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The Combined Use of an Attractive and a Repellent Sex Pheromonal Component by a Gregarious Parasitoid

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Abstract

Gregarious parasitoids usually clump their cocoons together and the adults emerge in a synchronized fashion. This makes it easy for them to find mating partners and most copulations indeed take place at the natal patch. Yet, males should leave such sites when females are no longer receptive. As yet, this decision-making process and the possible involvement of pheromones were poorly understood. Here we report on a remarkable use of attractive and repellent pheromones of the well-studied gregarious parasitoid species *Cotesia glomerata* (L.) (Hymenoptera: Braconidae). Virgin *C. glomerata* females were found to release an attractive as well as a repellent compound, which in combination arrest males on the natal patch, but after mating the females stop the production of the attractant and the males are repelled. The repellent compound was identified as heptanal, which was also released by males, probably reducing male-male competition on the natal patch. We also confirmed that the sex ratio of the emerging wasps can vary considerably among patches, depending on the relative quality of hosts and the number of females that parasitize a host. The newly revealed use of attractive and repellent pheromone compounds by *C. glomerata* possibly helps maximize mating success under these variable conditions.

Keywords Sex pheromones · Parasitoids · Mate location · Sex allocation · *Cotesia glomerata*

Introduction

Mate-seeking males tend to gather in habitats where they are likely to encounter receptive females. This can be at female emergence sites if females are receptive to mate shortly after emergence and if it is likely that sperm of the first mate is used to fertilize their eggs (Thornhill and Alcock 1983). Mating at the emergence site is very common among gregarious and quasi-gregarious parasitoids, the latter being solitary parasitoids that parasitize clumped hosts (Boulton et al. 2015; Godfray 1994). The competition among male wasps at the emergence site can be intense, and physical interactions can range from being mild to lethal (Boulton et al. 2015; Godfray 1994). For example, newly-emerged males of certain fig wasps and *Melittobia* parasitoids fight among themselves until just a single individual remains alive, and the winner will mate with all females in the emergence area (Herre et al. 1997; Matthews et al. 2009; Reece et al. 2007). More frequently, however, the competition involves male siblings, which engage in only mild fighting, and does not result in killing of the losers (Hamilton 1967; West et al. 2002). For instance, *Nasonia vitripennis* males push each other around to be near the emergence hole of a host pupa from where females will exit (Godfray 1994). In the extreme case of certain fig wasp species, males have evolved the abilities to mate inside a fig with pre-emerged females (Herre et al. 1997). In some other species, males enter a race: as soon as fig fruits become mature and open, males rush into the fruit and mate with all females inside within a few

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minutes (Godfray 1994). So far, studies on the mating behaviours of gregarious or quasi-gregarious parasitoids at natal patches have mainly focused on physical competition among males, and pheromone-mediated interactions have largely been neglected.

The gregarious parasitoid *Cotesia glomerata* has served as a model to study various aspects of mating systems and sex allocation theories (Elias et al. 2010; Harvey et al. 2013; Le Masurier 1991; Tanaka 2009; Zhou et al. 2006). Under field conditions, about 40% of newly emerged wasps leave their emergence sites without mating (Gu and Dorn 2003; Tagawa 2000; Tagawa and Kitano 1981). On average, the sex ratio of offspring is female-biased, but in the field 10% of the clutches have male-biased ratios (Gu and Dorn 2003; Hasan and Ansari 2010; Tagawa 2000). Some cocoon clutches collected from the field even show only male emergence (Gu and Dorn 2003). Superparasitism (multiple ovipositions on a host by different foundresses) also happens frequently in this species, especially when the hosts are present in low densities, and superparasitism has no apparent effect on parasitoid survival (Gu et al. 2003; Harvey 2000; Harvey et al. 2013), but it leads to a higher sex ratio (proportionally more males) (Gu et al. 2003; Tagawa 2000; Tanaka 2009). Since *Cotesia* females normally mate only once (De Freitas et al. 2004; Kimani and Overholt 1995), pheromones can be expected to serve as important cues for males to find virgin females. Indeed, Tagawa (1977) provided evidence that *C. glomerata* females synthesize and release a sex pheromone from a gland located on their abdomen and show that the last stage of the female cocoon (the so-called "black-eye" stage) already starts to attract newly-emerged males with this pheromone. In a previous study (Xu et al. 2014), we found that only virgin females of *C. glomerata* are attractive to males. Interestingly, males were repelled by mated females, as well as by males, which indicates that males and mated females also produce anti-aphrodisiac pheromones (Xu et al. 2014). These observations, which prompted the current study, indicate that the parasitoid uses a complex pheromone system that includes both attractive and repellent compounds. These compounds appear to be differentially released depending on the wasps' sex and mating status. Here we used chemical analyses, electrophysiological measurements, as well as behavioural assays, to identify the repellent compound, and to unravel how *C. glomerata* may use this repellent to help maximize its mating success.

Materials and Method

Insects

The parasitoid wasp *C. glomerata* was reared following the protocol described previously (Xu et al. 2014). *C. glomerata* and its host *Pieris brassicae* were originally collected from gardens in Neuchatel, Switzerland. *P. brassicae* caterpillars fed with Chinese cabbage (*Brassica rapa* var. *pekinesis*) served as hosts, which were parasitized by the female wasps at the first to second larval instar. Potted (ID = 30 cm) plants were grown in a greenhouse (25 °C, LD 16:8 h) for about one month before they were used as food of *P. brassicae* caterpillars. To obtain virgin adult wasps, parasitoid cocoons were placed individually in 1.5 mL centrifuge tubes until wasp emergence. Virgin females and males were kept separately in two Bugdorm-1 cages (30 × 30 × 30 cm, Mega View Science Education Services Co. Ltd., Taiwan), provided with honey and moist cotton wool in a 25 °C incubator (LD 16:8 h). In order to obtain mated wasps, 50 newly emerged females and 50 newly emerged males were placed together in another Bugdorm-1 cage, again with honey and moist cotton wool (Xu et al. 2017). Wasps were about three days old when they were used for behavioural assays or chemical extractions.

Headspace Sampling of Pheromones or Authentic Compound Mixtures

In order to select an efficient absorbent material to trap the wasp's pheromonal compounds, we tested filters with two kinds of absorbent that have different affinities for volatile compounds: SuperQ (25 mg, 80–100 mesh) (D'Alessandro and Turlings 2005) and polydimethylsiloxane (PDMS). The PDMS filters (OD = 6 mm, ID = 4 mm, length = 60 mm, Gerstel GmbH, Mülheim, Germany) are in principle designed for Gerstel thermal desorption devices.

C. glomerata parasitoids (100–200 individuals, either virgin or mated ones) or a mixture of purchased authentic compounds (each 800 ng in 800 μl dichloromethane pipetted onto a piece of filter paper, OD = 9 cm) were placed in a glass bottle (250 mL). Pure and moist air was pushed into the glass bottle at a speed of 1.1 L/min. A vacuum pump sucked the air from the glass bottle over the volatile traps at a speed of 0.8 L/min for two hours. The excess air (~0.3 L/min) went out through an additional opening in the glass bottle, thus preventing contamination with outside air. After collections, both SuperQ and PDMS filters were eluted with 150 μl dichloromethane (Biosolve LTD, HPLC grade). The samples were then stored at −80 °C until they were used for bioassays, fractionations or chemical analyses. Two internal standards (IS) (*n*-octane and nonyl acetate, each 200 ng combined in 10 μl dichloromethane) were added to each sample as reference and were used to quantify the collected compounds based on peak areas in the chromatograms. Headspace samples collected from empty bottles served as controls.

Solvent Extractions

Six virgin or mated wasps (either males or females) were placed in a freezer at −20 °C for one hour and then extracted

with dichloromethane (Biosolve LTD, HPLC-grade, 200 µl) for 10 min. Virgin wasps were dissected into heads, thoraxes (including legs and wings), and abdomens with a scalpel. The heads, thoraxes and abdomens of six wasps were placed in 50 µl, 100 µl and 100 µl dichloromethane respectively for 10 min. Equal volumes (~30 µl) of the supernatant liquids were removed with a syringe and stored at −80 °C until use.

Gas Chromatography-Electroantennographic Detection (GC-EAD) Analyses

The GC-EAD system consisted of a HP 6890 gas chromatograph (Agilent Technologies, Germany) equipped with a flame ionization detector (FID) and a polar column (ZB-WAX, 30 m, 0.32 mm ID, 0.25 µm film thickness, Phenomenex), and was coupled to an EAD setup (Syntech, Hilversum; Netherlands). Aliquots (2 µl) of samples were injected splitless into the GC injector (280 °C) at an initial column temperature of 40 °C, and then temperature was increased at a rate of 15 °C per minute to 250 °C. The GC effluent was split (split ratio FID: EAD = 1:3) by using a µFlow splitter (Gerstel, Mühlheim, Germany), and 25 ml/min of make-up gas (nitrogen) was added to carry separated compounds through two deactivated capillaries: one leading to the FID, and the other leading to the EAD setup. The outlet of the EAD entered into a purified and humidified constant airflow that was directed over the antennal preparation.

To prepare the antennae, *C. glomerata* males were anesthetized with CO_2, and their heads were pulled out from the body with forceps, and the two tips of both antennae were cut and inserted into one glass capillary. To close the electric circuit, the severed head was connected via the neck to another glass capillary. The capillaries were filled with insect Ringer solution (5 g NaCl; 0.42 g KCl; 0.19 g $CaCl_2$ in 1000 ml demineralised water). Each EAD test was replicated five times with different insect heads.

Gas Chromatography-Preparative Fraction Collector (GC-PFC) Analyses

The GC-PFC system consisted of a HP 6850 Series gas chromatograph (Agilent Technologies, Germany) equipped with an FID detector and a polar column (EC-WAX column, 30 m, 0.25 mm ID, 0.25 µm film thickness, Alltech), and was coupled to a Preparative Fraction Collector (PFC) setup (GERSTEL, Germany). Helium at constant flow (1.9 ml/min) was used as carrier gas. Aliquots (2 µl) of the headspace samples of *C. glomerata* (100–200 individuals) trapped on SuperQ filters were injected splitless into the GC injector (280 °C). About 30 injections were made. The oven temperature of the GC was initially 40 °C and was then increased at a rate of 15 °C per minute to 250 °C. The GC effluent was split (split ratio FID: PFC = 1:9) with the use of a four-arm splitter (GRAPHPACK 3D/2, Gerstel, Mülheim, Germany). The transfer line temperature and PFC chamber temperature were 280 and 300 °C, respectively. In order to get a better recovery of fractions, a gentle make-up flow (3.2 ml/min) of nitrogen was used to help carry the samples into the fractionation traps. The time intervals for the six fractions are given in Fig. 2a. In order to determine and identify pheromonal compounds in extracts, the attractiveness of different fractions to the male wasps was tested separately or in combinations.

Gas Chromatography–Mass Spectrometry (GC-MS) Analyses

In order to identify the headspace compounds of the wasp, samples were analysed with an Agilent 6890 gas chromatograph, coupled to an Agilent 5973 mass selective detector. A 2 µl aliquot of each sample was injected in splitless mode with an injector temperature of 280 °C onto a polar column (EC-WAX, 30 m, 0.25 mm ID, 0.25 µm film thickness, Alltech). Helium at constant flow (1.9 ml/min) was used as carrier gas. The oven temperature program was initially 40 °C, held for 3.5 min, and then temperature was increased at a rate of 15 °C per minute to 260 °C. Compounds were tentatively identified by mass spectrometry analysis: i.e. matching mass spectrum of samples with database in NIST mass spectral library. Then, key compounds were confirmed by injection of pure compounds purchased from Sigma-Aldrich or provided by colleagues.

Bioassays

The bioassays were performed in a four-arm olfactometer, as shown in Fig. 1a and described previously (D'Alessandro and Turlings 2005). Air entered into the central "wasp release chamber" via each arm at a flow of 0.6 L per minute (Fig. 1a). Six virgin males were released at the same time in this release chamber where they were allowed to choose among the arms for 30 min. Males were considered having made a choice, when they had been trapped in one of the "wasp trapping bulbs" at the end of each arm (Fig. 1a). When all males had made a choice, i.e. ended up in a "wasp trapping bulb", or after a maximum of 30 min, the result was recorded, and the respective group of wasps was removed from the olfactometer, and a new group of six wasps was released. The number of males that did not make a choice, i.e. stayed in the release chamber, was generally very low (about 20% of released wasps) and was excluded from the analyses. For each replicate, glassware was cleaned with the method described in a previous paper (Desurmont et al. 2016). The assays were repeated six times per odour combination, and each replication included four releases of a group of six males. Thus, in total 144 virgin males were tested for each odour combination. The positions of the treatments were systemically changed

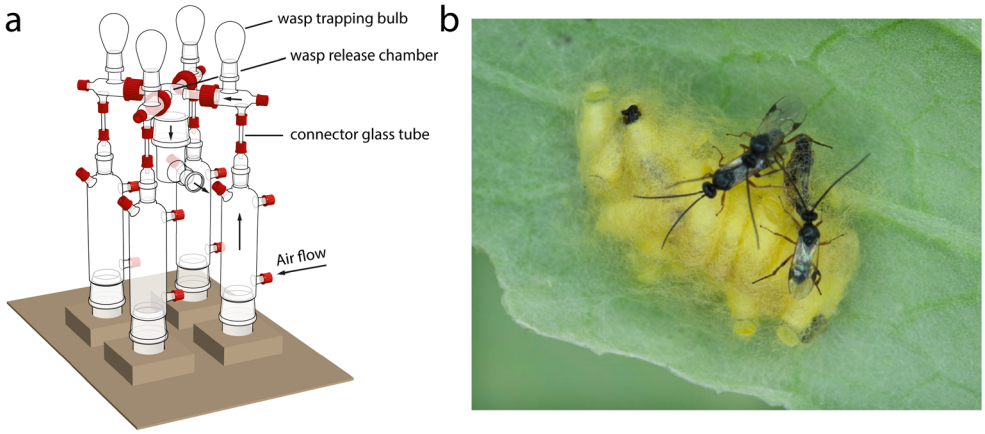

Fig. 1 **a** A drawing of the 4-arm olfactometer that was used for bioassays. A stream of purified and moist air was pushed through each arm and entered the "wasp release chamber". The samples were applied on a piece of filter paper (1 × 3 cm), which, after evaporation of the solvent, was rolled up and inserted in the "connector glass tube". A group of six virgin *C. glomerata* males was released in the "wasp release chamber", where they were allowed to make a choice among the different arms within 30 min. After wasps had entered one of the arms, they walked up into the "wasp trapping bulb" attracted by the light from a lamp above the centre of the olfactometer. **b** The gregarious parasitoid *C. glomerata* lays numerous eggs in each host caterpillar, and the last larval instars of the parasitoid pupate in cocoons that they construct into a patch next to their host. Adult males emerge first and mate with newly-emerged females on the natal patch with little competition with other males (see the Suppl. video)

(rotated) between releases to eliminate position effects (each treatment on each position once among replications).

A volume of 5 μl of the solvent extracts or headspace samples or of pure solvent (dichloromethane) was pipetted on a piece of filter paper (1 × 3 cm). If a treatment was a combination of two samples or more, then samples were mixed with the same total volume before being applied to filter paper (5 μl of mixture used). Before testing, the filter papers already treated with samples or solvent controls were put into a running fume hood for 15 min to allow solvent to evaporate (Steiner et al. 2007). Next, the paper was rolled up to fit into the "connector glass tube" (Fig. 1a). When the olfactometer was running, the air flow passed through the four pieces of filter paper in the "connector glass tube" of each arm and carried the volatile compounds to the "wasp release chamber" (Fig. 1a).

In a setup where we tested the attractiveness or repellence of an extract or synthetic compound(s) against a solvent control, two pieces of filter paper treated with the same samples were placed in two opposite arms of the olfactometer, with solvent controls in between. If there were two different treatments and two solvent controls, the two treatments were placed likewise at opposite arms, with controls in between. This design was selected to minimize male-male competition or repellence during the choice process. If there were three treatments and one solvent control, they were placed randomly into the arms of the olfactometer.

Parasitism Tests

Field investigations support the notion that the average sex ratio of *C. glomerata* is female biased, but it varies from patch to patch, and it is not uncommon that patches produce male-biased offspring or even only males (Gu and Dorn 2003; Tagawa 2000). On such patches with male-biased sex ratios, male-male competition for mating opportunities will be severe. To confirm this and to better understand the conditions under which females produce more males than females, we also studied the sex ratios of *C. glomerata* when offered hosts of different quality in the laboratory. To avoid a high proportion of diploid males caused by long-term laboratory rearing (Gu and Dorn 2003; Zhou et al. 2006), a new colony of *C. glomerata* was started with wasp cocoons collected from the field in Neuchatel, Switzerland, in September 2016. Its second generation was used for parasitism tests, which were carried out in insect rearing tents (40 × 40 × 60 cm) in the laboratory. Different numbers of mated foundresses (1, 3, 5, 7 and 10 mated females) were released to parasitize *P. brassicae* caterpillars (50 2nd instar larvae), which were feeding on a Chinese cabbage plant. They were allowed to parasitize for 24 h with a 16:8 h light:dark cycle at room temperature (about 25 °C), and the females had access to water and a honey solution. After 24 h, the wasps were removed from the tents, and the caterpillars were left on the cabbage plants until parasitoid pupation. The clutches of cocoons and pupae of surviving *P. brassicae* were harvested and counted. The cocoons from a test (i.e. a tent) were put in a Bugdorm-1 cage (30 × 30 × 30 cm, Mega View Science Education Services Co. Ltd., Taiwan), provided with honey and moist cotton wool at room temperature. After the wasp emerged from the cocoons, the males and the females were recorded and sex ratios were calculated. Each wasp density was replicated six times.

Statistics

For olfactometer data, statistical analyses were performed in R 3.0.2 with the package Lme 4 (Bates 2010). To test whether differences among responses of parasitoids to the treatments were significant, we used generalised linear mixed models (GLMMs) with Poisson distribution of error. The replicates were treated as random factor. Tukey's post-hoc test was performed for multiple comparisons, if necessary. The models were checked with the test of "overdisp" to estimate the residual deviation of the freedom factor, with considering the possible effects of over-dispersion caused, for instance, by positional biases or wasps affecting each others' responses (Davison and Ricard 2011). Each model was fitted by maximum quasilikelihood estimation in the software package R. To analyse the quantity of compounds, the data were analysed with One Way ANOVA followed with a Holm-Sidak test for multiple comparisons, performed with SigmaPlot 12.5. Statistical differences ($P < 0.05$) are indicated with different letters in the bar figures or with different numbers of asterisks, presenting different levels of significant differences in graphs (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). The results of the statistical tests are listed in Supplementary file 1.

Results

Only the headspace samples of virgin females collected on PDMS, but not those collected on SuperQ, were strongly attractive to males (Suppl. fig. 1). Surprisingly, SuperQ filters generally trapped compounds more efficiently than PDMS filters from the headspace of parasitoids, as well as the synthetic standards (Suppl. fig. 2). The difference in trapping efficiency was more significant for compounds with relatively lower molecular weights (Suppl. fig. 2). Possibly, the SuperQ filter collected more of the repellent compound(s) released by virgin females, which were less well trapped by PDMS filter. Consequently, for chemical analyses we used samples trapped with SuperQ.

Antennae of virgin males responded to a number of compounds that were non-sex-specific, i.e. released by both sexes, and did not depend on the mating status of the females (virgin or mated) (Fig. 2a–c). Confirmed with authentic standards, we identified the following compounds in the headspace samples of the wasp (Fig. 2b): heptanal, nonanal, nonadecane, eicosane, heneicosane, (*Z*)-10-heneicosene, tricosane, and (*Z*)-9-tricosene. Some compounds still remain unidentified (e.g. P3, P8, and P11 in Fig. 2b). Several compounds, such as heptanal and nonanal, evoked EAD responses in male antennae (Fig. 2a–c). A compound present in samples of virgin females, but apparently not in samples of virgin males or mated females, elicited a strong electroantennographic (EAG) response (marked with an arrow in Fig. 2a) in male antennae close to the end of the GC run. This compound occurred only in trace amounts in the samples and its identity remains unknown.

When using fractions of the extracts of virgin females for behavioural assays, only the combination of Fr5 and Fr6, rather than the combinations of Fr1 and Fr2, and of Fr3 and Fr4, was more attractive to males than the solvent control (Fig. 2d). In subsequent assays, this attractiveness was found to be only due to Fr6 (Fig. 2e). Only Fr6 of virgin females, not Fr6 of males or mated females was attractive to males (Fig. 2f), implying that the EAD active compound in Fr6 is the attractive pheromone that is only released in significant amounts by virgin females.

The combination of Fr1 and Fr6 of virgin females was attractive to males compared to the solvent control (Fig. 3a), but the combination was less attractive to males than Fr6 alone (Fig. 3b), suggesting that Fr1 contains repellent compound(s). When Fr1 of virgin females was replaced by Fr1 of virgin males, the repellence to males remained (Fig. 3g). Fr2, Fr3 or Fr4 of virgin females or virgin males all increased the attractiveness of Fr6 of virgin females (Figs. 3c–j). Fr5 of virgin females and virgin males did not affect the attractiveness of Fr6 of virgin females to males (Figs. 3f and k).

In Fr1, we identified two dominating aldehydes, heptanal and nonanal (Fig. 2 and Suppl. fig. 3). Heptanal was found to be strongly repellent to males (Fig. 4f). The combination of heptanal with Fr6 of virgin females or with nonanal were consistently less attractive to males than the relevant controls (Figs. 4a–g). Nonanal by itself did not attract males (Fig. 4c), but when combined with Fr6 of virgin females, it was more attractive to males than Fr6 alone (Fig. 4b). Nonanal therefore adds to the attractiveness of the essential compound(s) in Fr6, but is not attractive by itself.

The antennae of males responded differently to solvent extracts of heads, thoraxes and abdomens of virgin females: as highlighted in Fig. 5, only the extracts of abdomens evoked EAD responses at the retention time of the putative key component of sex pheromone, indicating that the unknown constituent was only released from the wasp's abdomen (Fig. 5a–c). Indeed, only abdomen extracts were more attractive than solvent controls (Fig. 5d). The low response to thorax extracts indicated repellency (Fig. 5d). Indeed, when comparing the attractiveness of the thorax extract of virgin females with that of head extracts and a solvent control, the repellent property of the thorax extract was confirmed (Fig. 5e). Heptanal and nonanal were detected in all sections of the wasp body and the amounts released by each body part appeared to increase in accordance with the size of each part (head < abdomen ≤ thorax) (Suppl. figs. 4a and b), suggesting these compounds are generically released from the cuticle. Heptanal was released by both males and females independent of mating status (Suppl. fig. 4c).

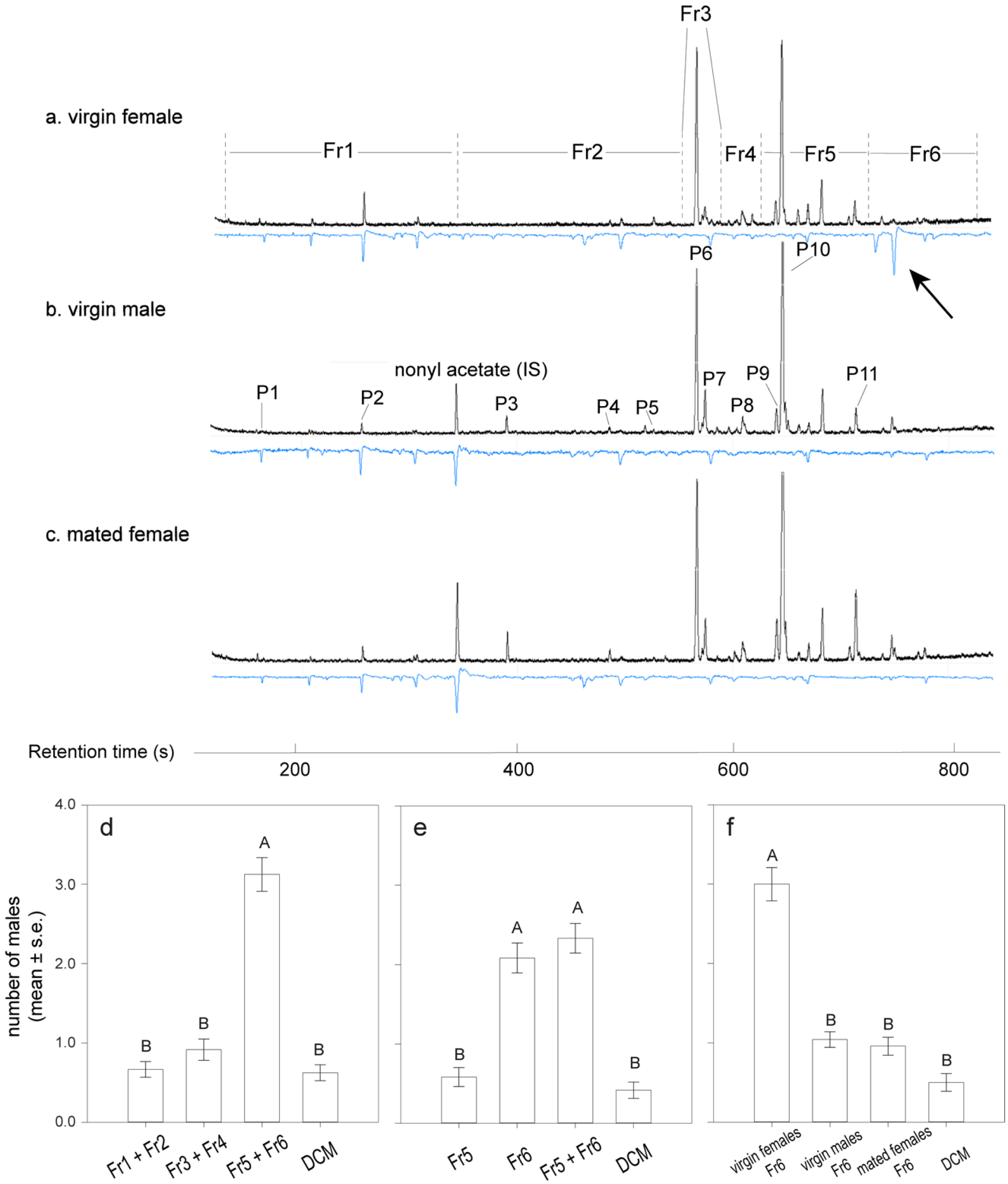

Fig. 2 Electrophysiological response of male antennae to the headspace volatiles of virgin females (**a**), virgin males (**b**), and mated females (**c**). The headspace extracts were fractionated with the use of a gas chromatography-preparative fraction collector (GC-PFC). The six fractions (Fr1–6) are indicated by dotted lines (**a**). The labelled peaks (**b**) represent: P1, heptanal; P2, nonanal; P3, unknown; P4, nonadecane; P5, eicosane; P6, heneicosane; P7, (*Z*)-10-heneicosene; P8, unknown; P9, tricosane; P10, (*Z*)-9-tricosene; P11, unknown. The arrow in (**a**) indicates a putative key component of sex pheromone. Attractiveness of fractions with virgin female volatiles to virgin males was tested (**d** and **e**). The relative attractiveness of Fr6 collected from virgin females, virgin males and mated females to virgin males are also shown (**f**). The letters above the bars indicate statistical differences ($P < 0.05$)

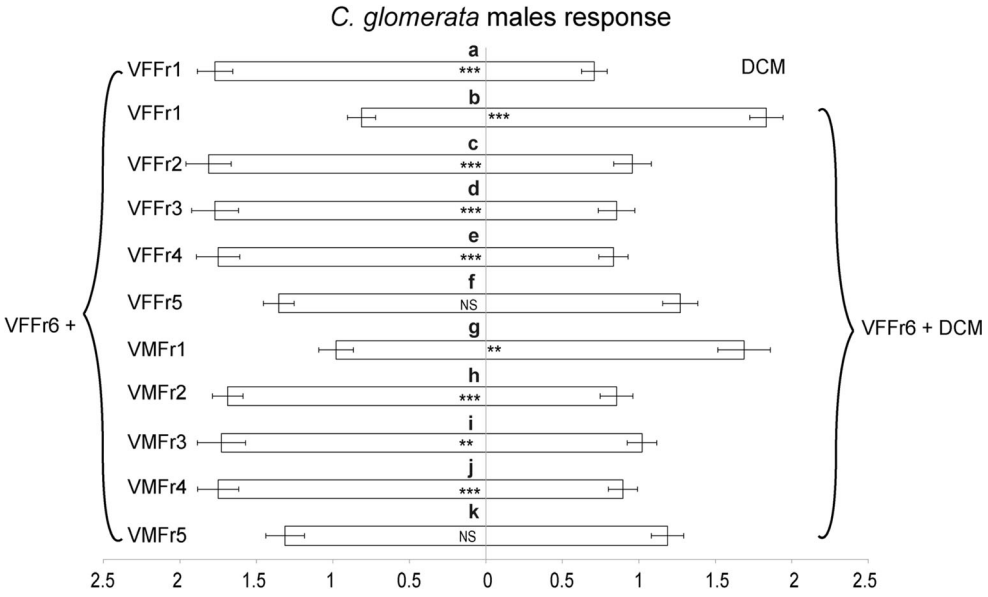

Fig. 3 The attractiveness or repellence of different fraction combinations to virgin males of *C. glomerata*. VF: virgin females; VM: virgin males; DCM: dichloromethane. NS, no statistical difference; "*" $P < 0.05$, "**" $P < 0.01$, "***" $P < 0.001$

In the experiment where different numbers of foundresses were offered 50 hosts to parasitize, the number of clutches of cocoons increased with increasing numbers of foundresses (Fig. 6a), as did the average number of cocoons per clutch (Fig. 6b). Correspondingly, the number of surviving *P. brassicae* (number of produced pupae) decreased with increasing numbers of foundresses (Suppl. fig. 5). Importantly, a higher proportion of males was produced with increasing numbers of foundresses (shifting from a female-biased ratio, 25% males, to a male-biased ratio, 67% males; Fig. 6c).

Discussion

The parasitic wasps are a very species-rich group (about 50,000 described species) and of great ecological importance in natural and agricultural ecosystems because they regulate the population density of their respective hosts (Godfray 1994). Our knowledge of the sexual communication systems in parasitoid wasps has lagged far behind that of other insect taxa (Godfray 1994; Ruther 2013). A recent review reveals that only for about 30 parasitic wasp species it is known that they use pheromones to communicate with conspecifics (Ruther 2013), and little is known about how gregarious parasitoid species use pheromones to optimize their mating success. Here we demonstrate that virgin females of the gregarious parasitoid *C. glomerata* release an attractive as well as a repellent pheromone. The repellent compound was identified as heptanal, and the key attractive pheromone was found to be produced only by virgin females. The combination of fractions with the repellent and the key attractant obtained from virgin females was found to be much more attractive than the solvent control (Fig. 3a),

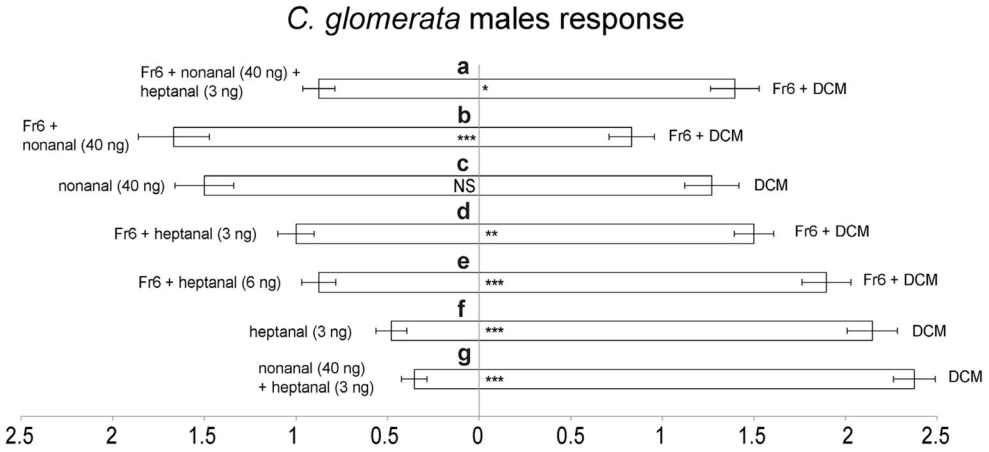

Fig. 4 The attractiveness or repellence of synthetic compounds to virgin males of *C. glomerata*. The two main constituents in Fr1, heptanal (3 ng), nonanal (40 ng) (equivalent to six individuals) were tested alone (**c** and **f**) or in combinations (**g**). Heptanal and nonanal were combined with Fr6 of virgin females to test for synergistic or additive effects in attracting virgin males (**a**, **b**, d and **e**). DCM: dichloromethane. NS, no statistical difference; "*" $P < 0.05$, "**" $P < 0.01$, "***" $P < 0.001$

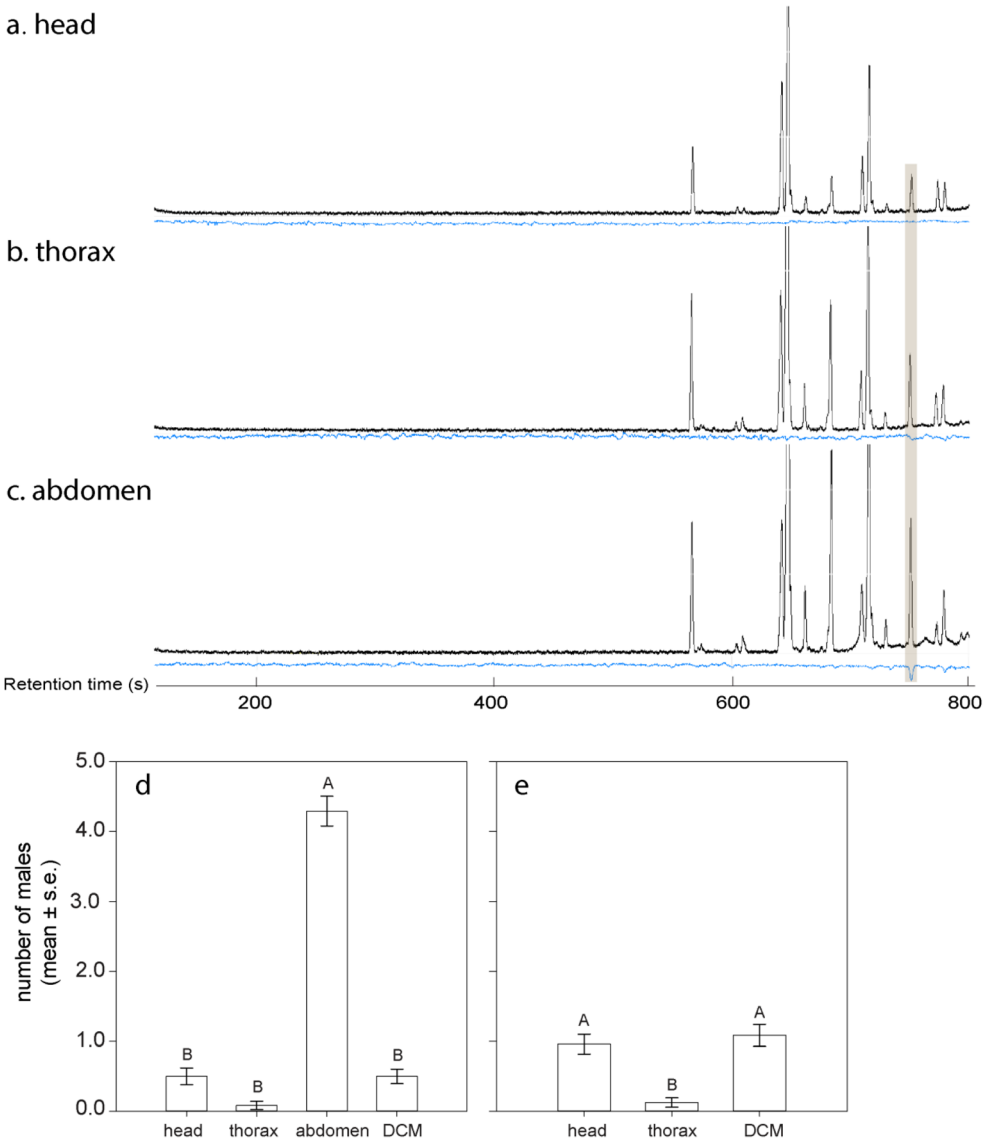

Fig. 5 Electrophysiological response of male antennae to solvent extracts of head, thorax and abdomen sections of virgin females (**a**, **b** and **c**). Only abdomen extracts evoked EAD responses at the retention time of the putative key component of sex pheromone (marked by the transparent grey line). Attraction of males to solvent extracts of heads, thoraxes and abdomens of virgin females (**d**). Extracts of thorax sections were less attractive to males than extracts of heads or solvent controls, implying repellence (**e**). The letters above the bar indicate statistical differences ($P < 0.05$)

implying that the repellent effect of heptanal is overridden by the attractive sex pheromone. After mating, the females appear to stop the production of the attractant, but keep releasing heptanal (Fig. 2 and Suppl. fig. 4c), which causes the males to be repelled, confirming the findings of Xu et al. (2014). Heptanal was also released by males, probably to reduce male-male competition on the natal patch.

Mating at the site where they reach adulthood or leaving such a site to mate elsewhere are opposing mating strategies in gregarious animals (Thornhill and Alcock 1983). It is known that the *C. glomerata* males usually emerge a few minutes before females and are arrested by an attractive sex pheromone that the females already emit at the cocoon stage (Gu and Dorn 2003; Tagawa 1977) (Suppl. video). The majority of newly emerged individuals of *C. glomerata* mates on their natal patch shortly after emergence (Gu and Dorn 2003; Tagawa and Kitano 1981). The frequency of such matings is largely influenced by the sex ratio on a patch, with a higher sex ratio (i.e. more male-male competition) triggering increased male dispersal (Gu et al. 2003; Tagawa and Kitano 1981). Our results show that this dispersal might be mediated by pheromones: we can expect that in cases where the sex ratio on a natal patch is relatively high (Fig. 6c), the high concentration of heptanal emitted by males may encourage some males to leave the natal patch before mating. In contrast, if the sex ratio is relatively low, we can expect that a certain proportion of newly-emerged females disperse before mating on natal patches (Gu and Dorn 2003). After dispersal, both virgin males and virgin females are strongly attracted by herbivore-induced plant volatiles (HIPVs), increasing the likelihood that they locate each other and mate before oviposition (Xu et al. 2017; Xu and Turlings 2018).

C. glomerata normally produces female-biased broods in the field (Gu and Dorn 2003). However, fierce competition for

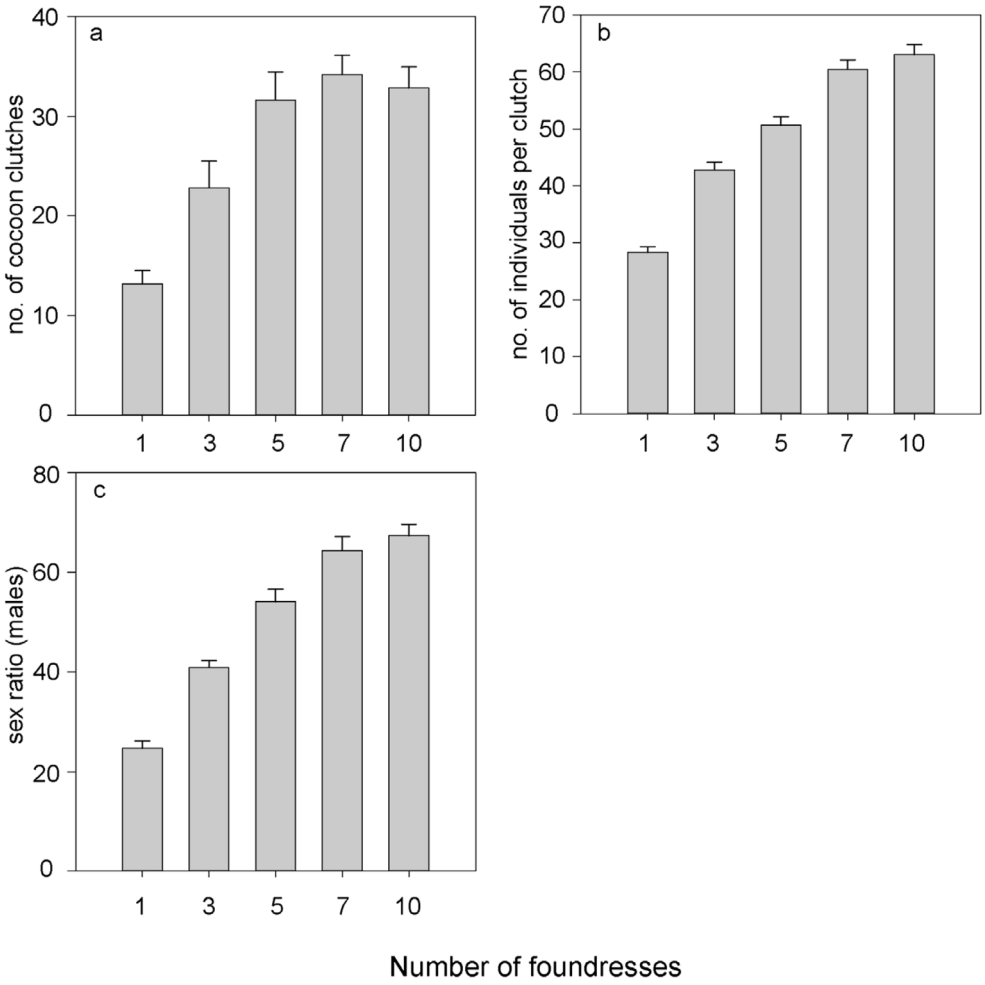

Fig. 6 Sex allocation by *C. glomerata*. The number of cocoon clutches (**a**), the number of cocoons per clutch (**b**), and the sex ratio of the emerged wasps (**c**) were obtained for different number of foundresses that were offered 50 caterpillars to parasitize

hosts can result in male-biased broods (Fig. 6c), as females that encounter hosts that are already parasitized may choose to produce more males to compete with unrelated males, but also because under limited resource conditions, males may show better survival rates than females (Boulton et al. 2015; West 2009). In *C. glomerata*, superparasitism occurs mainly when unparasitized hosts are rare (Gu et al. 2003), does not necessarily lead to increased mortality (Harvey et al. 2013), and results in a male-biased offspring (Gu et al. 2003). This will promote male-male competition and it may therefore be critical that adult males are able to assess the situation and can opt to disperse from a natal patch in order to search for mates elsewhere. Given the well-developed olfactory senses of insects, one likely strategy is to rely on pheromones, as is confirmed in our study.

For arthropods with less-developed wings, male-male competition can be very aggressive and even results in killing of the losers (Herre et al. 1997; Matthews et al. 2009; Reece et al. 2007), and females may even provoke male-male competition probably to select the strongest mating partner (Oku et al. 2015; Watson 1990). Both males and females of *C. glomerata* have well-developed wings and they are often found to leave their natal patch before mating (Gu and Dorn 2003; Tagawa 2000; Tagawa and Kitano 1981). Males that stay on the patch, probably because they are arrested by the pheromone released by emerging females, focus on finding these females rather than fight with other males (Suppl. video). Thus, it appears that this gregarious species has evolved a mating strategy that avoids fierce mating competition among siblings by pheromone-mediated dispersal.

In insects, anti-aphrodisiacs are used by mated females to dissuade males from mating attempts (Peso et al. 2015; Wyatt 2003). This might be also important for braconid parasitoids, in which females normally mate only once (Godfray 1994; Xu et al. 2014). As yet, the use of anti-aphrodisiac pheromones is largely unknown in parasitoid wasps, and only a few examples have been reported for other hymenopteran insects (Ayasse et al. 2001; Malouines 2017; Ruther 2013). In published examples, anti-aphrodisiac pheromones are believed to be merely biosynthesized by males either in specialized glands (Kohl et al. 2015; Larsdotter-Mellström et al. 2015; Schlechter-Helas et al. 2011) or produced by the cuticle (Scott 1986; Scott and Jackson 1988). They are purposely transferred from males to females during copulation

(Andersson et al. 2000; Gilbert 1976; Kohl et al. 2015; Larsdotter-Mellström et al. 2015; Malouines 2017; Scott 1986; Scott and Jackson 1988; Weiss et al. 2013). We report on the repellent volatile compound heptanal that is probably released from the cuticle of both male and female wasps independent of mating status (Suppl. fig. 4). There is no apparent transmission of this compound from males to females (Suppl. fig. 4c). This implies that *C. glomerata* employs an as yet unknown mating strategy, using cuticular volatile compounds to facilitate mate finding and dispersal decisions.

In conclusion, our results show that pheromones can act as essential cues for newly-emerged (quasi-)gregarious parasitoids in their decision to either stay at or disperse from natal patches to locate mates. The combined use of attractive and repellent pheromonal compounds appears to be an effective mating strategy that is likely to help maximize reproductive success in gregarious parasitoids.

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Author Contributions H.X., G.Z., S.D., T.D. and T.T. designed experiments; and H.X. and G.Z. performed bioassays. I.S., S.D., M.V.A., G.Z., L.C., and H.X. performed the electrophysiological analyses. H.X., G.Z., L.C. and G.R. did fractionation and chemical analyses. H.X. analysed data and made the Figs. H.X., T.D. and T.T. wrote an early version of manuscript. All authors commented on the manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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