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Identification of sex pheromone components in *Trissolcus brochymenae* females

Gianandrea Salerno [a], Alessia Iacovone [a], Silvia Carlin [b], Francesca Frati [a], Eric Conti [a,*,1], Gianfranco Anfora [b,1]

[a] Dipartimento di Scienze Agrarie e Ambientali, Università di Perugia, Borgo XX Giugno 74, 06121 Perugia, Italy
[b] Research and Innovation Centre, Fondazione Edmund Mach, Via E. Mach, 1-I-38010 San Michele all'Adige (TN), Italy

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ABSTRACT

Long- and short-range sex pheromones appear to play a crucial role in the mate finding and courtship behaviour of most parasitic Hymenoptera. Yet these parasitoids have been rarely investigated and only a few pheromones have been identified. Recent studies have shown that sexual communication of *Trissolcus brochymenae* (Hymenoptera: Platygastridae), a quasi-gregarious egg parasitoid of the harlequin bug, *Murgantia histrionica* (Heteroptera: Pentatomidae), starts before contact between males and females when chemical compounds produced by virgin females trigger the courtship behaviour of males. In the present study, the pheromone components involved in the short-range recognition of *T. brochymenae* females by males were investigated using electrophysiological and behavioural methods. Female body extracts were analyzed through EAG and GC-EAD and the active compounds were identified through GC–MS. The behavioural responses of virgin males to the GC-EAD active compounds were subsequently evaluated in closed arena bioassays. Two active compounds in EAG and behavioural tests, tetradecyl acetate and (Z)-11-hexadecen-1-yl acetate, were identified as sex pheromone components. Both compounds triggered intense male antennation and mount when applied to solvent-washed female cadavers. Dose–response tests showed different curves for the two compounds. This is the first study on the identification of sexual pheromones in Platygastridae.

1. Introduction

Sex pheromones are assumed to play a crucial role in the sexual communication of most parasitic Hymenoptera and yet have the involved chemical compounds been rarely investigated (Kainoh, 1999). There is evidence for sex pheromones in about 13 families of parasitic wasps (see Ref. in Salerno et al., 2012). In very few of these families, however, have the sex pheromones been chemically characterized or identified: Braconidae (Kainoh et al., 1991; Swedenborg and Jones, 1992; Swedenborg et al., 1994; Syvertsen et al., 1995; DeLury et al., 1999), Ichneumonidae (Shu and Jones, 1993; Eller et al., 1984; Hrdy and Sedivy, 1979; Robacker and Hendry, 1977), Charipidae (Micha et al., 1993; Höller et al., 1994), Pteromalidae (Ruther et al., 2007, 2008, 2011; Nichols et al., 2010), Bethylidae (Collatz et al., 2009), Eulophidae (Cônsoli et al., 2002).

Parasitic Hymenoptera utilize volatile sex pheromones for attraction at relatively long range (>2 cm) and compounds with less volatility at short range for species recognition and courtship (Quicke, 1997; Sullivan, 2002; Steiner et al., 2005, 2006; Ruther et al., 2011). Moreover, short-range pheromones, spread by antennal contact during courtship, are thought to be involved in species recognition and in the induction of female receptivity (Keeling et al., 2004; Romani et al., 2008).

Among insects and hymenopteran parasitoids in particular, most of the studies are focused on female produced pheromones (Keeling et al., 2004). However there is increasing evidence for production of sex pheromones also by males (Gonzalez et al., 1985; Matthews et al., 1985; Cônsoli et al., 2002; Ruther et al., 2007, 2011; Steiner and Ruther, 2009). It is not uncommon for male and female parasitoids to share same pheromones but in different quantities (Keeling et al., 2004; Ruther and Steiner, 2008).

This is the first study on the identification of sexual pheromones of Platygastridae (=Scelionidae, see Sharkey, 2007, and Murphy et al., 2007); investigations were focused on the pheromone components involved in inter-sexual interactions in *Trissolcus brochymenae* Ashmead (Hymenoptera: Platygastridae).

T. brochymenae is a small egg parasitoid recorded from 11 Nearctic pentatomid bug species (Johnson, 1984; Salerno, 2000; Conti et al., 2004), and it is mainly known as a potential biocontrol agent of the harlequin bug, *Murgantia histrionica* Hahn (Heteroptera: Pentatomidae) (Johnson, 1984), a pest of cabbage and other brassicaceous crops (McPherson, 1982). The sex ratio of *T. brochymenae* offspring is strongly female-biased, normally with only one male emerging from each parasitized egg mass. As in other platygastrid parasitoids, the male emerges earlier than the female

* Corresponding author. Tel.: +39 0755856029; fax: +39 0755856039.
E-mail address: econti@unipg.it (E. Conti).
[1] Authors share joint seniority.

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and when the females emerge, the male mounts each sister trying to make contact between his and the female's antennae. After the copula, the male remains on the egg mass waiting for other emerging sisters and the mated female leaves it (Salerno et al., 2012). This mating behaviour is similar to that described for *Trissolcus basalis* (Wollaston) (Wilson 1961; Bin et al., 1986; Clarke and Walter 1994; Loch and Walter 2002).

In the *Trissolcus* genus, sex pheromones have been shown to play a role in male recognition and acceptance during male–female antennal contact (Bin et al., 1986; Isidoro et al., 1996). Since *Trissolcus* species are proterandrous and quasi-gregarious parasitoids, it could be expected that females of these species do not use long-range volatile sex pheromones to attract males far from the emerging sites, unlike some other hymenopteran parasitoids (reviewed by Quicke, 1997; Sullivan, 2002). On the other hand, recent studies have shown that, at short range, the sexual communication in *T. brochymenae* is mediated by chemical compound(s) produced by virgin females (Salerno et al., 2012). Behavioural experiments showed that the polar component(s) of the pheromone plays a major role in triggering male courtship. Males courted and attempted copulation with 1- to 2-day-old female cadavers, but not with male cadavers, nor female cadavers rinsed in organic solvents of different polarities. Male responsiveness to female cadavers was re-established by treating cadavers with acetone extracts of females, but not with ether and hexane extracts (Salerno et al., 2012).

Short-range pheromones are involved in recognition and initiation of male courtship behaviour in many Hymenoptera species. The involved chemicals are mostly stable and low volatile, and thus, recognizable by contact or at few millimetres distance; the active compounds are usually cuticular lipids (reviewed by Quicke, 1997; Kainoh, 1999; Keeling et al., 2004). There are several parasitoid families in which such short-range female-derived sex pheromones have been reported: Chalcididae (Simser and Coppel, 1980; Mohamed and Coppel, 1987), Ichneumonidae (Shu and Jones, 1993), Braconidae (Vinson, 1978; Syvertsen et al., 1995; Danci et al., 2006), Pteromalidae (Yoshida, 1978; Sullivan, 2002; Steiner et al., 2005, 2006; Nichols et al., 2010; Ruther et al., 2011), Eulophidae (Finidori-Logli et al., 1996) and Platygastridae (Schwartz and Gerling, 1974; Salerno et al., 2012).

The aim of the present study was to identify the pheromone components involved in the short-range recognition of *T. brochymenae* females by virgin males, by using electroantennography (EAG) and gas chromatography coupled with electroantennographic detection (GC-EAD). The behavioural responses of virgin males to the GC-EAD active compounds were then studied by closed arena bioassays.

2. Materials and methods

2.1. Insects

Individuals of *T. brochymenae* used in our experiments were obtained from a laboratory strain originally collected near San Diego, California (USA) from eggs of *M. histrionica* that had been laid on *Isomeris arborea* Nutt. (Capparidaceae). Parasitoids were reared in a climatic chamber (25 ± 1 °C, 60 ± 5% RH, L16:D8) on eggs of *M. histrionica* in 85-ml glass tubes, and fed on small drops of sugar-water diet (Safavi, 1968). Individuals of *M. histrionica* were collected from cabbage in the Beltsville, Maryland (USA) area, and were reared as described by Conti et al. (2004). Both colonies were maintained in quarantine conditions in the Entomology laboratories of the University of Perugia (Italy). Virgin females were obtained from individual parasitized host eggs. To reduce parasitoid larval mortality, egg masses were split once the parasitoid entered the pupal stage, and each egg was placed singularly into a glass tube (Ø = 10 mm; *h* = 25 mm) with a small drop of Safavi diet, and kept under controlled conditions (25 ± 1 °C, 60 ± 5% RH, L16:D8) until adult emergence. Male wasps were obtained as progeny from host egg masses parasitized by virgin wasp females (haplodiploidy). In all experiments, 1- to 2-d-old virgin males of *T. brochymenae* isolated at the emergence site were tested. Before behavioural experiments, males were allowed to acclimatize in the bioassay room (25 ± 1 °C, 50 ± 10% RH) for at least 30 min, then tested from 09:00 to 13:00 h, and discarded after each bioassay.

2.2. Semiochemical extraction

According to the methodology used in a previous research (Salerno et al., 2012), the semiochemical extracts from virgin females were obtained by acetone extraction. For each solvent extraction, 20 wasps 0- to 2-d-old were killed by freezing at −4 °C for ∼30 min, transferred into a 1.1-ml conical bottomed glass vial and immersed in 60 μl of acetone (3 μl per female) (⩾99.8%; Sigma–Aldrich, St. Louis, MO, USA). The extract was kept at room temperature for ∼1 h. After removal of the insects, the resulting extract was evaporated under a gentle stream of nitrogen and redissolved in acetone (5 μl/female for EAG; 0.5 μl/female for GC-EAD and behavioural bioassays). Some of the female extracts were prepared for chemical quantification, and 500 ng of heptyl acetate (⩾98% purity, Sigma–Aldrich) diluted in 1 μl of acetone, was added as an internal standard in 100 μl of the female acetone extracts.

2.3. Chemicals

The electrophysiological active compounds were identified as (Z)-11-hexadecen-1-yl acetate and tetradecyl acetate. (Z)-11-hexadecen-1-yl acetate (95%) and octanal (99%) (used as a standard stimulus in order to normalize the electrophysiological responses) were purchased from Sigma–Aldrich and tetradecyl acetate (98%) was purchased from ISCA Technologies, Inc. (Riverside, CA, USA). For the dose-dependence experiments all the compounds were dissolved in paraffin oil (Fluka, Buchs, Switzerland).

2.4. Antennal preparation for EAG and GC-EAD analysis

Male parasitoids were anaesthetized by refrigerating them at about -4 °C for about 30–45 s. They were cut between pro- and mesothorax and half of the distal antennomere was also severed. The anterior part (constituted by antennae, head and prothorax) was used for the recordings. EAG and GC-EAD connections were made by inserting the cut end of the prothorax into a glass capillary (1.5 mm o.d., 1.2 mm i.d) grounding electrode in contact with a silver wire, filled with a Beadle–Ephrussi Ringer solution (Bjostad, 1998) containing 5 g/l of polyvinylpyrrolidone (Fluka). The recording electrode was a similar glass capillary brought into contact with the distal cut end of the antenna. The capillary tubes were drawn to a fine point using a microelectrode puller (Narishige PC-10, London, UK) to get an inner diameter wide enough to enable insertion of the preparation.

For odour presentation in EAG experiments, 10 μl of the test sample were applied to a small piece (2 cm^2) of filter paper (1300/80 Filter-Lab, Barcelona, Spain). When we used acetone extracts or chemicals dissolved in acetone, the solvent was allowed to evaporate for 60 s. Subsequently the filter paper was inserted in a Pasteur pipette (150 mm in length, Volac®) constituting an odor cartridge. The tip of the glass pipette was placed about 3 mm into a small hole in the wall of a L-shaped glass tube (130 mm long, 12 mm i.d.) oriented towards the antennal preparation (∼5 mm away from the preparation). The stimuli were provided as 1 s puffs of charcoal-filtered air into a continuous humidified main air stream, at 2100 ml/min continuous flow