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Role of volatile and contact pheromones in the mating behaviour of *Bagrada hilaris* (Heteroptera: Pentatomidae)

SALVATORE GUARINO[1], CLAUDIO DE PASQUALE[2], EZIO PERI[1], GIUSEPPE ALONZO[2] and STEFANO COLAZZA[1]*

[1]Department of S.En.Fi.Mi.Zo., Section of Acarology, Entomology and Zoology, Università di Palermo, Viale delle Scienze 13, 90128 Palermo, Italy; e-mail: colazza@unipa.it

[2]Department of I.T.A.F., Università di Palermo, Viale delle Scienze 13, 90128 Palermo, Italy

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Abstract. Volatiles and contact pheromones involved in the mating behaviour of the Painted bug, *Bagrada hilaris* Burmeister (Heteroptera: Pentatomidae), were investigated in behavioural and chemical experiments. Vertical open Y-shaped olfactometer bioassays showed that odour from males attract females but not males, while that from females did not attract either gender. Adult females were also attracted by hexane extracts of volatile compounds collected from males. In open arena bioassays, males displayed the characteristic steps of courtship behaviour in the presence of virgin females. Such courtship behaviour was displayed in the presence of females killed by freezing, but not in the presence of freeze-killed females washed with hexane. Gas chromatography-mass spectrometry (GC-MS) analysis of volatile compounds produced by cohorts of 20 *B. hilaris* adults and collected over 48 h showed that both males and females produce the compounds nonanal, decanal and (*E*)-2-octenyl acetate. Of these compounds males produce significantly more (*E*)-2-o-octenyl acetate, i.e. 186.74 ng and 67.53 ng for males and females respectively. These findings indicate this compound is possibly a long range volatile pheromone, and a complex lipophilic fraction of the adult cuticle possible contact pheromone involved in short range courtship behaviour.

INTRODUCTION

Mating behaviour in insects can be divided into two main phases, long-range mate location and close-range courtship (Thornhill & Alcock, 1983). Long-range mate location is the upwind orientation and approach of one sex towards the other, which brings the two sexes into close proximity; while close-range courtship is the interaction of both sexes when in close proximity, which can result in copulation (Thornhill & Alcock, 1983; Wertheim et al., 2005).

Studies on phytophagous species of Heteroptera show that the long-range mate location is mediated by sex and/or aggregation pheromones, consisting of a mixture of volatile chemicals, mainly esters, terpenoids and alcohols (Aldrich, 1996; Miklas et al., 2000). In many cases, pheromone compounds are produced only by one gender, as in *Nezara viridula* L., *Euschistus* spp., *Plautia stali* Scott, *Thyanta pallidovirens* Stal, *Piezodorus hybneri* Gmelin, *Biprorulus bibax* Breddin, *Chlorochroa sayi* Stal, *Thyanta perditor* F., *Thyanta pallidovirens* Stal and *Murgantia histrionica* Hahn (Baker et al., 1987; Aldrich et al., 1991, 1994; Brezot et al., 1994; James et al., 1994; Sugie et al., 1996; Millar, 1997; Borges et al., 1998; Leal et al., 1998; Ho & Millar 2001a; McBrien et al., 2002; Moraes et al., 2005; Zahn et al., 2008). In other cases, pheromones are produced by both sexes but with different gender-specific relative abundances, as in *Geocoris punctipes* Say and *Leptocorisa chinensis* Dallas (Leal et al., 1996; Marques et al., 2000). Once males and females are in close proximity, cues such as visual (Capone, 1995), acoustic (Cade, 1985) and/or close range contact pheromones (Ginzel et al., 2003) are involved in triggering the courtship behaviour. Studies on the close-range courtship behaviour of true bugs reveal the presence of specific behavioural steps, from contact to copulation, indicating that behavioural postures and chemical stimuli are implicated in successful mate recognition (Borges et al., 1987; Ho & Millar, 2001a). As is the case of the cuticular hydrocarbons that are responsible for the gender recognition in several species of non-social insects belonging to the orders Dyctioptera (Nojima et al., 1999), Coleoptera (Ginzel et al., 2003) and Diptera (Carlson et al., 1998). In some cases males and females produce different hydrocarbons, as in *Megacyllene cariae* Gahan (Ginzel et al., 2006), or the same hydrocarbons, but in gender-specific relative abundances, as in *Drosophila* spp. (Toolson & Kuper-Simbron, 1989; Cobb & Ferveur, 1996).

Bagrada hilaris (Burmeister), the commonly named Painted bug, is a pest of Cruciferous crops throughout Asia, Africa and on some islands of southern Europe such as Malta and Pantelleria (Italy) (CAB, 1981). On Pantelleria, Painted bug attacks caper plants, *Capparis spinosa* L., damaging leaves, stems and flower buds (Colazza et al., 2004). *B. hilaris* populations are usually sprayed annualy with 4–5 applications of pesticide, (Colazza et al., 2004). As a consequence, adults of *B. hilaris* are slowly becoming resistant to pesticides (Swaran Dhingra & Seema, 1998; Guarino et al., 2007).

* Corresponding author.

Little information is available on the chemical compounds involved in the mating behaviour of *B. hilaris*. Previous studies on the composition of the cuticular hydrocarbons of the Painted bug revealed linear and branched hydrocarbons, which are qualitatively similar, but present in different quantities in the two genders (De Pasquale et al., 2007). However, the significance of these quantitative differences in the mating behaviour is not defined. The main objectives of this study were to characterize the volatile pheromones used in long range mate location and the cues involved in the close range courtship of *B. hilaris*.

MATERIAL AND METHODS

Insects

A colony of *B. hilaris* was established and restocked regularly with insects collected from May to November 2006 from caper fields located on the island of Pantelleria. The insect colony was maintained on caper buds, cole-seed and cabbage depending on seasonal availability. Food was changed every 2–3 days. All stages were reared in an environmentally controlled room (25 ± 1°C, 70 ± 10% R.H., photoperiod 16L : 8D), inside wooden cages (25 × 25 × 40 cm) with two 5-cm Ø mesh-covered holes for ventilation. Separate rearing cages were used for nymphs and adults.

Collection of volatiles produced by adults

Cohorts of 20 virgin adult *B. hilaris* of the same sex and age (about 5 days old) and caper flower buds (used as food) were put into an horizontal all-glass apparatus 1 l in volume. Humidified and charcoal filtered air was drawn through the chambers (flow 0.4 l min^{-1}). Bugs were transferred carefully into the chambers to avoid the discharge of defensive secretions and were exposed to the air flow for 48 h at 25 ± 2°C. The volatiles were trapped in glass collectors (6 mm ID) filled with 70 mg of 18–35 mesh activated charcoal (Merck KGaA, Darmstadt, Germany) held in place by glass wool plugs. Collectors were prepared a few minutes before the start of a collection, then, at the end of the aeration period, were eluted with hexane (200 µl). The elutes were stored at –18°C until subjected to GC-MS analyses or used in the vertical open Y-shaped olfactometer bioassays. The experiments were done close to a window which provide a source of natural daylight, and two fluorescent light bulbs (Lival, 220V-11W, Finland) controlled by a timer located above the horizontal all-glass aeration apparatus to provide supplementary light source with a 16L : 8D photoperiod.

Chemical analyses

GC-MS analyses were performed using a Hewlett-Packard 5890 GC system interfaced with an HP 5973 quadrupole mass spectrometer detector. As a stationary phase an HP5-MS capillary column (5% diphenyl – 95% dimethylpolysiloxane 30 m – 0.2 mm, 0.25 µm film thickness, J&W Scientific, USA) was used. Injector and detector temperatures were 250°C and 270°C respectively. Helium was used as the carrier gas. The GC oven temperature program was 40°C for 5.00 min, than increased by 10°C/min to 280°C. Electron impact ionization spectra were obtained at 70 eV, recording mass spectra from 42 to 550 uma. Compound identification was carried out using a commercial NIST 98 mass spectra library search and by comparison with standard analytical grade compounds purchased from Sigma-Aldrich (USA). [The syntethic (*E*)-2-octenyl acetate was kindly provided by Prof. J.G. Millar.] Quantitative analysis was carried out only for (*E*)-2-octenyl acetate, the putative pheromone of this bug. For this analysis the elutes were diluted in 1 ml of hexane using a volumetric flask. Five point calibration curves in the 1–50 ng μl^{-1} range were used in order to evaluate the chromatographic response. Then, analytical extraction recovery experiments of (*E*)-2-octenyl acetate were carried out using 10 µl of hexane solutions (100 ng μl^{-1}) of the synthetic standard spotted on a disk of filter paper (3 cm diameter), placed inside the aeration apparatus described above, and aerated for 1 h (flow 0.4 l min^{-1}).

Vertical open Y-shaped olfactometer bioassays

Olfactory orientation evoked by volatiles of different origin was investigated using a vertical open Y-shaped olfactometer, which is a modification of the apparatus described by Visser & Piron (1998). Unlike to their olfactometer, the brass rod has an outer diameter of 5 mm, the length of the central wire stem is 16 cm and the flow rate was 0.4 l min^{-1}. Two glass chambers (125 ml) were used as the odour source holders, and during the different tests, at every fourth replicate, the odour sources were interchanged in order to avoid bias. At every switch, all the glass parts of the apparatus were washed with water and detergent, and then wiped with acetone and the brass rod cleaned with distilled water and acetone. After each experiment, the apparatus was cleaned with distilled water and acetone and oven dried for at least 20 min at 150°C. Light was provided by an electric lamp (Osram, 12V–35W, Germany) suspended 30 cm above the olfactometer. Bioassays were conducted at 25 ± 2°C and 50 ± 15% R.H. A single *B. hilaris* adult was carefully released at the bottom of the central stem of the olfactometer and allowed 15 min to respond. The first choice of the bug was recorded once the insect moved into one arm 5 cm past the junction. Bugs that didn't move into one of the two arms during the 15 min trial were scored as non responders and not included in the analysis.

The first experiments are carried out to determine the olfactory reactions of males and females of *B. hilaris* to the odour released by the same or opposite gender. The insects used for this experiment were sexed and kept separated for 3–4 days before the test. A group of 8 virgin 5-days-old sexually mature adults was placed in the glass chamber, while the other glass chamber was left empty as a control. The group of bugs used as the stimulus source was changed after each four replicates. The following four combinations were tested: males responding to female odour (N = 33), males responding to male odour (N = 34), females responding to female odour (N = 34), females responding to male odour (N = 32).

A second set of experiments was used to determine the olfactory response of females to hexane extracts of volatile compounds collected from male adults, as previously described. An aliquot of 1 µl of the extract was spotted on a disk of filter paper (2 cm diameter). The solvent was allowed to evaporate for 2 min. The control chamber was supplied with a filter paper disk (2 cm diameter) treated with 1 µl of hexane. Only sexually mature virgin females (> 5 days old, N = 11) were tested in this bioassay, using, as the odour source, male extract obtained from a 48hr aeration of a cohort of 20 male bugs.

Petri dish arena bioassays

Bioassays were carried out using a 5 cm diameter Petri dish as an arena, which was monitored by a Hitachi KP-D40 colour digital video camera connected to a Sharp VC-GH600SM VHS videocassette recorder. Insects used in this experiment were sexed and kept separate for 3–4 days before being tested. Virgin adult pairs of Painted bug were put individually in the Petri dish arena and recorded for 20 min (N = 22). Then, the different phases in the courtship were defined and their duration determined with the aid of a stop watch.

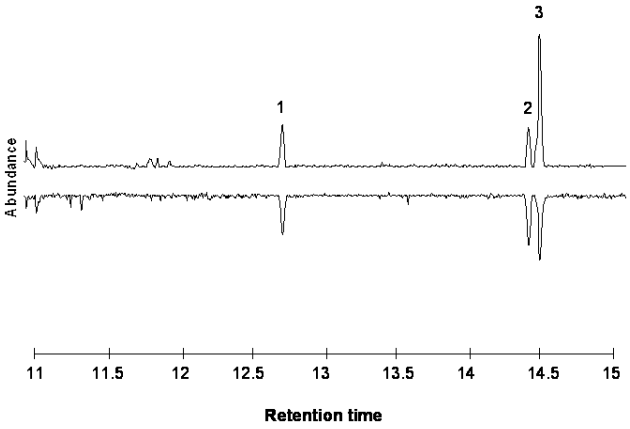

Fig. 1. Gas chromatograms of the volatile compounds collected from cohorts of 20 *B. hilaris* adults over a period of 48 h. Males (top) and females (bottom, inverted). 1 – nonanal; 2 – decanal; 3 – (*E*)-2-octenyl acetate.

In order to demonstrate that males of *B. hilaris* recognize females using a contact pheromone, females were killed by freezing (–18°C for 30 min) and then treated with different solvents or not as described below. After being killed by freezing, untreated females were allowed to warm up at room temperature and then presented to a male. The male's behaviour was recorded for 20 min, and scored as a positive response if it displayed courtship behaviour for more then 10 s after contacting the female with its antennae. This experiment was replicated 10 times. To determine the chemical fractions involved in mate recognition, freeze-killed females were immersed for 2 h in 2 ml of one of three solvents with different polarity: hexane, ethyl acetate or distilled water. Each treated female was exposed to a single male and the behaviour recorded as previously described. This experiment was replicated 10 times for each solvent.

Statistical analysis

Data on the number of responses of males and females to different treatments (live insects and air collected extracts versus controls) were analyzed using a χ^2 goodness of fit test (Sokal & Rohlf, 1995). The interest shown by male bugs towards females washed with different solvents was also analyzed using a χ^2 goodness of fit test (Sokal & Rohlf, 1995). While, the quantitative analysis of the differences in the amount of (*E*)-2-octenyl acetate recovered from males and females were compared using a *t*-test (Sokal & Rohlf, 1995). All the statistical analyses were performed using Statistica for Windows 6.0 (Stat Soft Italia, 1997).

RESULTS

Chemical analyses of adult volatiles

Main peaks of typical gas chromatograms of the aeration extracts of females and males of *B. hilaris* are shown in Fig. 1. Comparison of these extracts using GC-MS analyses revealed the same compounds as nonanal, decanal and (*E*)-2-octenyl acetate (Fig. 1). Quantitative analysis of (*E*)-2-octenyl acetate indicated that males produced an higher amount of this compound, i.e. groups of 20 males of *B. hilaris* produced an average of 186.74 ng per 48 h compared to the 67.53 ng produced by 20 females (t = –3.17; *N* = 5; P = 0.013).

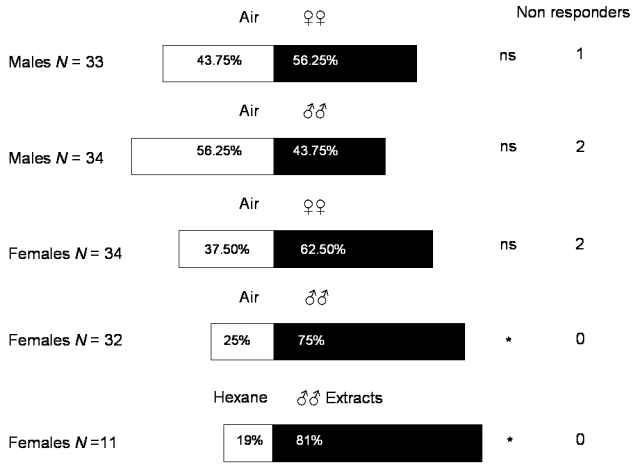

Fig. 2. First choice response (%) of *B. hilaris* adults in a vertical open Y-shaped olfactometer to different treatments. * = P < 0.05; ns = not significantly different (χ^2 test). *N* = number of replicates.

Vertical open Y-shaped olfactometer bioassays

The results of the vertical open Y-shaped olfactometer bioassays are shown in Fig. 2. Females of *B. hilaris* were more attracted to the odour from live males than from the control (χ^2 = 8; *N* = 32, P = 0.05). Females were not attracted to the odour of females (χ^2 = 2; *N* = 34, P > 0.05) and males showed no preference for either the odour of females (χ^2 = 0.5; *N* = 33, P > 0.05) or males (χ^2 = 0.5; *N* = 34, P > 0.05). Furthermore, females responded to the crude extract of males odour obtained from the air that passed over the males (χ^2 = 4.45, *N* = 11, P = 0.03).

Petri dish arena bioassays

The courtship behaviour that preceded copulation was divided into three phases. In the first phase, "contact phase", males antennate a female's body; in the second "mount-antennation phase", males mount a female and start to antennate the female's antennae and genitalia. Finally, in the "engagement" phase the male dismounts and start to copulate by putting his last pair of legs on the female's abdomen and coupling his genitalia with those of the female. For each phase the average time (± SD) was: 1.12 ± 0.93 s for contact, 14.78 ± 8.41 s for mount-antennation and 41.85 ± 18.31 s for engagement. After the contact phase, if the female was receptive, she adopted a posture with her head slightly lowered and abdomen raised, to allow the male to mate. Mating took place in about 77% of the replicates (*N* = 22). During mating, both female and male bugs stood in an end to end position, with their abdomens higher than their heads. Once copulation was complete one of the bugs swung from side to side to disengage from the other.

In the experiment with freeze-killed females of *B. hilaris*, males always attempted to mate unwashed cadavers (*N* = 10). After contacting cadavers treated with different solvents, 90% of the males attempted to mate with females previously washed in distilled water (χ^2 = 0.1; *N* = 10, P > 0.05); 40% when the females were washed in ethyl acetate (χ^2 = 3.6; *N* = 10, P > 0.05) and

only 20% when washed in hexane ($\chi^2 = 6.4$; $N = 10$, P = 0.01)

DISCUSSION AND CONCLUSIONS

The results of the vertical open Y-shaped olfactometer bioassays showed that females were attracted to odours produced by male and similarly attracted to a solvent extract of males volatiles. Chemical analysis of the odours of the two sexes of Painted bug indicated they are qualitatively similar. Quantitative analysis indicated that the odour of males contained more (*E*)-2-octenyl acetate than that of females. This suggest that females locate males from a distance partly or wholly by responding to (*E*)-2-octenyl acetate. This compound is also used as a sex and/or aggregation pheromone by other heteropteran species (Leal et al., 1996; Marques et al., 2000). For example, in the lygaeid *G. punctipes,* (*E*)-2-octenyl acetate is produced mainly from females and it plays a role as sex pheromone (Marques et al., 2000). Furthermore, in the alydid *L. chinensis*, a blend of (*E*)-2-octenyl acetate and octenol is produced by both sexes, but it is only attractive to males (Leal et al., 1996). Of the other chemical compounds in the Painted bug air collections, nonanal and decanal are commonly produced by other true bugs species (Borges et al., 2006). Further laboratory and field experiments are needed to determine the full role of (*E*)-2-octenyl acetate in the ecology of this species.

The close range courtship behaviour of the Painted bug is similar to that described for other species of true bugs, e.g. *N. viridula*, and *C. sayi* (Borges et al., 1987; Ho & Millar, 2001b). However, *B. hilaris* males showed a specific kind of mount and antennation of the female's antennae and genitalia, and they did not head butt the posterior end of females. The results of the Petri dish arena bioassays, using cadavers of females, indicate that males of the Painted bug recognize the opposite sex mainly by antennation of her cuticular surface. All the Painted bug males attempted to mate with cadavers of females, suggesting that the recognition was not cued only by mechanoreception or visual stimuli. The fact that the active compounds were partially removed by hexane, suggests that sex recognition is based on chemical cues in which the lipophilic fraction plays the main role. The role of cuticular hydrocarbons as contact pheromones in inducing close range courtship behaviour is described for species of Coleoptera (Ginzel et al., 2003; Ortiz-Domínguez et al., 2006; Ginzel et al., 2006), Diptera (Uebel et al., 1975; Carlson et al., 1984) and Hymenoptera (Howard & Baker, 2003). In *B. hilaris* the difference in the relative abundance of the cuticular hydrocarbons of males and females (De Pasquale et al., 2007), might mediate gender recognition in this species.

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