

CHAPTER 7

Host-Associated Kairomones Used for Host and Mate Finding in the Parasitoid *Lariophagus distinguendus* (Hymenoptera: Pteromalidae)

Abstract Males and females of the parasitic wasp *Lariophagus distinguendus* (Förster) respond to volatiles emitted by the larval faeces of one of their hosts, the granary weevil *Sitophilus granarius*. Previous studies have shown that attractive chemicals are emitted by astigmatid mites living in the host faeces and that these cues are attractive only for experienced parasitoids. In the present study we demonstrate that larval faeces of the host and headspace extracts of the faeces are attractive for both sexes of the parasitoid even when the mites were experimentally excluded from the beetle rearings. The response to volatiles from mite-free host faeces is innate. In order to elucidate the chemistry of this odour, headspace extracts were fractionated by adsorption chromatography. Tests using combinations of fractions of different polarities revealed that both the nonpolar pentane and the polar methanol fraction were necessary to maintain the attractiveness. This indicates that the attractive odour is composed of a complex blend of components with different polarities. The composition of the polar fraction was analysed by gas chromatography-mass spectrometry whereas structure elucidation of the nonpolar components was impossible so far. By orientating toward the same host-related volatiles used by females for host finding, *L. distinguendus* males may be arrested in patches of potentially high female density and thus, increase their chance of mating.

Key words *Lariophagus distinguendus*, mate finding, host finding, *Sitophilus granarius*, larval faeces, kairomone.

Introduction

Parasitic Hymenoptera use a variety of infochemicals for the location of food, mates or oviposition sites (Godfray, 1994). For host location and recognition, female parasitoids orientate toward volatile and non-volatile chemicals released by their host (e.g., cuticular hydrocarbons, pheromones) or its products (faeces, silk, exuviae), by the host's food plant (volatiles induced by feeding or oviposition) or by organisms associated with the host presence (bacteria, fungi) (Vet and Dicke, 1992; Quicke, 1997; Steidle and van Loon, 2002; Hilker and Meiners, 2006). Thereby, the response of the parasitoids to these host-related kairomones can be determined by physiological and genetic parameters but also be affected by environmental factors and particularly by experience (Vet et al., 1995). Numerous studies have demonstrated that parasitoids are able to associate profitable environmental cues such as volatiles with the presence of their hosts (Turlings et al., 1993; Vet et al., 1995).

Long-range orientation during mate finding of parasitic wasps is supposed to be mediated mainly by pheromones (Godfray, 1994; Quicke, 1997; Kainoh, 1999). Female-derived sex attractants of high volatility have been shown to attract males over long distances (e.g., Swedenborg et al., 1994; McNeil and Brodeur, 1995; Jewett and Carpenter, 1999). However, in several parasitoid species females appear to produce only relative low-volatile pheromones that stimulate male courtship behaviour but do not mediate long-range attraction (e.g., Finidori-Logli et al., 1996; Ruther et al., 2000; Steiner et al., 2006). In these species, males have to rely on other infochemicals than pheromones for long-range orientation toward females. One option for these males to reach the vicinity of females is to use the same host-associated volatiles as the females use for host finding. However, this aspect has been widely neglected in the literature so far. One of the very few parasitoid species in which a male response to host-associated volatiles has been demonstrated is *Lariophagus distinguendus* (Förster) (Hymenoptera, Pteromalidae), a polyphagous larval and pupal ectoparasitoid of stored product infesting beetles (Steidle and Schöller, 1997). Males and females of this parasitoid use volatiles emitted by the larval faeces of one of its hosts, the granary weevil *Sitophilus granarius* for host and mate finding (Steidle and Schöller, 1997; Ruther and Steidle, 2000). Remarkably, the response by the parasitoids is associatively learnt (Ruther and Steidle, 2000; Steidle et al., 2003). Furthermore, the active

compounds do not originate directly from the host faeces but from the host-associated astigmatid mite *Tyrophagus putrescentiae* (Schrank) living in the faeces (Ruther and Steidle, 2000). These astigmatid mites are especially abundant in so-called hot spots, sites of intense primary infestation by beetle pests (Sinha, 1961). However, hosts of *L. distinguendus* also occur in areas of lower infestation where moisture-sensitive mites are missing. Field observations demonstrate that hosts are located and parasitised by *L. distinguendus* under these conditions as well (Steidle, personal observation). Thus, the question arises which kairomones are used by *L. distinguendus* for host and mate finding when no mites are present.

The present paper aims to investigate kairomones for host and mate finding in *L. distinguendus* under conditions of low host infestation in the absence of host-associated mites. Therefore, we studied whether *L. distinguendus* males and females are attracted innately to larval faeces of the host *S. granarius* from cultures in which secondary infestation by astigmatid mites has been experimentally prevented. To characterise the chemical composition of potential kairomones, fractionated headspace extracts from larval host faeces were monitored for bioactivity and active fractions were analysed by coupled gas chromatography-mass spectrometry (GC-MS).

Methods and materials

Insect rearing To establish a mite-free *S. granarius* culture, batches of weevils were washed under warm water to free them from the astigmatid mites and dried with a hair dryer. Cultures of hosts and parasitoids were kept in the laboratory at a constant temperature of 25°C, a relative humidity of 55 ± 5% and a photoperiod of 16:8 h (L:D). Under these rearing conditions secondary infestation by moisture-sensitive astigmatid mites is prevented. Nevertheless, weevil cultures were regularly controlled for the presence of astigmatid mites both optically with a stereo microscope and chemically by GC-MS analyses of headspace extracts from larval faeces (see below). Only larval faeces that did not contain typical mite volatiles such as neral, geranial, neryl formate and tridecane (Kuwahara, 1991; Ruther and Steidle, 2000) were used for behavioural experiments and chemical analyses.

To obtain host larvae of known age, 100 ml of adult *S. granarius* were kept on 1000 ml wheat grain (*Triticum aestivum* L., variety Batis) with about 14% moisture content in plastic containers (19 cm width x 19 cm length x 6 cm height). Weevils were allowed to lay eggs in the grains for 7 days and were then removed. To rear *L. distinguendus*, approximately 100 freshly emerged parasitoids were placed in Petri dishes (9 cm diameter x 1 cm height) with 60 ml weevil-infested grains containing 21- to 28-day-old host larvae and kept there until their death. This host age is known to be optimal for parasitisation by *L. distinguendus* (van den Assem, 1971). Parasitoids emerged 18 to 21 days after host larvae had been parasitised.

Insects for bioassays Only males and females without any mating or oviposition experience were used in our experiments. To obtain naïve individuals parasitised grains containing parasitoids shortly before emergence were kept individually in 1.5 ml microcentrifuge tubes. After emergence, parasitoids were held in single-sex groups of at most 15 individuals in Petri dishes outfitted with moistened filter paper. One hour before experiments, parasitoids were individually placed in microcentrifuge tubes for acclimation at room temperature. Individuals tested were 1 to 2 days old.

Static four-chamber olfactometer The behavioural response of inexperienced *L. distinguendus* to potential volatile attractants in the larval host faeces was investigated by using a static four-chamber olfactometer as described by Ruther and Steidle (2000). No airflow was generated. In one chamber, the odour sample was placed in a small Petri dish (5.5 cm diameter). The opposite chamber was used as control chamber and the remaining two chambers adjacent to the test chamber were considered as buffer zones. The olfactometer was covered with a walking arena (19 cm diameter x 1 cm height) made of plastic gauze (mesh 0.5 mm) and finally with a glass plate to prevent parasitoids from escaping. At the start of each bioassay, a single parasitic wasp was released into the arena and the time it spent within each of the four sectors above the chambers (arrestment time) was recorded for 10 min by using the Observer programme 3.0 (Noldus, Wageningen, The Netherlands). Parasitoids that walked for less than 50% of the total observation time were assumed to be unmotivated and not included in the statistical analysis. The olfactometer was rotated clockwise by 90° after each replicate to prevent biased results due to possible side preferences of the parasitoids. Walking arena

and glass plate was regularly cleaned with ethanol and demineralised water. Odour sources were changed after five individuals had been tested.

Experiment 1: Activity of the larval faeces This experiment was performed to investigate the attractiveness of mite-free host larval faeces to *L. distinguendus* males and females. For this purpose, larval faeces from *S. granarius* were obtained by sieving grains infested by 21- to 28-day-old weevil larvae. In the experiment, 150 mg host faeces were offered to parasitoids of both sexes, whereas the control chamber contained only brownish filter paper discs (4 cm diameter) ($N = 17$ for females; $N = 22$ for males).

Experiment 2: Activity of headspace extracts of the larval faeces Volatiles emitted by the mite-free faeces were collected using the closed-loop stripping (CLS) technique. For this purpose, volatiles emitted from 5 g larval faeces were trapped for 4 h on a 1 mm charcoal layer (5 mg) of a CLS-adsorption tube (65 mm length x 5 mm diameter) (Gränicher & Quartero, Daumazan, France). Circulating air leaving the vacuum pump was purified before entering the sample container using another adsorption tube (75 mm length x 6 mm diameter) containing 150 mg activated charcoal. The volatiles were eluted twice with 25 μ l dichloromethane each. The combined eluates were applied on a filter paper disc and the solvent was allowed to evaporate for 15 min. Then the treated filter paper disc was offered to male and female parasitoids in the olfactometer. The control chamber contained a filter paper disc treated only with pure solvent ($N = 31$ for females; $N = 30$ for males).

Experiment 3: Activity of fractions Active headspace extracts of larval faeces (prepared as described in experiment 2) were fractionated by adsorption chromatography on a 500 mg silica gel cartridge (IST, Mid-Glamorgan, UK). For this purpose, 400 μ l headspace extract were concentrated to 80 μ l under a stream of nitrogen and then applied to the column. Compounds were eluted successively with 2 ml each of pentane, 10% dichloromethane in pentane, dichloromethane and methanol. Resulting fractions were concentrated to (a) 100 μ l (b) 133 μ l and (c) 200 μ l. In experiment a, the individual fractions were reunited and tested for attractiveness to investigate whether the activity of extracts is still present after fractionation of headspace extracts ($N = 31$). Since it was assumed that the host-associated kairomone consists of two or more compounds of different polarity, the fractions were assayed in experiment b in subtractive combinations for behavioural

activity. Testing of fractions in subtractive combinations has been shown to be the most efficient approach to narrow down active components of multicomponent mixtures (Byers, 1992). Fractions were combined in four mixtures each with one of the four fractions not present. If the exclusion of one fraction had no effect on the attractiveness of the mixture, it was concluded that this fraction did not contain compounds involved in the long-range orientation of *L. distinguendus*. The following combinations of fractions were applied on filter paper discs and offered in the olfactometer: (1) pentane, 10% dichloromethane in pentane and dichloromethane ($N = 22$); (2) pentane, 10% dichloromethane in pentane and methanol ($N = 25$); (3) pentane, dichloromethane and methanol ($N = 27$); (4) 10% dichloromethane in pentane, dichloromethane and methanol ($N = 21$). Based on the results of experiment b (see below), only the putatively active fractions (pentane and methanol) were combined in experiment c and tested for behavioural activity ($N = 29$). The remaining chambers in experiment a, b and c contained paper discs treated with the adequate mixture of pure solvents.

Chemical analysis Pentane and methanol fractions of active headspace extracts from the host larval faeces (prepared as described in experiment 3) were analysed by coupled gas chromatography-mass spectrometry (GC-MS) on a Fisons GC 8060 with a Fisons MD 800 Quadrupole MS (Thermo Finnigan, Egelsbach, Germany) using two columns of different polarities (DB-Wax and DB-5ms, 30 m x 0.32 mm, film thickness 0.25 μm J & W Scientific, Folsom, CA, USA). Helium was used as carrier gas (inlet pressure 10 kPa); the injector temperature was 240°C. The temperature programme started at 40°C and raised 3°C/min to 240°C (DB-Wax) or 280°C (DB-5ms). The MS was operated in EI mode at 70 eV, the mass range was m/z 35-450 with a scan time of 0.9 s and an interscan delay of 0.1 s. Due to the low volatile amounts in the headspace extracts, pentane and methanol fractions were carefully concentrated to one twenty-fifth of the original volume under a gentle stream of nitrogen. One μl of each fraction was injected alone and together with a series of *n*-alkanes with chain lengths between 7 and 26 carbon units (Sigma-Aldrich, Steinheim, Germany) to calculate the linear retention indices (LRI). Single peaks were identified by comparison of the mass spectra and LRI with those of authentic reference compounds.

Statistical analysis Statistical analyses were done by using the Statistica 4.5 scientific software (StatSoft, Hamburg, Germany). Arrestment time of males spent

in the sectors above the test and control chamber of the olfactometer were compared by Wilcoxon-matched pairs test.

Results

Experiment 1: Activity of the larval faeces Both naïve *L. distinguendus* males and females were attracted to the odour of mite-free larval faeces from the host *S. granarius* (Fig. 1a). Parasitoids spent significantly more time in the test sector than in the opposite control sector.

Experiment 2: Activity of headspace extracts of the larval faeces Arrestment time of naïve *L. distinguendus* males and females was significant higher in the sector above the filter paper treated with the larval faeces extract when compared to the control sector (Fig. 1b). Thus, volatile compounds in the larval faeces of *S. granarius* remained attractive to *L. distinguendus* after being trapped by CLS and extracted with dichloromethane.

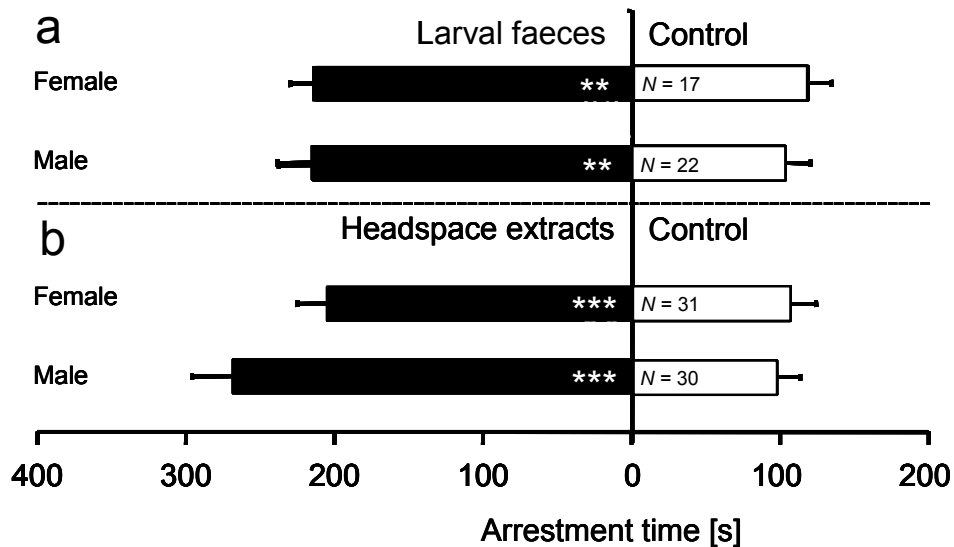


Fig. 1 Mean arrestment time (\pm SE) of naïve *L. distinguendus* males and females in the sectors of a four-chamber olfactometer during a 10 min observation time. The test chamber contained (a) 150 mg mite-free larval faeces from the host *S. granarius* or (b) filter paper disc treated with 30 μ l headspace extract of the larval host faeces. The opposite control chamber contained (a) brownish filter paper discs or (b) discs treated with the pure solvent. Asterisks indicate significant differences at $P < 0.01$ (**) or $P < 0.001$ (***) (analysed by Wilcoxon-matched pairs test).

Experiment 3: Activity of fractions In experiment a, where all fractions of the active headspace extract were reunited, naïve *L. distinguendus* females spent significantly more time in the test sector than in the control sector (Fig. 2a). Thus, the responsible chemicals passed adsorption chromatography without loss of activity. In experiment b, where the fractions were offered in subtractive combinations, only mixtures containing both pentane and methanol fractions were attractive to female parasitoids (Fig. 2b), whereas fractions of 10% dichloromethane and pure dichloromethane could be omitted without any loss of kairomone activity. In experiment c, a mixture containing only the active pentane and methanol fraction was offered. Again, females of *L. distinguendus* preferred the test sector to the control sector (Fig. 2c). The results clearly showed that host-related kairomones used by *L. distinguendus* were present in the pentane and methanol fraction and therefore must consist of both nonpolar and polar compounds.

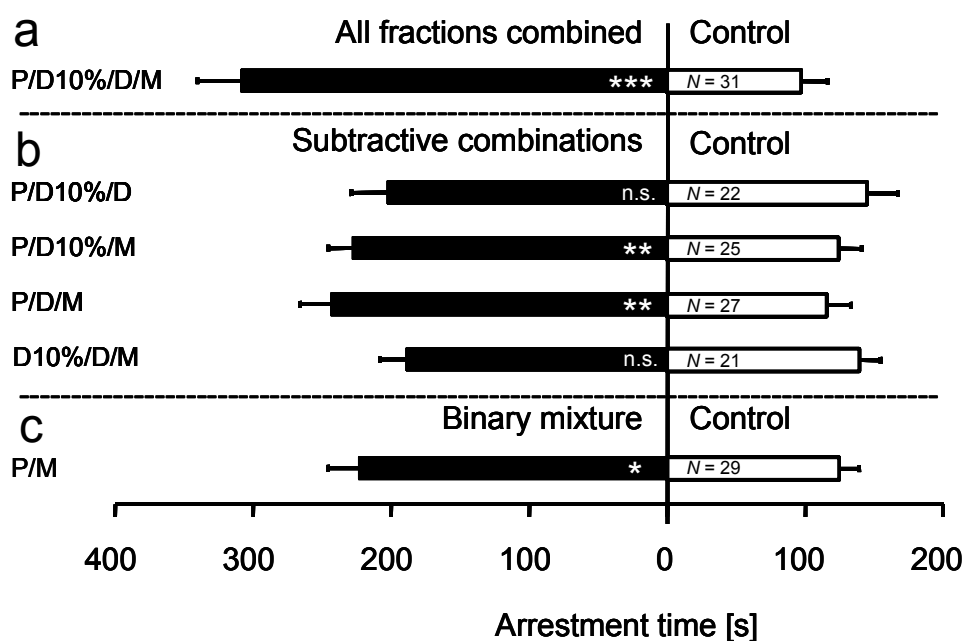


Fig. 2 Mean arrestment time (\pm SE) of naïve *L. distinguendus* females in the sectors of a four-chamber olfactometer during a 10 min observation time. The test chamber contained (a) a mixture of all fractions from the headspace extract of the host larval faeces, (b) mixtures of fractions in subtractive combinations or (c) a binary mixture of putatively active fractions. The opposite control chambers contained the adequate mixture of pure solvents. P = pentane; D10% = 10% dichloromethane in pentane; D = dichloromethane; M = methanol; n.s = not significant. Asterisks indicate significant differences at $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***) (analysed by Wilcoxon-matched pairs test).

Chemical analysis The components identified in the methanol fraction are listed in Table 1. They comprise mainly alcohols with two diastereomers of 2,3-butanediol, 1-octen-3-ol and acetoin as major components. Chemicals in the bioactive pentane fraction could not be identified in the present study. The major compounds were structurally related hydrocarbons with m/z 202 as mol peak (Fig. 3). However, mass spectra did not match any available library and literature data. Since all volatile components in the pentane fraction occurred only in minute amounts, preparative extraction and structure elucidation by standard procedures was not possible so far.

Discussion

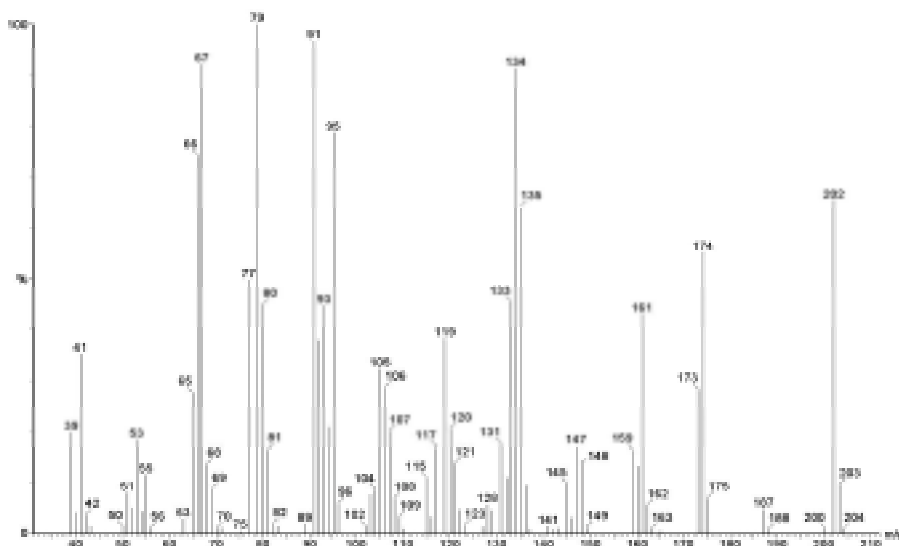
Our data demonstrate that females of *L. distinguendus* are attracted to odours from the faeces of their hosts, larvae of the granary weevil *S. granarius*. This confirms earlier results showing that host-related kairomones play a crucial role in the long-range orientation for host finding in *L. distinguendus* (Steidle and Schöller, 1997; Steidle et al., 2001a,b). So far, it was unclear if these kairomones only originate from host-associated mites (Ruther and Steidle, 2000) or if they are also released from the faeces of the weevil larvae. This aspect was completely neglected in earlier work on the innate use of kairomones for host-location in *L. distinguendus* (Steidle and Schöller, 1997; Steidle et al., 2001b). The present study using mite-free weevil cultures reveals that faeces are innately attractive also in the absence of mites. Therefore, chemicals released by the host faeces themselves must be responsible for the attraction.

With respect to the identity of the chemicals involved, our studies with different fractions of mite-free faeces extracts revealed that only mixtures containing both the pentane and the methanol fraction were attractive to the female parasitoids in the olfactometer. This demonstrates that the kairomone is composed of a complex blend of components with different polarities.

Remarkably, mite-free larval faeces from *S. granarius* are not only attractive to females but also to naive males of *L. distinguendus*. Because no female-derived sex attractant was found in previous studies (Ruther and Steidle, 2000), it seems likely that males rely on these host-associated volatiles when orientating toward

Table 1 Composition of the bioactive methanol fraction from a headspace extract of the larval host faeces.

No.	Compound	Relative peak area [%]
1	3-pentanol	1.6
2	2-pentanol	3.6
3	2-methylbutane-1-ol	1.7
4	1-pentanol	0.8
5	acetoin	11.3
6	1-hexanol	2.7
7	cyclohexanol	1.5
8	nonanal	1.0
9	1-octen-3-ol	29.5
10	decanal	3.0
11	2,3-butanediol (1. isomer)	23.9
12	2,3-butanediol (2. isomer)	14.4
13	4-pentanolide	0.6
14	4-butanolide	2.7
15	1,3 butanediol	0.4
16	2-phenylethanol	1.4

**Fig. 3** Mass spectrum of the unknown major component of the bioactive pentane fraction from a headspace extract of the host larval faeces.

females during mate finding. According to Ruther et al. (2002a), the chemicals involved can be classified as sexual kairomones, since males use them for sexual purposes only. There are two possibilities conceivable how *L. distinguendus* males use these kairomones. First, males often wait for females to emerge from the grain and subsequent mating occurs at the site of emergence. Therefore, males should search for females in the area of host patches. The perception of the larval host faeces may help males to get information about their position within a host patch and signal them when they are about to leave the site of female emergence. Second, it is also possible that males disperse and increase their probability of finding additional mating opportunities by entering new host patches to look for host seeking females outside the site of their own emergence. Thereby, habitats contaminated with host larval faeces reliably indicate the presence of potent areas for female oviposition. Several studies on other insect species have previously demonstrated that kairomones are involved in the sexual communication of insects. Host-related volatiles have been shown to enhance synergistically male responsiveness to female sex pheromones (Light et al., 1993), induce the release and the production of sex pheromones (McNeil and Delisle, 1989; Raina et al., 1992) or even act as direct cues for mate finding of males (Ruther et al., 2002b; Reinecke et al., 2002; Tooker et al., 2002). However, the present study is one of the first to demonstrate the use of host-related kairomones for mate finding in parasitic Hymenoptera.

In conclusion, host and mate finding in *L. distinguendus* obviously involves two categories of chemical cues in the host faeces. First, as shown in this study, a combination of nonpolar and polar cues which originate directly from the host and which are innately attractive to the wasps. Second, there are cues which are released as volatile pheromones from host-associated mites which are associatively learnt by male and female wasps during mating and host encounters, respectively (Ruther and Steidle, 2000).

Generally, the study of infochemicals mediating host and mate finding in natural enemies of insect pests is of economic significance in biological control (Cox, 2004). The application of these volatiles enables the monitoring of parasitoid's population density in pest-infested stored grain (Robacker et al., 1976) and may also increase the efficacy of the parasitisation rate (Godfray, 1994). Thus, future investigations will deal with the identification of those chemical cues that are

present in the larval faeces of hosts of *L. distinguendus* that are innately attractive to the wasps. The distribution of these chemicals in storage facilities might help to retain released wasps in cases of low granary weevil infestation and improve the effect of the control measure.

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