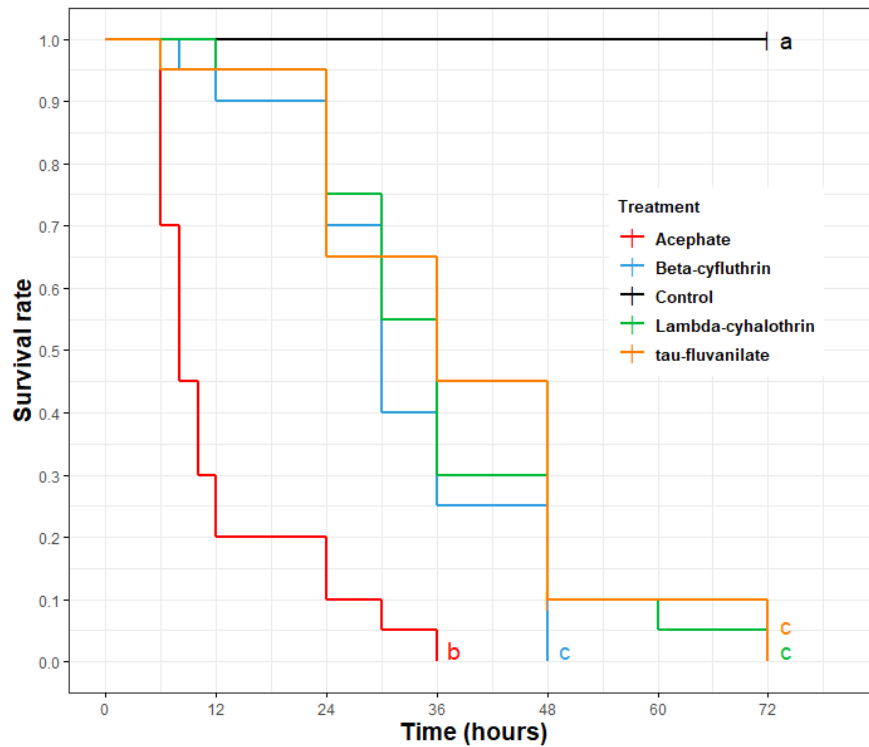


Figure 1. Survival curves of *Bathycoelia distincta* exposed to acephate (Ace® 750SP; 750 g a.i.kg-1; Nulandis), beta-cyfluthrin (Buldock® Beta 125 SC; 125 g a.i.L-1; Bayer (Pty) Ltd), lambda-cyhalothrin (Karate Zeon® 10 CS; 100 g a.i.L-1; Syngenta South Africa Ltd), tau-fluvalinate (Klartan® 240 EW; 240 g a.i.L-1; Adama South Africa (Pty) Ltd), and water (control). The same letters within each parameter indicate that treatments are not significantly different from each other at $P > 0.05$.

Concentration-mortality bioassays

Standard probit analysis of concentration-mortality data showed that after 48h of exposure to lambda-cyhalothrin, the LC_{50} value was estimated at 157 $\mu\text{g/ml}$ (Table 1). The LC_{10} and LC_{30} values were estimated at 22.8 and 71.2 $\mu\text{g/ml}$, respectively, and were used for the subsequent assessment of sublethal effects.

Table 1. Estimates of the lethal concentrations of Lambda-cyhalothrin against *B. distincta*

LC	$\mu\text{g/ml}$	LCL-UCL	Slope $\pm$ SE	χ^2	df	n
LC10	22.8	4.22-48.9				
LC30	71.2	27.5-128	1.53-0.181	10.6	5	240
LC50	157	82.9-302				

SE = standard error, LCL = 95% lower confidence level, UCL = 95% upper confidence level, χ^2 = chi-square statistic, df = degrees of freedom, n = total number of stink bugs used.

Horizontal movement

Effects of exposure to insecticide residues were observed on the distance walked immediately after insecticide exposure (Figure 2A, $\chi^2 = 29.70$, df = 4, $P < 0.001$) and *post-hoc* analyses evidence different trends between treatments. After 10 min of insecticide exposure, *B. distincta* adults moved significantly greater distance for three insecticides beta-cyfluthrin, lambda-cyhalothrin and tau-fluvalinate than acephate or the control (Dunn's test, $P < 0.05$). The distance walked by adults exposed to acephate for 10 min was not significantly different from the control (Dunn's test, $P > 0.05$). Effects of exposure to insecticide residues were also observed on the angular velocity (Figure 2B, $F = 10.48$, df = 4, $P < 0.001$). The angular velocity significantly decreased for the pyrethroids beta-cyfluthrin, lambda-cyhalothrin and tau-fluvalinate compared to acephate or the control (Tukey HSD, $P < 0.05$). The angular velocity between adults exposed to acephate was not significantly different from the control (Tukey HSD, $P > 0.05$).

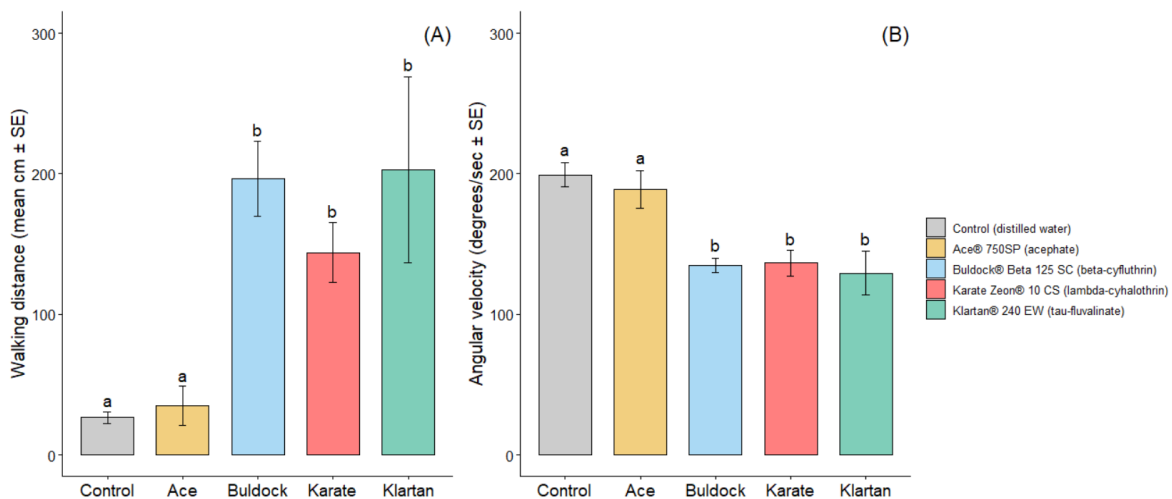


Figure 2. Distance walked (A) and angular velocity (B) after 10 min exposure of adults *B. distincta* to commercial insecticide formulations. The same lowercase letters within each parameter indicate that treatments are not significantly different ($P > 0.05$, Dunn's and Tukey's multiple comparisons tests).

Video recordings show that movement patterns consisted mainly of circular paths along the edge of the arena which were different from those observed in acephate or control adults (Figure 3).

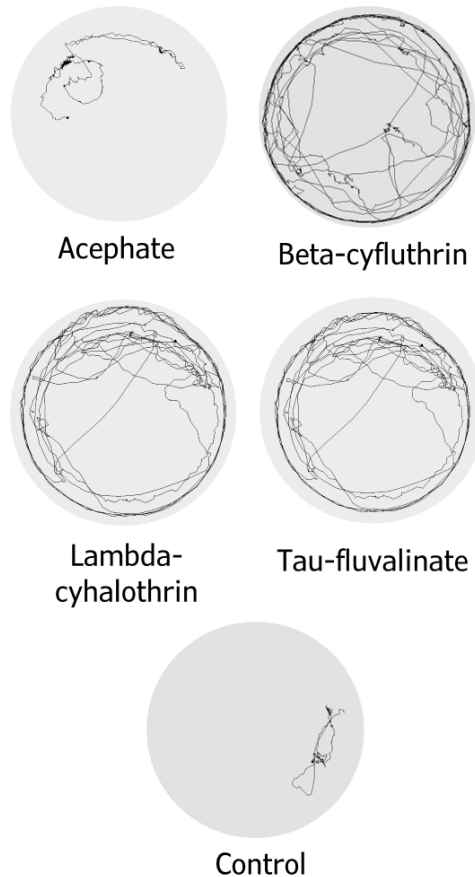


Figure 3. Representative tracks showing the walking pattern of *B. distincta* in petri-dishes after 10 min exposure to various commercial insecticide formulations: acephate (Ace® 750SP; 750 g a.i.kg-1; Nulandis), beta-cyfluthrin (Buldock® Beta 125 SC; 125 g a.i.L-1; Bayer (Pty) Ltd), lambda-cyhalothrin (Karate Zeon® 10 CS; 100 g a.i.L-1; Syngenta South Africa Ltd), tau-fluvalinate (Klartan® 240 EW; 240 g a.i.L-1; Adama South Africa (Pty) Ltd), and control (water).

Sublethal effects of lambda-cyhalothrin on the F0 generation

Adult *B. distincta* longevity was significantly affected by treatments ($F = 10.77$, $df = 2$, $P < 0.001$) and sex ($F = 9.04$, $df=1$, $P < 0.001$) (Figure 4A). LC_{30} significantly reduced adult male longevity when compared with the LC_{10} and control treatments (Figure 4A, Tukey's HSD tests, $\alpha = 0.05$). Exposure to sublethal concentrations of lambda-cyhalothrin significantly affected the fecundity of *B. distincta* (Figure 4B, $F = 8.55$, $df = 2$, $P < 0.001$). The number of eggs produced per female was significantly lower when stink bugs were

exposed to LC₃₀ (40 ± 24 eggs laid) compared to control treatments (170 ± 28 eggs laid) (Tukey HSD, $P < 0.05$). No significant difference was observed in the fecundity between the LC₁₀ and LC₃₀ or control treatment (Tukey HSD, $P > 0.05$).

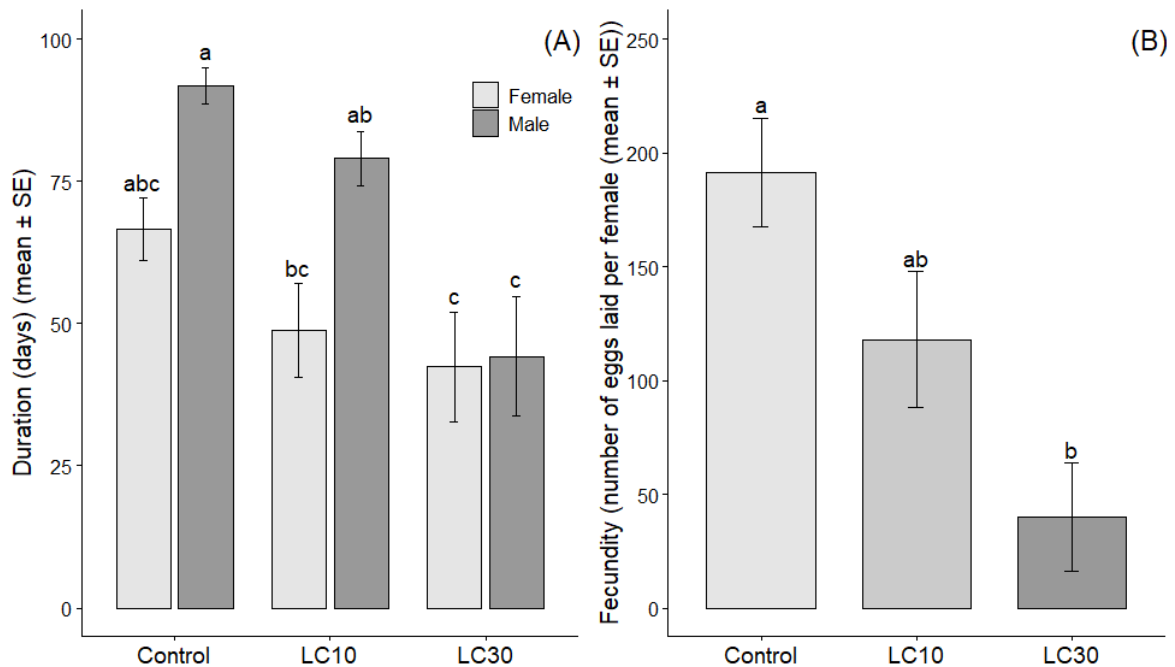


Figure 4. Effects of sublethal concentrations (LC₁₀ and LC₃₀) of lambda-cyhalothrin on the longevity of adults (A) and the fecundity (B) of the F0 generation of *Bathycoelia distincta*. Data are mean $\pm$ standard errors (SE), and the same lowercase letters within each parameter indicate that treatments are not significantly different ($P > 0.05$, Tukey's HSD test).

Sublethal effects of lambda-cyhalothrin on the F1 generation

The development duration, hatching rate, survival of nymphs and sex ratio of the F1 generation of *B. distincta* are shown in Figure 5. Both LC₁₀ and LC₃₀ had no significant effect on the hatching rate of eggs (Figure 5A, $\chi^2 = 1.93$, $df = 2$, $P > 0.05$). Sublethal concentrations of lambda-cyhalothrin had significant effects on the total development time from egg to the adult stage (Figure 5B, $\chi^2 = 31.64$, $df = 2$, $P < 0.001$). The total developmental time from egg to the adult stage was significantly shorter for LC₁₀ and LC₃₀ compared to the control treatment (Dunn's test $P < 0.05$). The LC₁₀ and LC₃₀ significantly decreased the developmental duration time at the egg stage ($\chi^2 = 22.67$, $df = 2$, $P < 0.001$) and of the 4th ($\chi^2 = 23.64$, $df = 2$, $P < 0.001$) and 5th instar ($\chi^2 = 1.514$, $df = 2$, $P < 0.01$) compared to the control treatment (Figure 5C). In comparison, the LC₃₀ significantly increased the

development time of the 3rd instar compared to the control, whereas the LC₁₀ significantly decreased it compared to the control ($\chi^2 = 24.50$, $df = 2$, $P < 0.001$). Nymphal survival was lower during the 2nd instar when stink bugs were exposed to LC₁₀ compared to the control treatment (Figure 5D, $\chi^2 = 6.02$, $df = 2$, $P < 0.05$). No significant difference was observed in the sex ratio of the F1 generation (determined at the adult stage) between the various treatments (Figure 5E, $F = 0.59$, $df = 2$, $P > 0.05$).

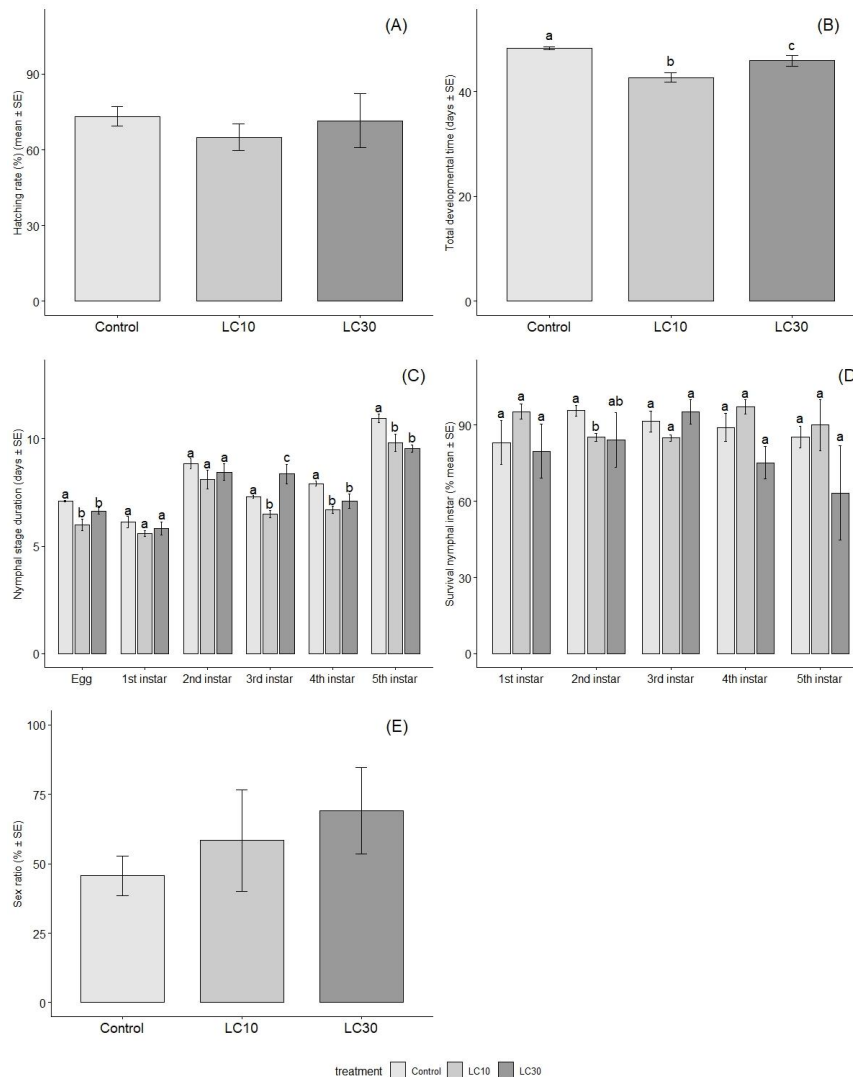


Figure 5. Effects of sublethal concentrations (LC₁₀ and LC₃₀) of lambda-cyhalothrin on the hatching rate of eggs (A), development duration of nymphs (B), total developmental time until adult stage (C), mortality of nymphs (D) and sex ratio (E) of the F1 generation of *Bathycoelia distincta*. Data are mean ± standard errors (SE), and the same lowercase letters within each parameter indicate that treatments are not significantly different from each other at $P > 0.05$.

4.5 Discussion

In this study, the toxicity and behavioural effects of four commercial insecticides used to control the stink bug pest *B. distincta* in macadamia orchards, were evaluated. The bioassays revealed that the organophosphate acephate was more toxic ($LT_{50} = 8h$) than the pyrethroids lambda-cyhalothrin ($LT_{50} = 36h$), beta-cyfluthrin ($LT_{50} = 30h$) and tau-fluvalinate ($LT_{50} = 36h$). In the behavioural bioassays, the pyrethroids induced an increase of the distance walked and a decrease of angular velocity, suggesting that commercial application of pyrethroids might result in a higher dispersal and low mortality. The effects of sublethal concentrations (LC_{10} and LC_{30}) of the active ingredient of lambda-cyhalothrin were subsequently assessed on the F0 and F1 generation of *B. distincta*. Knowledge regarding the sublethal effects of insecticides is relevant for insect pest management since the target species are often exposed to sublethal concentrations of these compounds for longer periods than to lethal concentrations as a consequence of insecticide degradation. In the F0 generation, the LC_{30} significantly reduced male longevity and female fecundity. For the F1 generation, sublethal concentrations (LC_{10} and LC_{30}) of lambda-cyhalothrin significantly accelerated the egg stage, and the 3rd, 4th and 5th instar developmental time compared to the control, resulting in a shorter developmental time.

In our study, toxicity bioassays revealed that *B. distincta* is more tolerant to pyrethroids than the organophosphate. The toxicity of pyrethroids and organophosphate insecticides to the Pentatomidae can be variable among species (Blackman et al., 2015; Cira et al., 2017; Lee, Short, et al., 2014; Leskey et al., 2012; Nielsen et al., 2008; Pazini et al., 2019; Snodgrass et al., 2005; Tillman & Mullinix, 2004). For example, *H. halys* was able to recover after exposure to various pyrethroids (Leskey et al. 2012; Nielsen et al. 2008), whereas Tillman and Mullinix (2004) showed that *E. servus* and *P. maculiventris* from Georgia were not able to recover from pyrethroid exposure. In the southeastern United States, *Euschistus servus* was more tolerant to pyrethroid and organophosphate insecticide exposures compared to *Chinavia hilaris* (Say), and *Nezara viridula* (L) (Snodgrass et al. 2005). The difference in tolerance observed in this study could therefore be a *B. distincta* specific trait, and other species associated with macadamia orchards might respond differently.

The higher tolerance of *B. distincta* to the commercial pyrethroids than the organophosphate could also be linked to management practices since the egg masses were collected from commercial macadamia orchards. For example, intensive and repeated applications of acephate and lambda-cyhalothrin linked to the expansion of soybean production in Brazil, reduced the susceptibility of *E. heros* to these insecticides considerably (Sosa-Gómez et al., 2020; Somavilla et al., 2020; Tuelher et al., 2018). In fact, the recommended doses of acephate increased four-fold in 14 years (Sosa-Gómez et al., 2020). Similarly, the rapid expansion of macadamia planted areas in South Africa has led to the increase of insecticide applications, especially pyrethroids, and a decline in the susceptibility in *B. distincta* populations has been suggested (Schoeman 2014a). Although this study constitutes the first toxicity assays conducted on *B. distincta*, future research should also evaluate individuals from populations with variable exposure histories.

Changes in locomotory behaviour of *B. distincta* were observed after brief exposure to commercial insecticide formulations. These effects are not surprising since neurotoxic insecticides were used which can trigger distinct behavioural responses (Desneux et al., 2007). In our study, sublethal doses of the pyrethroids increased the distance walked and decreased the angular velocity of *B. distincta* movements, while no differences were observed between bugs exposed to organophosphate and bugs without exposure. Lee et al. (2013) obtained similar results where pyrethroid insecticides caused immediate neurotoxicity on *H. halys* that resulted in rapid and uncoordinated movement compared to organophosphates where the effects were slower. Alterations in walking behaviour has been shown to be a strategy to overcome the action of insecticides (Haddi et al., 2015; Morales et al., 2013) and it is possible that surviving *B. distincta* could disperse from insecticide-contaminated areas, since stink bugs are highly mobile (Lee et al., 2014). Thus, further studies should test behavioural responses over longer time periods (i.e., a few hours) and test whether *B. distincta* can leave areas treated with pyrethroid and organophosphate residues.

In this study, the two sublethal concentrations (LC₁₀ and LC₃₀) of lambda-cyhalothrin did not impact female longevity whereas the LC₃₀ decreased male longevity significantly. Similar results have been observed in the mirid bug *A. lucorum*, where the authors suggested that the susceptibility to insecticides may be correlated to size and weight (Tan et al., 2012). However, Nielsen et al. (2008) tested several insecticides and observed that *H. halys* males

were less susceptible to thiamethoxam than females despite having smaller body mass. It is therefore unclear if body mass could explain the decreased male *B. distincta* longevity to lambda-cyhalothrin observed in this study. Other parameters such as detoxifying enzymes or target-site insensitivity may be involved. Previous studies have shown that detoxification of insecticides in pentatomids is associated with P450 monooxygenases, glutathione-S-transferases, α -esterase, and β -esterase enzymes (Bansal & Michel, 2018; Boff et al., 2022; Mittapelly et al., 2019; Sosa-Gómez et al., 2009; Sosa-Gómez et al., 2020; Sparks et al., 2020), and differential expression of P450s genes among sexes can occur (Huber et al., 2007; Musasia et al., 2013; Zuo & Chen, 2014). Thus, detoxification mechanisms might also occur in *B. distincta* and enzyme expression levels should be investigated in future.

The fecundity of females exposed to LC₃₀ of lambda-cyhalothrin was significantly reduced compared to the control. Several studies have demonstrated effects of sublethal concentrations of insecticides on fecundity (Bao et al., 2009; Wu et al., 2022) where females exposed can exhibit compensatory effects, resulting in a higher reproductive performance and a shorter life span (Santos et al., 2015; Vilca Mallqui et al., 2014). This has been demonstrated in female *E. heros* with sublethal concentrations of imidacloprid which increased their fecundity and fertility rates but reduced their longevity (Santos et al., 2015). In addition, another study showed that sublethal exposure of imidacloprid can increase the mating frequency of males and induced higher fecundity rates (Haddi et al., 2016). In our study no effect was observed on the hatching rate of eggs from females exposed and nonexposed. Further studies are required to determine if female *B. distincta* exposed to LC₃₀ were mated or not. Female and male reproductive organs and their morphology (e.g., testicles, sperm mobility, sperm storage, ovaries, ovarian cells, etc.) should also be investigated in future to determine the effects of sublethal exposure of lambda-cyhalothrin on the reproductive capacity of *B. distincta*, in order to explain the significant differences in fertility observed in this study.

Sublethal exposure of the parent generation (F0) to lambda-cyhalothrin stimulated F1 nymphal development in *B. distincta*, resulting in a shorter total developmental time to the adult stage and low overall mortality. Acceleration of development has been documented and the effect seems dependent on the insecticide, as well as the pest. For example, sublethal concentrations of imidacloprid shortened nymphal development in *A. gossypii* (Koo et al.,

2015; Yuan et al., 2017) and *A. glycines* (Qu et al., 2015) whereas in other species such as *Rhopalosiphum padi* (L.) (Li et al., 2018) or *Myzus persicae* (Sulzer) (Zeng et al., 2016), the duration of nymphal development was extended. Our results suggest that a low dose of lambda-cyhalothrin on *B. distincta* can accelerate development in the F1 generation which could increase the population size of this pest and likely cause resurgence and outbreaks in the field (Cordeiro et al., 2013; Guedes & Cutler, 2014) which may explain the decline of susceptibility in *B. distincta* populations observed previously (Schoeman 2014a).

In this study, various commercial insecticide formulations were tested on *B. distincta* adults and the sublethal effects of the active ingredient lambda-cyhalothrin were investigated in the F0 and F1 generation of *B. distincta*. Brief exposure to pyrethroids at field application rates stimulated the walking behaviour of *B. distincta* whereas at sublethal concentrations the pyrethroid lambda-cyhalothrin accelerated the development of offspring. Our results suggest that it is important to consider changes in mobility and effects that sublethal concentrations of insecticide could have on the development and reproduction of the target pest. These behavioural changes should be taken into consideration for IPM programs in macadamia orchards. Future experiments should be expanded to greenhouse and field conditions to confirm these results and to determine the mechanism of action of pyrethroids on *B. distincta*.

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Chapter 5

Evaluation of commercial lures for capturing stink bug species (Pentatomidae) associated with macadamia orchards in South Africa

5.1 Abstract

The South African macadamia industry is severely affected by a complex of stink bugs (Pentatomidae). Finding simple and reliable management tools for these species is imperative. No commercial pheromone products are available to monitor and control the stink bug species typically found in macadamia orchards. However, some cross-attraction with commercial lures has been observed within the Pentatomidae and may occur between stink bugs present in macadamia orchards in South Africa. The objective of this study was to screen commercially available lures for attraction of stink bugs associated with macadamia orchards. Four lures were tested, the Pherocon® CAP CSB, Pherocon® BMSB, Pherocon® GSB and Pherocon® BMSB DUAL (BMSB+GSB)) and trap catches compared to an unbaited control (no lure). Two trials were conducted in Limpopo and Mpumalanga over two periods, corresponding to phenological stages of macadamia nut development (from nut set to harvest). In Limpopo, the effectiveness of sticky traps attached directly in the trees and baited with the different lures were tested. In Mpumalanga, the lures were tested using two trap types, clear sticky traps on wooden stakes and black pyramidal traps. In parallel, the population density of *Bathycoelia distincta* and the lure release rates were also monitored. In trial 1, 47 stink bugs were captured (41 *Parachinavia prunasis*, 6 *B. distincta*, 1 *Nezara viridula*). In trial 2, 15 stink bugs were captured (5 *Coenomorpha nervosa*, 3 *B. distincta*, 2 *Caura rufiventris*, 4 unidentified bugs). The Pherocon® BMSB DUAL lures captured significantly more bugs than the other lures in both trials. No effect was observed between the type of traps in trial 2. A low density of stink bugs was recorded in both trials and the lure release rates confirmed the release of pheromone during the trials. The results suggest that the commercial lures tested are not attractive for stink bug species associated with macadamia orchards. Further experiments, including identification of pheromones of species present in orchards, need to be conducted before pheromone-baited traps can be implemented in the IPM program of the macadamia industry.

5.2 Introduction

There are a considerable number of economically important pest species of stink bugs (Hemiptera: Pentatomidae) (McPherson 2018; McPherson & McPherson 2000; Schaefer & Panizzi 2000). Phytophagous and polyphagous, pentatomids are a major concern for several agricultural commodities, destroying cultivated crops by direct feeding, causing pre-mature abortion or deformation of seeds, nuts and fruiting bodies (McPherson & McPherson 2000; Schaefer & Panizzi 2000). The feeding wounds can also indirectly result in losses by providing entry points for fungal and bacterial diseases, resulting in additional crop losses (McPherson & McPherson 2000; Schaefer & Panizzi 2000).

Stink bug pest management relies mainly on the application of synthetic organic insecticides. However, the intensive use of chemicals is accompanied by a negative impact on the environment and human health (Ali et al. 2021; Le Goff & Giraudo 2019). It has also led to the development of resistance towards pyrethroids and organophosphates in some stink bug populations (Sosa-Gómez et al. 2020). For example, significant differences in susceptibility to acephate has been observed between different populations of the Neotropical brown stink bug, *Euschistus heros* (Fabricius) in Brazil between two soybean growing seasons, where some individuals were up to 15-fold less susceptible to this insecticide. Additionally, application of insecticide is often incompatible with biological control and establishment of natural enemies (Lowenstein et al. 2019), leading to the need for sustainable alternatives such as the utilisation of semiochemicals.

Pentatomidae produce more than one type of pheromone that can potentially be used for management with some of them already commercially available (El-Sayed 2019; Millar 2005; Weber et al. 2018). For example, the compound methyl (2*E*,4*Z*)-2,4-decadienoate (MDD) was identified as the major male-produced aggregation pheromone component in several *Euschistus* species including *E. conspersus* (Uhler), *E. tristigma* (Say), *E. politus* (Uhler), *E. servus* (Say) and *E. ictericus* (L.) (Aldrich et al. 1991). This compound is also the major compound in the putative sex pheromone of *E. heros* (Aldrich et al. 1994; Borges & Aldrich 1994). Since its discovery, the MDD has been used to monitor populations of *E.*

servus and *E. tristigmus* in apple and peach orchards (Leskey and Hogmire 2005; Hogmire and Leskey 2006), and *E. conspersus* in processing tomatoes (Cullen and Zalom 2000). The aggregation pheromone of *P. stali*, methyl (*E,E,Z*)-2,4,6-decatrienoate or MDT has a similar chemical structure (Sugie et al. 1996). This has allowed the development of pheromone-based trapping methods to monitor this species in fruit trees (Adachi et al. 2007; Yamanaka et al. 2011). Similarly, the aggregation pheromone of *H. halys* has been identified as (3*S*,6*S*,7*R*,10*S*)-10,11-epoxy-1-bisabolen-3-ol and (3*R*,6*S*,7*R*,10*S*)-10,11-epoxy-1-bisabolen-3-ol (murgantiol or PHER) (Khrimian et al. 2014). This pheromone has been used successfully to monitor the abundance and phenology of *H. halys* across the United States (Leskey et al. 2015) and an attract and kill system has been developed recently in commercial apple orchards (Morrison et al. 2019).

The phenomenon of cross-attraction to heterospecific pheromones has been observed between various pentatomid species. For example, in the field *H. halys* and *Chinavia hilaris* (Say) are attracted to the aggregation pheromone of *P. stali* (Aldrich et al. 2007; Aldrich et al. 2009; Tillman et al. 2010). Furthermore, it has been reported that the combination of the PHER and MDT lures can attract more *H. halys* than either the aggregation pheromone or MDT alone (Weber et al. 2014; Weber et al. 2020). A recent study demonstrated that an analogous trienoate, ethyl (2*E*,4*E*,6*Z*)-decatrienoate (EDT), can enhance *H. halys* captures when combined with murgantiol (Rice et al. 2018a). MDT can also attract several other pentatomid species such as *Acrosternum hilare* (Say), *Podisus maculiventris* (Say), *E. tristigmus*, *Banasa* spp. (Say) or *Thyanta* spp. (Aldrich et al. 2007). Similarly, traps baited with *E. heros* pheromone have caught other Pentatomidae species such as *Piezodorus guildinii* (Westwood) and *Edessa meditabunda* (F.) in the field (Borges et al. 2011; Borges et al. 1998).

In South Africa, various stink bug species occur in macadamia orchards (Schoeman 2013; Schoeman 2018; Sonnekus et al. 2021; Van den Berg et al. 1999), causing nut damage worth approximately 200 million ZAR in losses annually (Schoeman 2013; Taylor et al. 2018). This complex includes species such as *Bathycoelia distincta* (Distant) (two-spotted stink bug), *Nezara viridula* (L.) (Green vegetable bug), *Chinavia pallidoconspersa* (Stål) (yellow-edged stink bug, formerly *Nezara pallidoconspersa*), *Parachinavia prunasis* (Dallas)

(formerly *Acrosternum prunasis* and *Nezara prunasis*), and *Pseudatelus raptorius* (Germar) (powdery stink bug, formerly *Atelocera raptorial*) (van den Berg et al. 1999; Schoeman 2018; Sonnekus et al. 2021). The presence and abundance of these species vary over time in correlation with the phenological stages of the macadamia tree throughout the season (Schoeman 2018). For example, some species such as *C. pallidoconspersa* and *P. prunasis* have been observed mostly during winter compared to *B. distincta* and *N. viridula* that are present more during the summer (Schoeman 2018). In contrast, these species were found commonly throughout the season (Sonnekus et al. 2022). Among the different species, *B. distincta* is regarded as the most abundant and one of the most damaging pests of the macadamia industry (Schoeman 2013; Schoeman 2018; Sonnekus et al. 2022; Van den Berg et al. 1999). This species can cause kernel damage throughout the entire season (Bruwer et al. 2021; Schoeman 2020), but other species such as *N. viridula*, *C. nervosa*, *C. pallidoconspersa*, *P. raptorius* and *Farnya* sp. are also considered to be damaging stink bug pests for macadamia orchards (Bruwer et al. 2021; Schoeman 2020).

In South African macadamia orchards, IPM programs are still mainly focused on the utilisation of insecticides (Schoeman 2014b). Although the alarm pheromone of *B. distincta* has been recently described (Pal et al. 2022), no commercial pheromone products are available to monitor and control the stink bug species typically found in macadamia orchards. However, some cross-attraction with the commercial lures MDT, PHER and MDD has been observed within the Pentatomidae and may occur for stink bugs present in macadamia orchards in South Africa as well. In this study, we present the results of a field trial using the commercial lures Pherocon® CAP CSB (containing MDD), Pherocon® BMSB (containing PHER), Pherocon® GSB (containing MDT) and Pherocon® BMSB DUAL (BMSB+GSB), to assess the attraction to pheromone-baited traps of *B. distincta* and other common stink bug species associated with macadamia orchards. We evaluated stink bug captures on two sites over two periods corresponding to phenological stages of nut development (from nut set to harvest). At the end of the macadamia growing season in 2021/2022, we compared the effectiveness of sticky traps attached directly in the tree and baited with these lures. For the start of the 2022/2023 season, we compared the effectiveness of clear sticky traps deployed on wooden stakes and standard pyramidal traps baited with the three commercial lures. This study will provide an indication of the potential for

utilisation of pheromone-baited traps with commercial lures in macadamia orchards for various species and if further research in terms of development of specific attractant needs to be conducted for stink bugs present on macadamia orchards in South Africa.

5.3 Materials and methods

Study sites

Traps were deployed in two commercial macadamia orchards located in Limpopo (Levubu, South Africa, 23°03'13"S, 30°14'02"E) and Mpumalanga (White River, South Africa 25°19'21.9"S 30°57'45.1"E). The block in Limpopo was bordered by mixed vegetation consisting primarily of Mexican weeping pine (*Pinus patula*) and avocado trees (*Persea americana*), whereas in Mpumalanga eucalypt trees were bordering the block. Traps were set out in Limpopo for a period of seven weeks between March 2022 to mid-April 2022 corresponding to the end of the macadamia growing season when the macadamia nuts are fully expanded and close to harvest. In Mpumalanga, traps were deployed at the beginning of the new macadamia growing season, between October 2022 to mid-December 2022 for a period of 10 weeks. During this period, macadamia nuts are still in development and pea sized.

Lure and trap deployment

In Limpopo (trial 1), four treatments were tested: Pherocon® CAP CSB lure (containing MDD), Pherocon® BMSB lure (containing *H. halys* aggregation pheromone murgantiol), Pherocon® GSB lure (containing MDT) and the Pherocon® BMSB DUAL lure (BMSB+GSB lures). Each lure was attached to a STKY™ Dual Panel Adhesive trap (double-sided clear sticky traps 15.2 x 30.5 cm) above the centre of the trap. Lures and sticky traps were supplied by Trécé® Inc. (Adair, OK, USA). A control was also included, consisting of a sticky trap without lure. Twelve replicates of each treatment were included, each replicate consisting of a row of traps with the different treatments randomly assigned. In total, 60 traps were deployed on the site. The traps were hung to the trees at approximatively 175 cm (Figure 1A). Each trap was separated by 35 m within rows and rows were spaced 20 m apart.



Figure 1. Traps deployed during the two study periods: (A) a double-side clear sticky trap attached to branch, (B) a standard black pyramid trap and (C) a double-sided clear sticky card attached to the top of a wooden stake. Traps were baited with four commercial lures, namely Pherocon® CAP CSB lure, Pherocon® BMSB lure, Pherocon® GSB lure and the Pherocon® BMSB DUAL lure (combination of BMSB+GSB lure).

At the Mpumalanga site (trial 2), the same lures as in trial 1 were tested, but with two different trap types. Each lure was attached to a STKY™ Dual Panel Adhesive trap (double-sided clear sticky traps 15.2 x 30.5 cm) which was placed at the top of a 1.5 m wooden stake (10 mm diameter), with approximately 0.20 m of the stake buried in the soil (Figure 1C). In addition to the sticky trap, a standard Dead-Inn black pyramidal trap (130 x 40 cm, Crop Watch, Komatipoort, Mpumalanga, South Africa) with a white collection jar at the top was also tested (Figure 1B). The lures were deployed inside collection jars along with a block of dichlorovous (Insect Science, Tzaneen, Mpumalanga, South Africa) as a killing agent to prevent insect escape. A control, with no lure, was included for each trap type. There were eight replicates of each treatment (lure and type combination, including control) representing a total of 80 traps deployed on the site. Treatments were randomized in each replicate. Traps were separated by 35 m within rows and rows were spaced 20 m apart.

Traps were monitored weekly in Limpopo and every two weeks in Mpumalanga. Damaged or missing traps were replaced as needed. The BMSB and GSB lures were replaced after seven weeks and CSB lures were replaced after four weeks. Pentatomids and other species were collected and conserved in 70% ethanol for transportation and storage until identification. To confirm the presence of *B. distincta* in the orchard, the standard protocol for scouting used by commercial growers was used, where ten randomly selected macadamia trees were sprayed with insecticide weekly and the number of *B. distincta* collected per tree were counted.

Pheromone dispenser release rate

Although, the concentration and diffusion rate of the lures was proprietary information of Trécé® Inc. company, the release rate of the lures was checked under controlled condition. The volatiles from the CSB, BMSB, GSB lures were collected on a SPME fibre (n = 3) and release rates were determined via the method described by Guignard et al. (2020). The dual lure (BMSB+GSB) is a combination of the BMSB and GSB and was not included in the analysis. All lures were placed on a cleaned and oven dried (110°C overnight) metallic grid where each lure occupied a 2 cm² spot and was separated from the next lure by an empty 2 cm² square. Lures were left on the plate in a closed climate-controlled room at 25°C at ambient humidity. Release rates of the three different pheromone dispensers were measured immediately after opening (T = 0 days) and weekly, until 7 weeks for the BMSB and GSB lures and until 4 weeks for the CSB lures. The release rate of the pheromone dispenser displayed in the field during the trial 1 (n = 3) were also checked after 4 weeks for the CSB lures, and 7 weeks for the GSB and BMSB lures.

Lures were placed in a clean and oven dried hermetic glass jar placed in a 25 °C controlled water bath for measurement. The relative release rates of the lures were performed with a SPME fibre (dvb/car/pdms, Supelco 57328-U) inserted through a septum in a glass jar for 10 min. Three replicates per type of lure were analysed, and all the analyses were conducted with the same SPME fibre during the study period. A blank (empty glass jar) was performed at the beginning of each series of analysis. The volatiles were analysed by GC-MS (Agilent 7890B coupled to a 5977B MSD) equipped with an HP-5MS UI column (30 m × 0.250 mm I.D. × 0.25 µm film; 122-7032; Agilent). The SPME fibre was desorbed through a SPME

inlet liner at 250°C for 4 min in splitless mode (vent time at one minute and flow 20 mL/min). Constant column flow of 1.2 mL/min (helium) was used. The oven temperature was maintained at 50°C for 2 min, ramped at 10°C min⁻¹ and then maintained at 300°C for 2 min (total run, 29 min). Electron impact ionization (70 e-V) was used to generate ions. The mass spectrophotometer was operated in scan mode (*m/z* 45-550). The ion source and MS quadrupole temperature were heated at 230°C and 150°C, respectively.

Statistical analysis

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. The number of stink bug captures was analysed using a generalized linear model with a zero-inflated (ZI) Poisson distribution because of an overabundance of zero observations in the dataset and with the lure treatment as fixed explanatory variable. A Tukey's post hoc comparisons was conducted afterward. In field trial 2, the same GLM ZI Poisson model and post hoc analysis was conducted, and with the lure treatment as fixed explanatory variable and type of trap was used as a random variable.

5.4 Results

Stink bug captures compared to lure and trap type

Trial 1: Comparison of lures using sticky traps

In total, 47 stink bugs were captured between March and April 2022 in Limpopo (6.71 ± 1.43 insects captured per week) (Figure 2A). The highest number of bugs captured was observed during weeks 2 and 3, with 13 and 11 stink bugs captured, respectively during weeks 2 and 3 (Figure 2A). The type of lure significantly affected the captures of stink bugs on clear sticky traps (Figure 3A, GLM ZI Poisson $\chi^2 = 12.22$; *df* = 4; $P = 0.016$). The BMSB (1.00 ± 0.58 bugs captured) and GSB (1.57 ± 0.48 bugs captured) attracted significantly more bugs than the control treatment (0.43 ± 0.20 bugs captured) (Tukey HSD, $P < 0.05$). In addition, the dual lure BMSB+GSB captured significantly more bugs (3.43 ± 0.61 bugs captured) than either the BMSB (1.00 ± 0.58 bugs captured) or GSB (1.57 ± 0.48 bugs

captured) lure alone. No significant differences were found between CSB lure, BMSB lure and the control treatment.

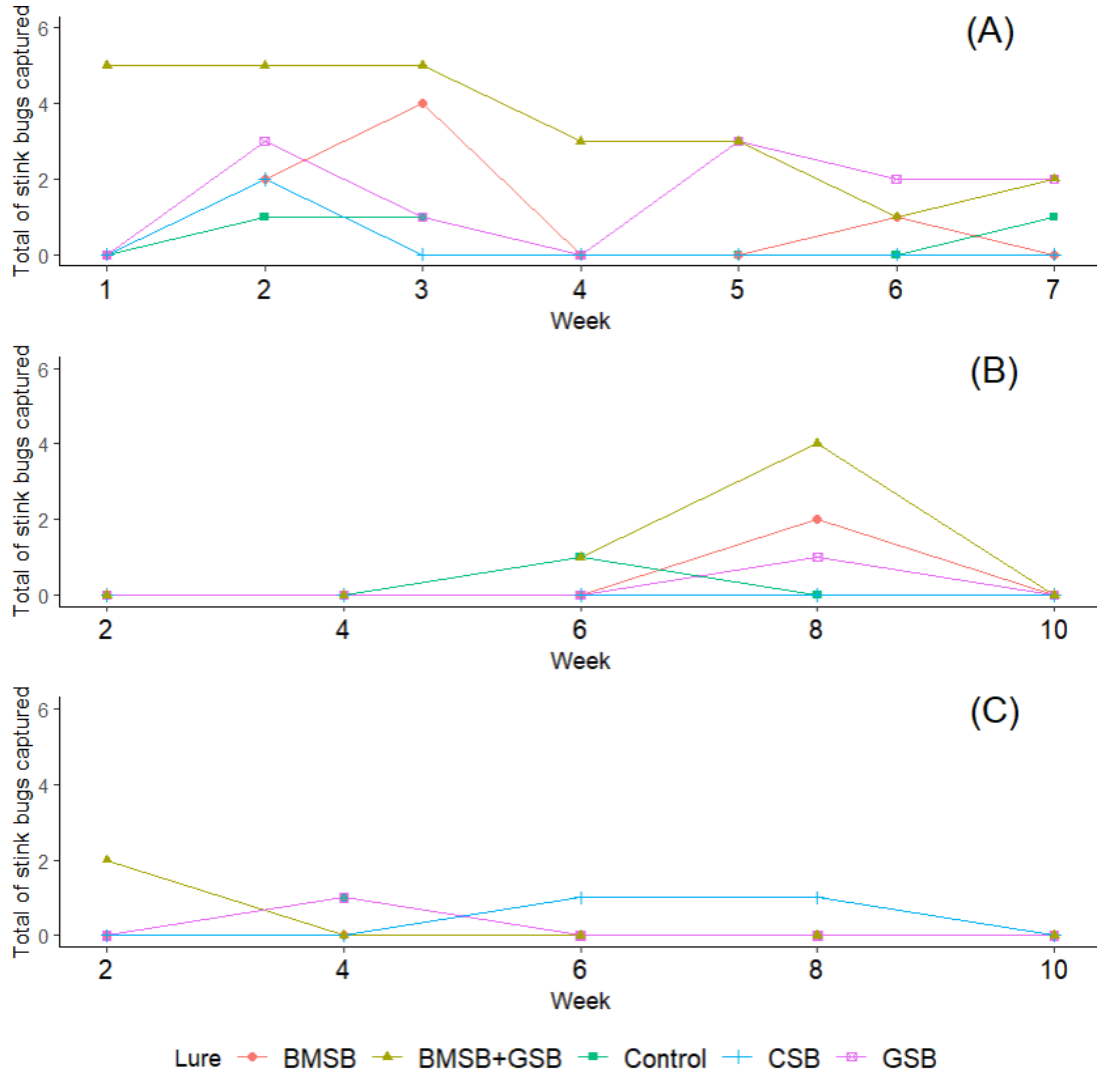


Figure 2. Number of weekly captures of stink bugs in total in trial 1 with sticky traps hung in macadamia trees (A), in trial 2 with sticky traps attached to wooden stakes (B) and in trial 2 with pyramid traps (C). The control remained unbaited, while the CSB refers to the Pherocon® CAP CSB lures, GSB refers to the Pherocon® GSB lures, BMSB refers to the Pherocon® BMSB lures, and BMSB+GSB refers to the Pherocon® BMSB DUAL lures (combination of BMSB+GSB lure).

A total of six *B. distincta* were captured, including five adults and one nymph. Forty-one other insects were captured and identified as *Parachinavia prunasis* (40 bugs captured) and *Nezara viridula* (one individual). Between March and April 2022, scouting revealed that the

average number of *B. distincta* present in the macadamia orchard in Limpopo ranged from 0 – 2 bugs/10 trees (Table 1A).

Table 1. Numbers of *B. distincta* recovered in macadamia orchard via scouting method during the seven weeks of experiment in Limpopo (A) and Mpumalanga (B).

(A)

week	<i>B. distincta</i> per 10 trees
1	2
2	1
3	0
4	0
5	1
6	0
7	0

(B)

week	<i>B. distincta</i> per 10 trees
1	2
2	2
3	7
4	2
5	0
6	0
7	0
8	0
9	0
10	0

Trial 2: Comparison of lures using sticky traps on wooden stakes and pyramidal traps

In total, 15 stink bugs were captured, with 1.20 ± 0.32 and 1.80 ± 1.15 bugs captured every two weeks on sticky traps deployed on wooden stakes and pyramid traps, respectively (Figure 2B and 2C). On sticky traps attached to wooden stakes, the number of captures was the highest during week 8, with seven stink bugs captured in total (Figure 2B). In comparison, the number of captures on the pyramidal traps were lower with a maximum of two bugs captured after the two first weeks (Figure 2C). Overall, no significant effect of the type of lure (GLM ZI Poisson $\chi^2 = 3.34$; $df = 4$; $P > 0.05$) and the type of traps was detected (GLM ZI Poisson $\chi^2 = 0.28$; $df = 1$; $P > 0.05$) (Figure 3B).

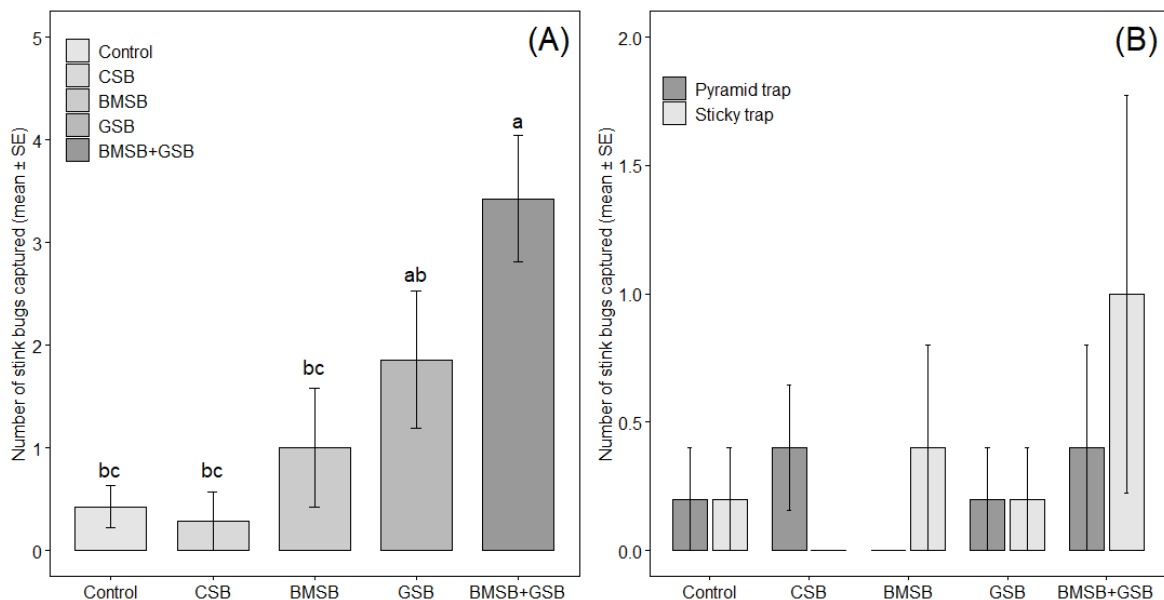


Figure 3. Total number of stink bugs captured (mean $\pm$ SE) during trial 1 in Limpopo (A) and trial 2 in Mpumalanga (B). Letters indicate significant differences between lure tested (GLM ZI Poisson, $P < 0.05$). In the trial 2, no significant differences were observed between pyramid and sticky traps for the same lure tested. The control remained unbaited, while the CSB refers to the Pherocon® CAP CSB lures, GSB refers to the Pherocon® GSB lures, BMSB refers to the Pherocon® BMSB lures, and the BMSB+GSB refers to the Pherocon® BMSB DUAL lures (combination of BMSB+GSB lure).

In terms of species captured during trial 2, only three nymphs of *B. distincta* were captured. The other stink bugs captured were identified as *Coenomorpha nervosa* (five bugs captured),

Carbula recurva (one bug captured), *Caura rufiventris* (two bugs captured) and four unidentified individuals. Scouting data between October and December 2022 indicated that the average number of *B. distincta* present in the macadamia orchard in Mpumalanga ranged from 0 – 7 bugs/10 trees (Table 1B).

Pheromone lure release rates

Under controlled condition, chemical analysis of the three different lures showed that after one week the relative amount released by the lures decreased by 34.04 ± 5.91 % for the CSB (Figure 4A), 37.52 ± 5.83 % for the GSB (Figure 4B) and 12.57 ± 1.44 % for the BMSB (Figure 4C).

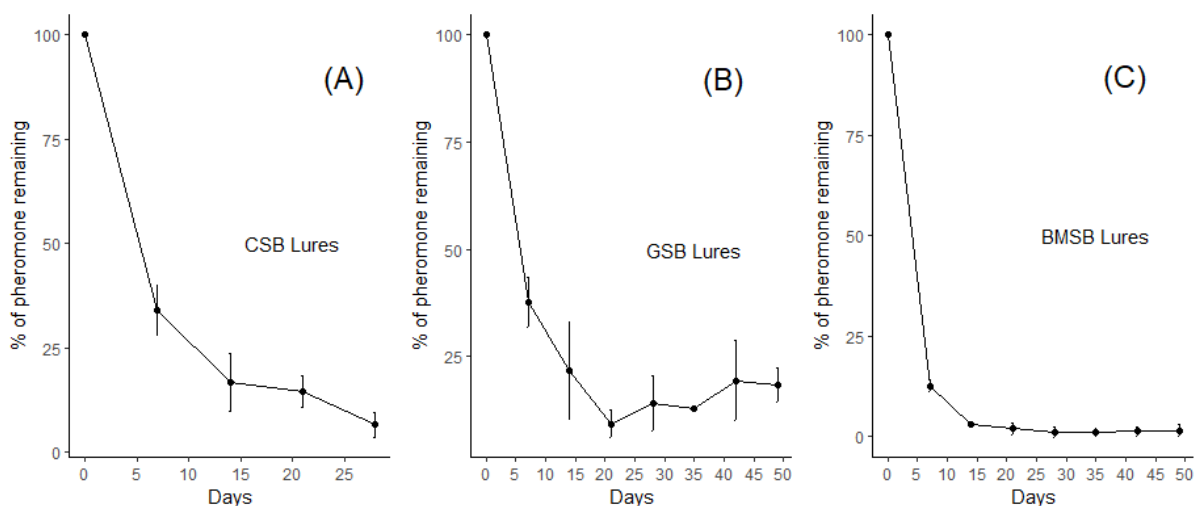


Figure 4. Percentage of pheromone that remained in CSB lure dispensers (A), GSB lure dispensers (B) and BMSB lure dispensers (C) measured under controlled conditions. The CSB refers to the Pherocon® CAP CSB lures, GSB refers to the Pherocon® GSB lures, and BMSB refers to the Pherocon® BMSB lures.

After 4 weeks in the field, the percentage of remaining pheromone from the CSB lures were 16.16 ± 2.70 % (Figure 5). After 7 weeks, 25.92 ± 9.86 % and 26.55 ± 5.09 % of pheromones were remaining from the GSB and BMSB respectively (Figure 5).

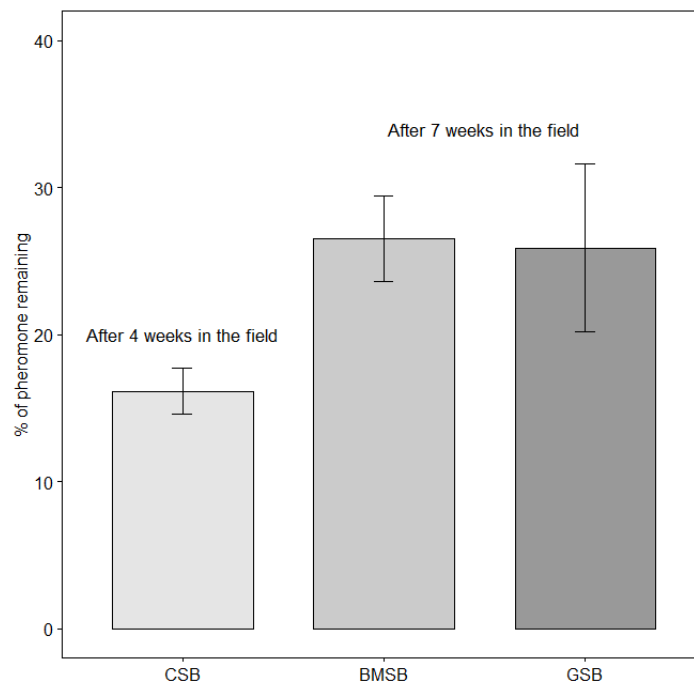


Figure 5. Percentage of pheromone that remained in lure dispensers displayed in the field during the trial 1. The CSB lures were measured after 4 weeks and the GSB and BMSB after 7 weeks. The CSB refers to the Pherocon® CAP CSB lures, GSB refers to the Pherocon® GSB lures, and BMSB refers to the Pherocon® BMSB lures.

5.5 Discussion

In this study, we tested the attraction of four commercial lures on stink bugs commonly found in macadamia orchards in South Africa. Two trials were carried out with these lures, one trial in Limpopo between March and April 2022 and a second trial in Mpumalanga between October and December 2022. Significant differences were observed between stink bug captures and type of lure; the dual lure BMSB+GSB captured more stink bugs in both trials. No significant differences were observed between the type of traps tested in trial 2. In terms of captures, the overall capture rate was low, however, the abundance of *B. distincta* was also low during both trials. Furthermore, the release rate of the lures was tested under controlled conditions, and chemical analysis confirmed that the compounds can be released during 7 weeks for the CBS and up to 10 weeks for the GSB and BMSB lures.

The dual lure BMSB+GSB attracted significantly more stink bugs, mostly represented by *P. prunasis* in trial 1 and by *C. nervosa* in trial 2. The phenomenon of cross attraction has been observed in the Pentatomidae, where pentatomid species can be cross attracted to pheromone blends of other species (Weber et al. 2018). For example, methyl (2*E*,4*E*,6*Z*)-2,4,6-decatrienoate, the aggregation pheromone of *P. stali* (Sugie et al. 1996) can cross attract several species from the Nezarini tribe (Adachi et al., 2007; Aldrich et al. 2009; Tillman et al. 2010). In South Africa, three species of the Nezarini tribe are present in the macadamia orchards, namely *N. viridula*, *C. pallidoconspersa* and *P. prunasis* (Schoeman 2013, 2014b; Sonnekus et al. 2021). Thus, attraction of *P. prunasis* by methyl (2*E*,4*E*,6*Z*)-2,4,6-decatrienoate on the sticky traps in trial 1 may represent another example of cross attraction considering the number of captures observed.

Previous studies have shown that sticky traps baited with pheromones can successfully attract insect pests, constituting a reliable method for monitoring agricultural pests such as stink bugs (Acebes-Doria et al. 2018; Morrison et al. 2017; Toyama et al. 2015). For example, in 2016 and 2017, clear sticky traps on a wooden post and baited with PHER and MDT pheromones were able to detect the presence of nymphs and adults *H. halys* even at relatively low population densities (2016: 1.93 ± 0.16 adults/trap/week, 2017: 0.88 ± 0.06 adults/trap/week) (Acebes-Doria et al. 2018). Thus, it is possible that the low number of captures obtained here could be attributed to the low abundance of adults in the orchards during the two study periods. In our study, we reported a *B. distincta* population density ranging between 0-2 insects/10 trees in Limpopo and 0-7 insects/10 trees in Mpumalanga which is low for each period of the season compared to previous studies (Schoeman 2013; Schoeman 2018). In Mpumalanga, up to 59 *B. distincta* individuals per month were recovered between November 2010 and March 2013 (Schoeman 2013), representing an abundance of 6.82 to 8.64 insects/10 trees for *B. distincta* (Schoeman 2018). Furthermore, the chemical sprays used for scouting could have affected our trials and might be a factor to consider for explaining the stink bug abundance observed.

In this study, sticky traps attached to wooden stakes and attached directly to tree branches, as well as black pyramidal traps were tested. Positioning and deployment strategies are important factors to consider for trapping effectiveness (Drummond et al. 1984; Knight &

Light 2005). Previous studies have tested the effect of trap height for *H. halys*, but overall results showed a significant decrease of sensitivity compared to traps deployed at ground level (Leskey et al. 2012; Morrison et al. 2015; Rice et al. 2018b). Standard pyramid traps hung in trees capture fewer insects, especially nymphs (Morrison et al. 2015). Similar patterns have been observed with sticky traps deployed directly in trees (Rice et al. 2018b) or attached to wooden posts on the ground (Acebes-Doria et al. 2018). This is probably because stink bugs have a natural tendency to climb up vertical surfaces, showing negative geotaxis (Millar et al. 2010; Morrison et al. 2015). Furthermore, plant architecture may obscure the visibility of the trap (Koivula et al. 2003; Morrison & Szendrei 2013) and affect the trap retention capacity as well as the diffusion of the semiochemicals by creating an area of arrestment (Blaauw et al. 2017; Morrison et al. 2016). For example, adults and nymphs of *H. halys* are attracted to the PHER and MDT pheromones but are subsequently arrested by these stimuli within approximately 2.5 m of the stimulus source regardless of dose or release rate (Morrison et al. 2016). Deploying a pyramidal or sticky trap in an open space such as the ground, rather than in a tree, reduces the number of structures on which stink bugs could be arrested before they reach the trap (Acebes-Doria et al. 2018; Morrison et al. 2015).

In trial 1, sticky traps attached directly to macadamia branches captured stink bugs. We were expecting to capture more bugs in trial 2 with sticky traps attached to a wooden stake that we positioned in a more open area to allow semiochemical diffusion, but the number of captures was low. Macadamia trees are very dense and tall and stink bugs such as *B. distincta* seems to shelter in the canopy (Schoeman 2014a), which might explain why we observed more captures in trial 1. However, no sticky traps attached directly to macadamia trees were tested in trial 2. Therefore, future trials including both trap positions need to be tested at the same time to confirm if the position of the trap is an important factor for stink bugs in macadamia orchards. Although the two types of sticky traps were deployed during two different trials, our results suggest that sticky traps might be more efficient than the pyramidal traps.

Chemical analyses revealed that the lures tested remained active throughout the trials. However, the release rates decreased over time among the lures tested. The release from the lure dispensers can be influenced by many factors such as vapor pressure of compounds,

chemical affinity to the materials used and solvents, and temperature, leading to a decline of the release rate over time (Kuenen & Siegel 2015; Kovanci et al. 2006). Considering the difference of materials between the lures tested these factors might explain the variation in decrease observed between the lures. In addition, release rate is an important parameter that can influence the trapping success. Captures of *H. halys* were higher with traps baited with fresh lures with a high release rate of MDT than with traps baited with aged lure and low release rate (Joseph et al. 2013), which was also observed in Acebes-Doria et al. (2018) study. Considering that higher temperatures can be encountered in the field compared with the laboratory measurements, this could explain the fluctuations of captures observed some weeks. Nevertheless, the low number of captures observed in this study could be also attributed to incorrect stereochemistry of the compounds, incorrect ratios, and unsuitable release rates for species associated with macadamia orchards.

In summary, the results presented here suggest that the four commercial lures tested here are not attractive for stink bug pest species associated with macadamia orchards in South Africa. More studies need to focus on the identification of pheromones and formulations of specific lures to target stink bugs of importance in macadamia orchards, including *B. distincta*. Other practical issues also need to be addressed before new pheromone lures can be tested in macadamia orchards. For example, the two trials were done on commercial farms where intense chemical spray programs are conducted which influenced our results. To prove that pheromone-baited traps can attract stink bugs, future experiments need to be conducted in pesticide-free orchards. Additional factors, such as cost associated with the deployment of pheromone lures and traps, including labor and time required to monitor the pheromone-baited traps, also need to be considered.

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Chapter 6

General discussion and conclusions

The implementation of a pest management program is complex and requires an in depth understanding of the biology of the targeted pest. In this thesis, specific aspects of the biology and ecology of *Bathycoelia distincta* (Pentatomidae) were studied in order to improve our ability to control it. A number of findings from this thesis have implications in terms for pest management in the macadamia industry of South Africa.

6.1 Implications

In this thesis, no evidence of genetic differentiation was observed between individuals from the three major macadamia production areas in South Africa. This result was unexpected since some of the sampling locations were particularly far from each other. The absence of cryptic species and high gene flow was also demonstrated, and these findings suggest high dispersal capabilities of this pest in its native range, likely linked to the rapid expansion of macadamia plantations in South Africa. This information is useful for pest management programs since the effectiveness of pest control can vary among genetically distinct populations. These findings also constitute a starting point for future genetic studies on this species.

In South Africa, the management of *B. distincta* is still limited primarily to the utilisation of neurotoxic insecticides, mostly organophosphates and pyrethroids. The utilisation of semiochemicals for control of this pest has never been investigated before, and incorporation of semiochemicals could lead towards more environmentally friendly pest control solutions. Thus, some aspects of the chemical ecology of *B. distincta* with the identification of the alarm pheromone were investigated in this thesis. This pheromone was characterised providing an insight into the alarm system in this pest, which could be integrated into IPM programs in the future and will also help future chemical ecology work on *B. distincta*.

Since the utilisation of insecticides is prevalent in macadamia orchards, this thesis also investigated the lethal and sublethal effects of various commercial insecticides commonly used in the management of *B. distincta* to determine the susceptibility of this pest. Lethal and sublethal exposure showed that pyrethroids are less toxic for this pest and can induce an increase of the pest movement compared to the organophosphate tested. The sublethal effects of the pyrethroid lambda-cyhalothrin, the insecticide most commonly used by macadamia

growers, were also investigated on biological parameters of two generations (F0 and F1) of *B. distincta*. The findings revealed that sublethal concentrations of lambda-cyhalothrin can accelerate the development of this pest. These results highlighted the importance of considering the sublethal effects of insecticides in pest management programs and might explain why it is currently difficult to control *B. distincta* in macadamia orchards.

Finally, this study tested various traps baited with commercial lures as a management tool for stink bugs associated with macadamia orchards. The results of these trials suggested that the lures tested are not attractive for stink bugs present in macadamia orchards in South Africa. This study revealed the importance to identify specific pheromones to target stink bugs such as *B. distincta* before this control strategy in macadamia orchards in South Africa can be implemented.

6.2 Future directions and research needs

The results of this thesis have the potential to improve the management of *B. distincta* but also provide a better understanding of this pest associated with macadamia orchards in South Africa.

The population genetic study provided an insight regarding the movement of this pest, linked with the expansion of the macadamia industry. Nevertheless, further analysis with other genetic markers should be considered. For example, microsatellite markers could shed light on genetic differentiation that may occur between farms that employ different management practices. Such population genetic differences should also be investigated between the Limpopo province, the area where the pest was first discovered, and KwaZulu-Natal, the province which contains the largest area of newly established macadamia plantations. The description of *B. distincta* genome should also be determined which could lead to the development of RNAi and CRISPR-Cas9 tools for future integrated management strategies.

Genomic and transcriptomic data can also help to determine the insecticide resistance status of this pest. The data generated in this thesis suggest that *B. distincta* is less susceptible to pyrethroids than organophosphate. Thus, the mechanism of action of pyrethroids needs to be investigated. The activity of detoxification enzymes as well as the genes involved must

be studied among stink bug populations from various macadamia growing regions. This could also help in the future to prevent and monitor the insecticide resistance of *B. distincta* in South Africa.

In terms of pest management control, more behavioural tests must be conducted to determine the optimal ratio and concentration of the alarm pheromone compounds to induce an alarm effect on *B. distincta*. Semi-field experiments should also be considered to investigate the potential of this pheromone in the field. Other pheromone such as the aggregation pheromone of *B. distincta* needs to be characterised and tested in the field. Tactics such as attract and kill or push and pull systems have potential and need to be tested in macadamia orchards. Furthermore, recent studies have showed that plant volatiles in combination with aggregation pheromone can improve trapping and trap cropping methods. Thus, future studies should also look at macadamia plant volatiles for improving trapping methods for *B. distincta*.

Various other aspects should also be investigated. Although, we observed that this pest has a high capacity of dispersion, this species overwintering sites remain unknown. Scouting surveys showed that stink bugs arrive in the macadamia orchards as adults early in the nut season, but more studies must be conducted to determine the area of dispersion of this pest and what the other food sources of *B. distincta* are. The development of the biological control is also important. The presence of parasitoids on *B. distincta* has been observed in macadamia orchards and more research should focus on this aspect. Finally, the disruption of symbiont acquisition is an interesting option to control stink bugs, and this should be tested for *B. distincta*.

6.3 Conclusions

The research conducted on *B. distincta* significantly improved our understanding of the ecology and management of this stink bug species in macadamia orchards in South Africa. In this thesis, various aspects linked to the integrated pest management were investigated and provide a foundation for future management tools. Currently, the macadamia industry is facing a dilemma with the utilisation of insecticides and the need to develop alternative management option for stink bug pests is crucial for the future of the industry. An

interdisciplinary approach is needed to understand the ecology of a targeted pest in order to implement an adequate management program. Although much more is needed to completely understand *B. distincta*, this thesis aimed to contribute to both the future management of this pest, and the understanding of the fundamental mechanisms underlying genetic, chemical and physiological aspects in stink bugs.

