The mate-seeking strategies of four braconid parasitoids (Hymenoptera: Braconidae)

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"The mate-seeking strategies of four braconid parasitoids (Hymenoptera : Braconidae)"

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Summary

Parasitic wasps are widely used as biological control agents. To optimize their applications in agriculture, we need to understand how they locate hosts and mate. Parasitic wasps have about 50,000 described species, but less than 30 species have so far been tested in terms of their use of pheromones. In this thesis, I used a combination of bioassays, chemical analyses, and electrophysiological measurements to study the sexual communication in four braconid parasitoids: *Cotesia glomerata* (L.), *Cotesia marginiventris* (Cresson), *Microplitis rufiventris* Kokujev and *Microplitis mediator* (Haliday). Virgin females of both *Cotesia* species release sex pheromones to attract conspecific males, which are not attractive to the other species. In *M. rufiventris*, virgin males are attractive to females, whereas males and females of *M. mediator* exhibit attraction to both sexes. The sex pheromones of the gregarious parasitoid *C. glomerata* and the congeneric solitary species *C. marginiventris* comprise both specific components and non-specific components. Some non-specific compounds (such as heptanal and nonanal) are produced by all body parts (heads, thoraxes and abdomens), indicating they are probably constituents of cuticular lipids (CLs). Some of these non-specific compounds are components of sex pheromones of the two *Cotesia* species, but only work synergistically with other components of their sex pheromone. The pheromone specificity of two *Cotesia* species appears to be determined by two specific compounds, which are released only in very small amounts, and which we, so far, failed to identify. Interestingly, the nonspecific CLs, which originally are probably part of the cuticular barrier to avoid desiccation and infection by entomopathgens, may have evolved pheromonal functions linked to the specific biology of the two *Cotesia* species. Heptanal is an anti-aphrodisiac in the gregarious species *C. glomerata*, probably reducing natal mate competition among sibling males, but in the solitary species *C. marginiventris*, heptanal is a sex pheromone constituent that synergistically enhances the attractiveness of other sex pheromone components. To my knowledge, this thesis presents the first study showing that CLs can evolve into distinct pheromonal functions. The sex ratio (percentage of males) of the gregarious parasitoid *C. glomerata* ranges from 25-67% depending on how ovipositing females perceive the quality and size of a host patch . In this species, mating on natal patches (the hosts from which the wasps emerge) is probably strongly influenced by pheromones: males normally emerge a bit earlier than sibling females, and males that emerge after that are arrested by emerging virgin females with sex pheromones, but repelled by other males and mated females, which produce the anti-aphrodisiac heptanal. By using a combination of attractive sex pheromones and the anti-aphrodisiac, the proportion of

males mating on the emergence sites probably varies according to the sex ratio (i.e. the level of male-male competition). Since the pheromones of the four braconid parasitoid species seem to work in a relatively short range, locating mate may be much more of a challenge for the studied solitary parasitoid species, as well as for those individuals of gregarious *C. glomerata* that leave their natal patch and try to find mates in other patches. Interestingly, virgin parasitoids of both sexes of all four braconid parasitoids were found to be strongly attracted by herbivore-induced plant volatiles (HIPVs), implying that host-damaged plants probably serve as rendezvous sites for mate-seeking individuals, in addition to being sites where the females find hosts for their offspring. Similar strategies have been reported for herbivorous insects and pollinators: host plant volatiles stimulate these insects to produce (or release) pheromones, and/or synergistically increased the attractiveness of pheromones to mate-seeking conspecifics. Based on the chemical properties (volatility) of typical insect pheromones and those of relevant plant volatiles, as well as recent theoretical and experimental advances in our understanding of the odour plumes that they form, I propose that insect pheromones and plant volatiles serve complimentary functions in mate location. I postulate that in many insects the use of plant volatiles has evolved into an efficient foraging strategy to not only find food (or host), but also mates.

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CHAPTER 1 Introduction and thesis outline

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The parasitic wasps are a very species-rich group (about 50,000 described species) and of great importance in natural and agricultural ecosystems because they regulate the population density of their host species [1]. The larvae of parasitoid wasps develop by feeding on the bodies of other arthropods, normally insect species. Parasitism normally results in the death of the hosts, which contributes to the importance of various species as biological control agents in agriculture [1-3]. Parasitoids of herbivores are known to use herbivore-induced plant volatiles (HIPVs) to locate their host [4]. This host location strategy has received ample attention and is the subject of many studies [5,6]. In comparison, our knowledge of their sexual communications has lagged far behind that of other insect taxa [7].

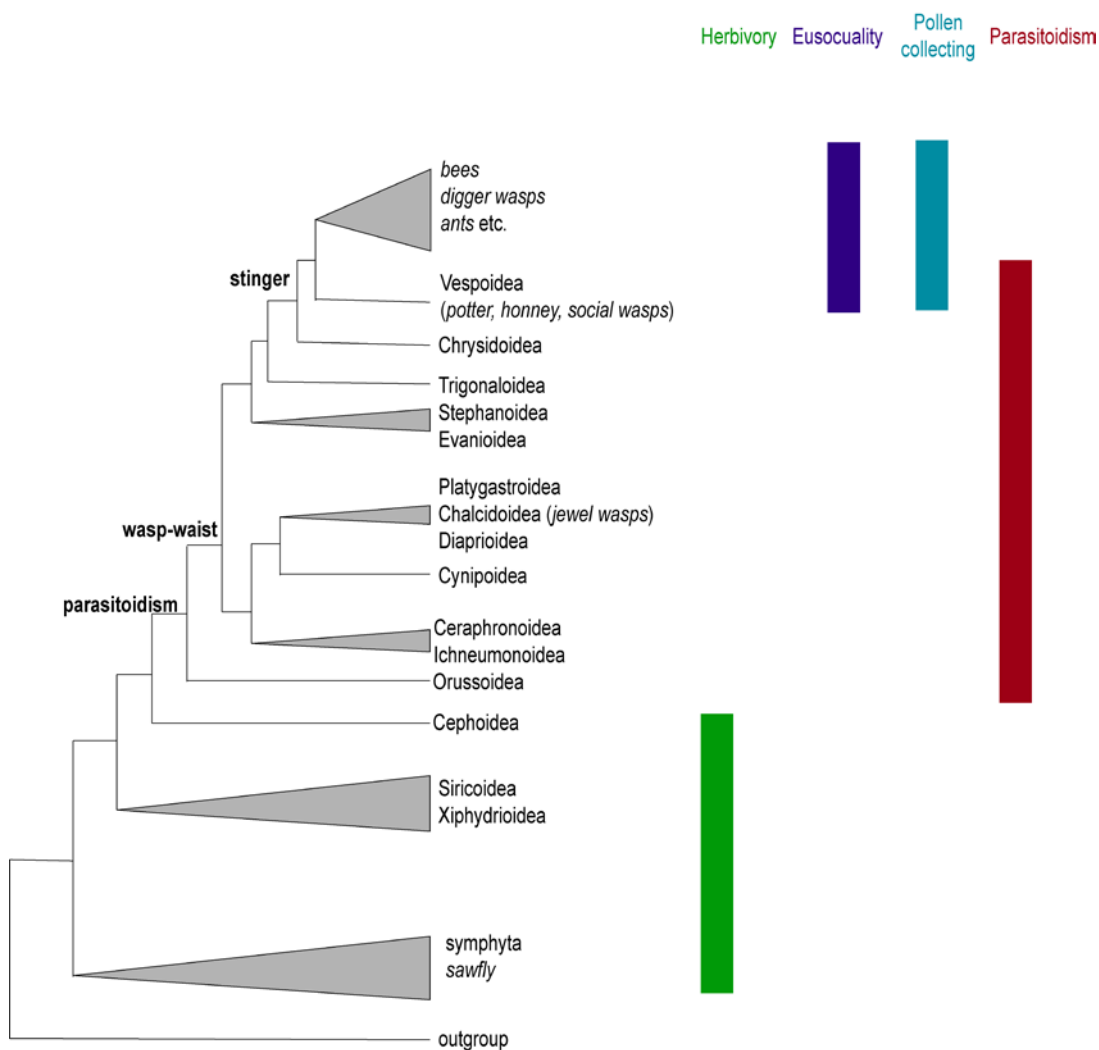**Figure 1** The phylogeny of Hymenoptera (modified from [1,8]). Parasitic wasps have evolved from their herbivorous ancestors (i.e. sawfly). Parasitic wasps are a species-rich group and appear in many families of Hymenoptera. When ovipositor evolves into stinger, hymenopterans start to engage in many new behaviours, such as predation, social behaviours, nectar feeding and pollen collecting behaviours.

A review by Ruther [7] reveals that fewer than 30 parasitic wasp species are known to release pheromones to communicate with conspecifics. Some parasitoid species produce and release highly volatile pheromones from specific glands or organs: for example, *Melittodia digitata*, *Nasonia* spp. and *Leptopilina heterotoma* etc. [7,9,10]. However, more commonly, the sex pheromone compounds of parasitoids are of relatively high molecular weight and mainly serve as short-range attractions over distances of maximally a few centimetres [1,7]. As yet, it is difficult to judge if the scarce examples of long-range sex pheromones is because they are truly rare in parasitoid wasps, or because they just have received little attention [1,2]. The production of volatile pheromones can be metabolically costly [1,7], and one hypothesis proposes that this could explain the scarcity of highly volatile pheromones in parasitic wasps: with the haplodiploid sex determination systems (unfertilized eggs developing to males, and fertilized eggs developing to females), female wasps possibly gain less from mating than diploid insects when both males and females are of equal value, which possibly leads certain parasitoid species to cease emitting long-range pheromones in natural selection [1]. This hypothesis remains untested in solitary parasitoid species, because it is very difficult to investigate the offspring sex ratio of solitary parasitoids in nature. For the gregarious parasitoid *Cotesia glomerata*, field investigations surprisingly find that the overwhelming majority of females successfully mate either with sibling males on their own natal patches, or with males of other patches after they have dispersed from their emergence sites [11]. How this and other parasitic wasps successfully locate mate even without a long-range pheromone remains poorly understood.

Many insects appear to facilitate mate-finding by mating at their emergence sites, at female feeding sites, at oviposition sites or at landmarks [12]. In these cases, the spatial locations of hosts and other resources in the environment shape the distribution of adult parasitoids and this may determine the type of mating strategy that they have adopted [1]. For example, gregarious parasitoids usually clump their cocoons together on a host and wasps show synchronized emergence as adults. This means that the broods are normally subject to local mate competition and inbreeding [2,13,14]. By comparison, the offspring of solitary parasitoids are more widely dispersed and face an entirely different challenge to find suitable mate [14]. These differences may have resulted in distinct mate finding strategies. It is likely that mated females of gregarious parasitoids are regularly disturbed by the nearby males, whereas the ones of solitary species are more likely to find a host without encountering males. So far, very little is known about possible differences in mate finding strategies between gregarious and solitary parasitoids.

In this thesis, I used behavioral bioassays, chemical analyses, and electrophysiological measurements to study: 1) the use of pheromones by four braconid parasitoids, 2) the pheromonal-mediated mating strategy of the gregarious parasitoid *C. glomerata*, 3) the differences in mating behaviours and pheromone uses between a gregarious and a solitary *Cotesia* species and 4) the plant-produced volatile cues that parasitoids use for mate location at relatively long distances.

Study systems

We used four braconid parasitoids (*Cotesia glomerata*, *Cotesia marginiventris*, *Microplitis rufiventris* and *Microplitis mediator*) to study their mate-seeking systems. The four species are endoparasitoid species which lay eggs in the body of the lepidopteran caterpillars (1-3 instar). The four species are koinobiont parasitoids, which means that newly parasitized hosts remain alive and feeding on plants until the last instar of parasitoid larvae emerge from the host body [1]. The four species have been or have a great potential to be used as biological control agents in crop protection. For example, the gregarious parasitoid *C. glomerata*, native to Europe and Asia, was imported to USA in 1883 to control the invasive pest *Pieris rapae* [15]. Similarly, *C. marginiventris,* which is currently distributed throughout North, Central, and South America, is an important solitary endoparasitoid of pestiferous noctuids and has been considered for augmentative biological control of these caterpillars on vegetables grown in greenhouse in Canada and Spain [16]. Both solitary species *M. rufiventris* (originally distributed in North Africa) and *M. mediator* (distributed in Europe and China) parasitized on several noctuid species [17,18]. And *M. mediator* was released to control the main pest *Helicoverpa armigera* on cotton plants in the field in China, and the damaged cotton boll and bud in the parasitoid-released field was 80% less than the control field [17].

The four parasitoids have been studied extensively, particularly in the context of their host searching behaviours with HIPVs. For example, in *Microplitis* species, males and females are both responsive to HIPVs in behavioural or electrophysiological tests [19-22]. For *C. marginiventris,* both males and females were attracted to certain green leaf volatiles (GLVs) and HIPVs [22]. In the gregarious parasitoid *C. glomerata,* more than half of newly-emerged females leave their natal patch without mating with siblings, but nevertheless most females successfully find mates and produced both female and male offspring in the field [23], which probably resulted from that mate-seeking females and males use pheromone and HIPVs in

combination for mate location after they had dispersed from emergence sites [24]. Nevertheless, much less is known about their mate finding behaviours with pheromones.

Four braconid parasitoids were reared with their respective hosts in our laboratory at the University of Neuchatel, Switzerland (Table 1). The *C. glomerata* rearing was started with individuals that had emerged from *Pieris brassicae* caterpillars collected from cabbage plants grown in gardens around Neuchatel, Switzerland. The offspring was reared on *P. brassicae* (first–second instar) that was fed on cabbage plants. *C. marginiventris* were initially obtained from the United States Department of Agriculture-Agricultural Research Service (USDA-ARS), Biological Control and Mass Rearing Research Unit (Stoneville, MI, USA), occasionally replenished with individuals from field collections in Mexico and reared as described in a previous study [25]. The hosts were about three-day old *Spodoptera littoralis* caterpillars (first–second instars), which were fed with a wheat germ-based artificial diet. The eggs of *S. littoralis* were provided by Syngenta (Stein, Switzerland). The parasitoid *M. mediator* was reared on first instar caterpillars of cabbage moth *Mamestra brassicae*, which were fed on Chinese cabbage. *M. brassicae* eggs were provided by Forschungsinstitut für biologischen Landbau (FiBL), Frick, Switzerland. The parasitoid *M. rufiventris* was reared on *S. littoralis* caterpillars (first–second instars) kept in square plastic boxes (15 × 13 × 5 cm) and fed with a wheat germ-based artificial diet. In order to get virgin wasps, each parasitoid cocoon was placed in a 1.5 ml centrifuge tube until the wasp emerged. Then, virgin females and virgin males were kept separately in two Bugdorm-1 cages (30 × 30 × 30 cm, Mega View Science Education Services Co. Ltd, Taiwan). In order to obtain mated wasps, about 50 females and 50 males of the same parasitoid species were put into the same Bugdorm-1 cage. The wasps were provided with honey and moist cotton wool, and the cages were stored in an incubator at 25°C (LD 16 : 8 h) for about three days before each test.

Figure 2 The parasitoid species used in the thesis.

Table 1. The tritrophic systems used in this thesis

Parasitic wasps	host species	host-food plant	gregarious or solitary
C. glomerata	P. brassicae	Brassica rapa	gregarious
C. marginiventris	S. littoralis	Zea mays	solitary
M. rufiventris	S. littoralis	Zea mays	solitary
M. mediator	M. brassicae	Brassica rapa	solitary

Outline of this thesis

In the Chapter 2, I investigated the use of sex pheromone by two *Cotesia* species and found that only virgin females released sex pheromones to attract conspecific males. The males of the gregarious species *C. glomerata* were repelled not only by conspecifc males and mated females, but also by virgin females of the congeneric species *C. marginiventris*. The results indicated the two *Cotesia* species use their own specific sex pheromones. The anti-aphrodisiac compound(s) of the gregarious parasitoid *C. glomerata* were probably not specific and also produced by *C. marginiventris*.

In the Chapter 3, I studied the pheromone-controlled mating system of the gregarious parasitoid *C. glomerata* in more detail. Virgin females were found to release a combination of an attractive pheromones and an anti-aphrodisiac compound which was identified as heptanal. The combination of these compounds arrested males on natal patches, but after mating the females appear to cease the production of the attractant and the males were repelled. The production of heptanal was not a sex-specific constituent of cuticular lipids (CLs) and was also released by males, probably to reduce male-male competition on the natal patch. Depending on the quality of a host patch, the sex ratio of the wasps emerging from host can range from 30-70% males. The use of pheromones by *C. glomerata* may help to maximize mating success. In case of a female-biased sex ratio emergence from a host, sibling males may perceive enough of the attractive pheromone to be encouraged to stay at their natal patches until most females have been inseminated. In contrast, male-biased offspring, which is typically produced when females parasitizes a host patch of relatively poor quality, may lead to male dispersal from natal patches because of the dominant presence of heptanal.

In the Chapter 4, I compared the use of pheromones by the solitary and gregarious *Cotesia* species. The virgin females of the solitary parasitoid *C. marginiventris* also produced heptanal. Interestingly, in this species heptanal was a component of female pheromone. It was not attractive to males by itself, but worked as a synergist of other components of the female-produced sex pheromone. In both *Cotesia* species, certain nonspecific compounds synergistically enhance the attractiveness of the main constituents of the species-specific sex pheromone. This specificity was probably achieved by two compounds that evoked strong electroantennographic (EAG) responses in the male antennae of the respective species. Overall, the results presented in this chapter imply that cuticular lipids can evolve into distinct pheromonal functions.

In the Chapter 5, I studied, in addition to the two *Cotesia* species, the use of pheromones by two *Microplitis* species, and found that the two species had distinctally different sex pheromone systems. Virgin males of *Microplitis rufiventris* were found to release a sex pheromone to attract females, and *Microplitis mediator* males and females exhibited attractions to both sexes. Importantly, females and males of all four braconid wasps were strongly attracted by HIPVs, independent of mating status. The wasps preferred the combination of HIPVs and pheromones over plant odours alone, except *M. mediator*, which appears to mainly use HIPVs for mate location. This appears to be the first study to show that braconid parasitoids use HIPVs and pheromones in combination to locate mates, which might explains the absence of long-range sex pheromones in certain parasitoid species.

In the Chapter 6, I review the current knowledge on the role of plant volatiles in mate location by insects. It is argued that this role is more important than commonly assumed. The combined use of plant volatiles and pheromones can be highly effective in mate-seeking processes. When studied on their respective chemical properties (volatility), as well as recent theoretical and experimental advances on odour plumes, it is suggested that they operate at different distances. It is postulated that in many insects the use of plant volatiles to find food (or host), as well as mates has evolved as a highly efficient foraging strategy.

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CHAPTER 2 Exceptional use of sex pheromones by parasitoids of the genus *Cotesia*: males are strongly attracted to virgin females, but are no longer attracted to or even repelled by mated females

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Abstract

Sex pheromones have rarely been studied in parasitoids, and it remains largely unknown how male and female parasitoids locate each other. We investigated possible attraction (and repellency) between the sexes of two braconid wasps belonging to the same genus, the gregarious parasitoid, *Cotesia glomerata* (L.), and the solitary parasitoid, *Cotesia marginiventris* (Cresson). Males of both species were strongly attracted to conspecific virgin females. Interestingly, in *C. glomerata*, the males were repelled by mated females, as well as by males of their own species. This repellency of mated females was only evident hours after mating, implying a change in pheromone composition. Males of *C. marginiventris* were also no longer attracted, but not repelled, by mated females. Females of both species showed no attraction to the odors of conspecific individuals, male or female, and *C. glomerata* females even appeared to be repelled by mated males. Moreover, the pheromones were found to be highly specific, as males were not attracted by females of the other species. Males of *Cotesia glomerata* even avoided the pheromones of female *Cotesia marginiventris*, indicating the recognition of non-conspecific pheromones. We discuss these unique responses in the context of optimal mate finding strategies in parasitoids.

Keywords: parasitoids; mate finding strategy; sex pheromones; repellency; *Cotesia glomerata*; *Cotesia marginiventris*; gregarious; solitary

1. Introduction

The reproductive success of parasitoids is tightly linked to the females' ability to find hosts for their offspring, but optimal foraging for food and mates is also essential to maximize adult lifespan and fitness. Having a haplo-diploid sex determination system, female hymenopteran parasitoids are able to reproduce without mating, but this will result in only male progeny. Hence, newly emerged females of parasitic wasps are facing the trade-off decision of either locating hosts as soon as possible, producing only male offspring, or first investing time and energy into mating before ovipositing, as this would allow them to produce both males and females. Generally, the latter option is observed to be favored [1-4]. Therefore, an effective mate-finding strategy can be expected in parasitoids. Indeed, the relatively few studies that have investigated mate finding in parasitoids have found evidence for sex pheromones (e.g., [5-8]). Mate finding in parasitoids is mainly based on pheromones released by females, although on some occasions, the roles are reversed, with the males also releasing sex pheromones [9,10]. After mating, females switch from releasing pheromones or searching for males to searching for hosts [3,11,12]. This switch may be immediate or take more than 24 hours after mating [3,11,13]. For example, mated females of *Nasonia vitripennis* stop responding to the pheromones released by males just five minutes after mating [8]. Mated females of *Cotesia vestalis* are strongly attracted by host-induced plant volatiles, to which virgin females, by contrast, are indifferent [12]. The fact that mated females cease to release pheromones gives other, still virgin females more opportunities to attract mates, and the mated females are less harassed by males and, therefore, can better concentrate on host location [8].

Gregarious parasitoids, when they leave their host, usually clump their cocoons together, and wasps show synchronized emergence as adults. This means that the broods are normally subject to local mate competition and probably inbreeding [9,14]. By comparison, the offspring of the solitary parasitoids are more widely dispersed and face an entirely different challenge to find suitable mates. These differences may have resulted in distinct mate finding strategies. It is likely that mated females of gregarious parasitoids may be readily disturbed by the nearby males, whereas solitary parasitoids are more likely to find a host without encountering other males. Little is known about possible differences in mating behaviors between gregarious and solitary parasitoids.

Only a few studies have dealt with the use of pheromones by parasitic wasps, especially in the context of specificity. For the genus, *Melittobia*, it is known that males of

certain species are also attractive to females of closely-related species [15]. The braconids, *Cotesia flavipes, Cotesia sesamiae* and *Cotesia chilonis*, all larval parasitoids with stem-boring lepidopteran hosts, mate interspecifically, and in olfactometer assays, males of *C. sesamiae* have been found to be slightly attracted to virgin *C. chilonis* females [16]. The pteromalid pupal parasitoid, *Trichomalopsis sarcophagae*, shares the same pheromone components with members of the sister genus, *Nasonia*, and in the case of *N. vitripennis*, specificity is assured through the production of an additional stereoisomer component [17].

Cotesia* is a very species-rich genus in the Braconidae (Hymenoptera), with an estimated number of nearly one thousand species distributed worldwide [18]. The group is significant for both practical applications in biological control and fundamental ecological research [18]. Females normally mate once, while males can mate several times throughout their life [16,19]. Given the species richness, it is surprising that studies on the pheromones in the group are so rare. What is known is, for example, that both sexes of *C. flavipes* attract each other by volatile and contact pheromones [16,19], and females of *C. rubecula* attract males with air-borne chemicals [20]. By contrast, mate finding in *C. sesamiae* and *C. chilonis* is not based on the release of volatile pheromones [16]. In *C. glomerata*, a model species for parasitoid research, there is some evidence from a field study that sex pheromones are not only produced by mature females, as males are already attracted before the females emerge from their cocoons [21,22]. Another well-studied *Cotesia* species is the generalist *C. marginiventris*, which is frequently used as an inundative biological control agent, but its pheromones and mating behaviors are as yet unexplored. In this study, we studied the mate finding behavior of gregarious *C. glomerata* and solitary *C. marginiventris* using a series of six-arm olfactometer assays. Thus, we obtained detailed information on the conspecific and heterospecific attraction for both sexes.

2. Experimental Section

2.1. Wasps

The two endoparasitoids were reared in our laboratory at the University of Neuchatel. The *C. glomerata* rearing was started with individuals that had emerged from *Pieris brassicae* caterpillars collected from cabbage plants grown in gardens around Neuchatel, Switzerland. The offspring was reared on *P. brassicae* (first–second instar) that was fed on cabbage. *C. marginiventris* were initially obtained from the United States Department of Agriculture-Agricultural Research Service (USDA-ARS), Biological Control and Mass Rearing Research Unit (Stoneville, MI, USA), occasionally replenished with individuals from field collections in Mexico and reared as described in Tamò *et al.* [23]. The hosts were about three-day old *Spodoptera littoralis* caterpillars (first–second instars), which were fed with a wheat germ-based artificial diet. The eggs of *S. littoralis* were provided by Syngenta (Stein, Switzerland). To obtain virgin adult wasps, each parasitoid cocoon was placed in a 1.5-mL centrifuge tube until a wasp emerged. Then, 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) for three days before each test. To obtain mated individuals, one female and one male were placed in a Petri dish (90 × 15 mm) and then tested immediately or stored in Bugdorm-1 cages with honey and moist cotton wool in a 25 °C incubator for about 18 or 40 hours before tests.

2.2. Bioassay

The bioassays were carried out in a six-arm olfactometer, as described by Turlings *et al.* [24]. Each arm had an air flow of 0.6 L per minute that entered the central release chamber (Figure 1). Six virgin wasps were released at the same time in this central chamber, where they were allowed to choose among the arms within 30 minutes. From previous studies (*i.e.*, [23,24]), we know that the wasps are initially attracted to the light above the olfactometer. When attracted by an odor, they will enter the respective arm and walk until they encounter metal screens that prevent them from walking further into the arm. At this point, they move upwards into a glass bulb that is located just before the metal screen (Figure 1). They will readily stay in these bulbs for the duration of the experiment. Thirty minutes after their release, the wasps in the bulbs were counted and removed, before a new group of six wasps was released.
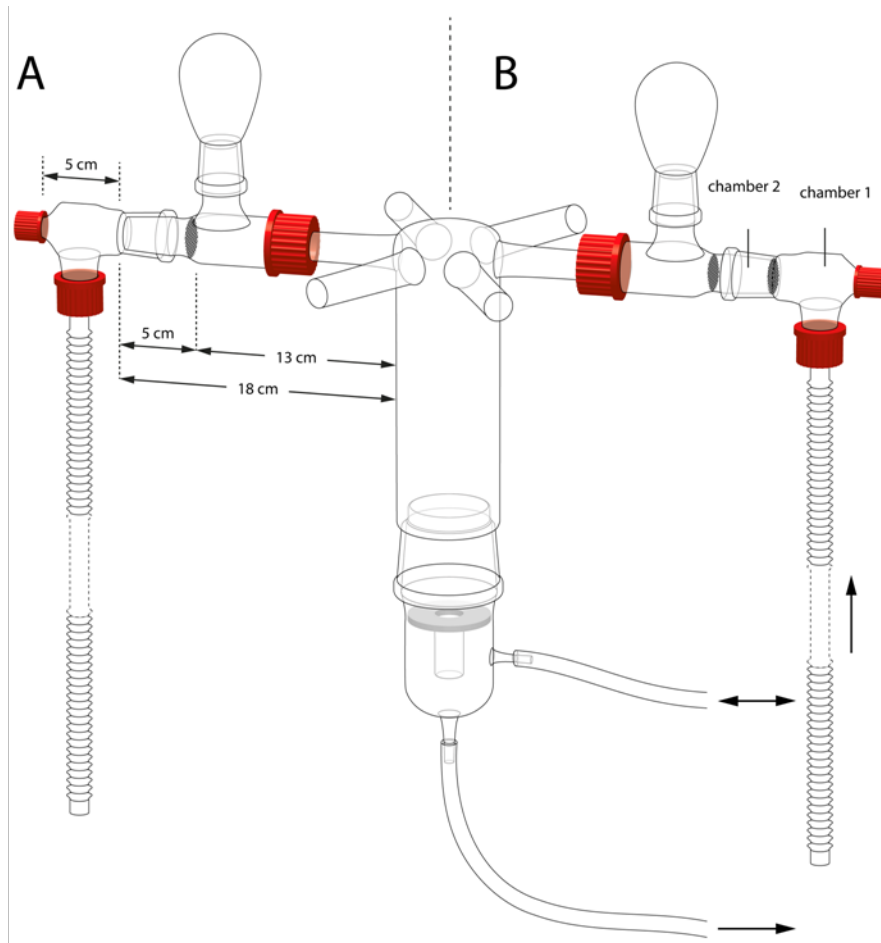
Figure 1. The six-arm olfactometer as it was used to test for attractiveness (**A**) and repellency (**B**). For the repellency tests, Chamber 1 contained virgin females (the source of attraction) and Chamber 2 either no wasps (control) or mated females or males (the source of potential deterrents).

Test for the presence of female pheromones: Six virgin females, six newly mated females and six unmated males were placed into three non-adjacent arms, respectively, and the three control arms in between were left empty. Several overlapping layers of metal meshes kept the "treatment" wasps from entering the central chamber, prevented the choosing wasps from approaching the pheromone source and reduced any potential visible cues (Figure 1A). Unmated males were released in the central chamber. The test was repeated four times, with six releases of six unmated males ($4 \times 6 \times 6$) on each experimental day, for a total of 144 wasps per experiment. Between replicates, the positions of the different treatments in the setup were changed.

Test for the presence of male pheromones: Six unmated males, six newly mated males and six virgin females were placed as odor sources in the olfactometer with the same design

as above. There were four releases of six virgin females per replicate. This assay was replicated four times, with a total of 96 wasps.

Test for repellency of mated females: To test if the observed poor responses to already mated females in the previous experiments were due to repellency, we combined odor sources as follows. Six virgin females were combined with six mated females; six virgin females were combined with six unmated males; and six virgin females were kept alone in each of the three non-adjacent arms separated by empty arms (control). The combined groups of six wasps were placed in two chambers in the olfactometer, as shown in Figure 1B: Chamber 1 contained the virgin females, and Chamber 2 included the six mated females (0, 18 or 40 hours after mating), or six unmated males, respectively, or was left empty. Each test was replicated four times with new wasps as odor sources and 6×6 unmated males each time (144 wasps).

Test for species specificity of *Cotesia* pheromones: Six virgin females of *C. glomerata* and *C. marginiventris* were placed into two opposite arms, and the remaining four arms were left empty. Unmated males (6×6) of either species were released into the central chamber to test their responses to these odor sources. An additional test was done to assess the responses to the interspecific pheromone in the absence of the conspecific pheromone. For this, only one arm of the olfactometer contained six virgin *C. glomerata* or *C. marginiventris* females, while the other five arms were left empty. Unmated males of the other species (6×6) were released into the central chamber. Each test was repeated four times (144 wasps).

Statistics: To test whether the differences among the responses of the parasitoids to the treatments were significant, we used generalized linear models (GLMs) with the assumption that the arms are equally chosen by parasitoids without stimuli [24]. The models take into account the possible effects of over-dispersion caused, for instance, by positional biases or wasps affecting each other's responses [25]. Each model was fitted by maximum quasi-likelihood estimation in the software package, R. In the figures, the number of wasps choosing empty arms was divided by the number of empty arms present in the setup to make it comparable to the other treatments.

3. Results

In both *Cotesia* species, virgin females were strongly attractive to males, whereas olfactometer arms containing mated females or males did not differ significantly from empty control arms in the number of male wasps that entered (Figure 2). Virgin females were not attracted to conspecific males (irrespective of mating status) or to virgin females (Figure 3). The overall pattern of responses was very similar for both species, except for the fact that virgin females of *C. glomerata* seem to avoid the arm containing mated males, which was not the case for *C. marginiventris* (Figure 3A).

After mating, females lost their attractiveness to males (Figures 2 and 4). In *C. glomerata*, mated females were even strongly repellent to the males several hours after mating (Figure 4C). No such repellency was found for *C. marginiventris* (Figures 4D and E). Virgin males were also repellent to males themselves in *C. glomerata*, but not in *C. marginiventris* (Figure 4).

In the cross-attraction tests, males of *C. glomerata* and *C. marginiventris* were not attracted by females of the other species, indicating that the pheromones are highly specific (Figure 5). Males of *C. glomerata* even avoided the odor of *C. marginiventris* females (Figure 5B), whereas males of *C. marginiventris* did not respond to female *C. glomerata* (Figure 5D).
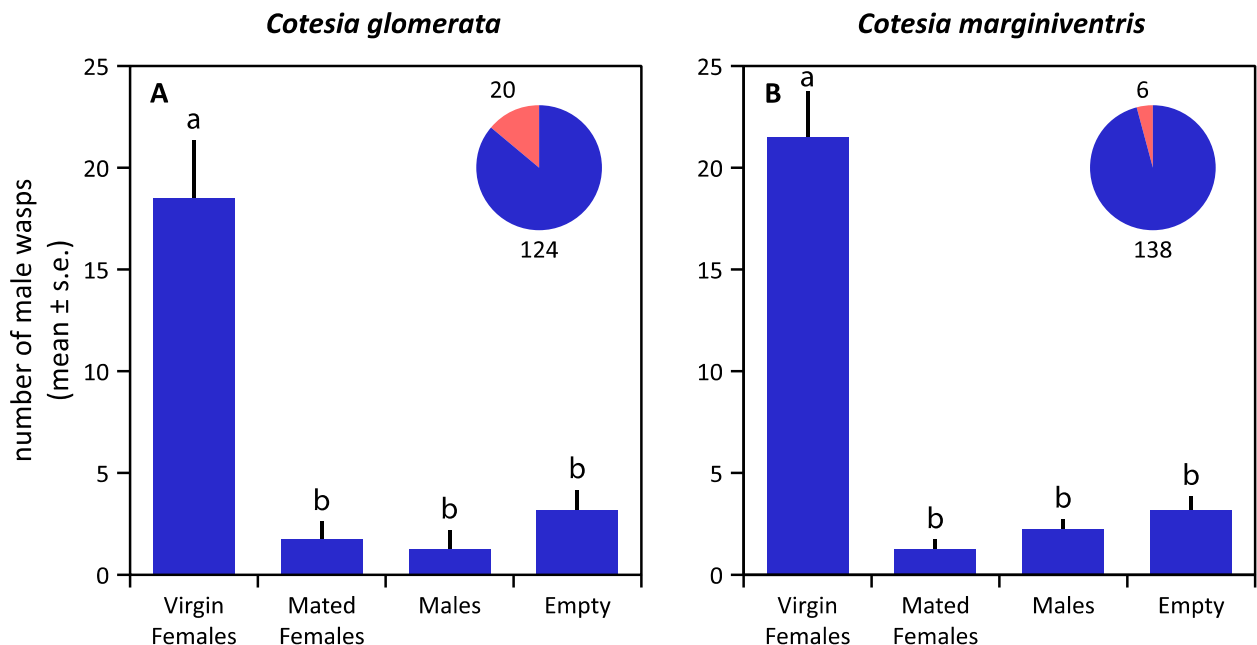

Figure 2. Responses of unmated males to conspecific virgin females, mated females and males in *C. glomerata* (**A**) and *C. marginiventris* (**B**) in a six-arm olfactometer. The proportion of wasps that showed no response, *i.e.*, that did not choose an arm, is given in red in the pie chart, supplemented with the absolute numbers of wasps. Statistical differences ($p < 0.05$) are indicated with different letters above the bars.
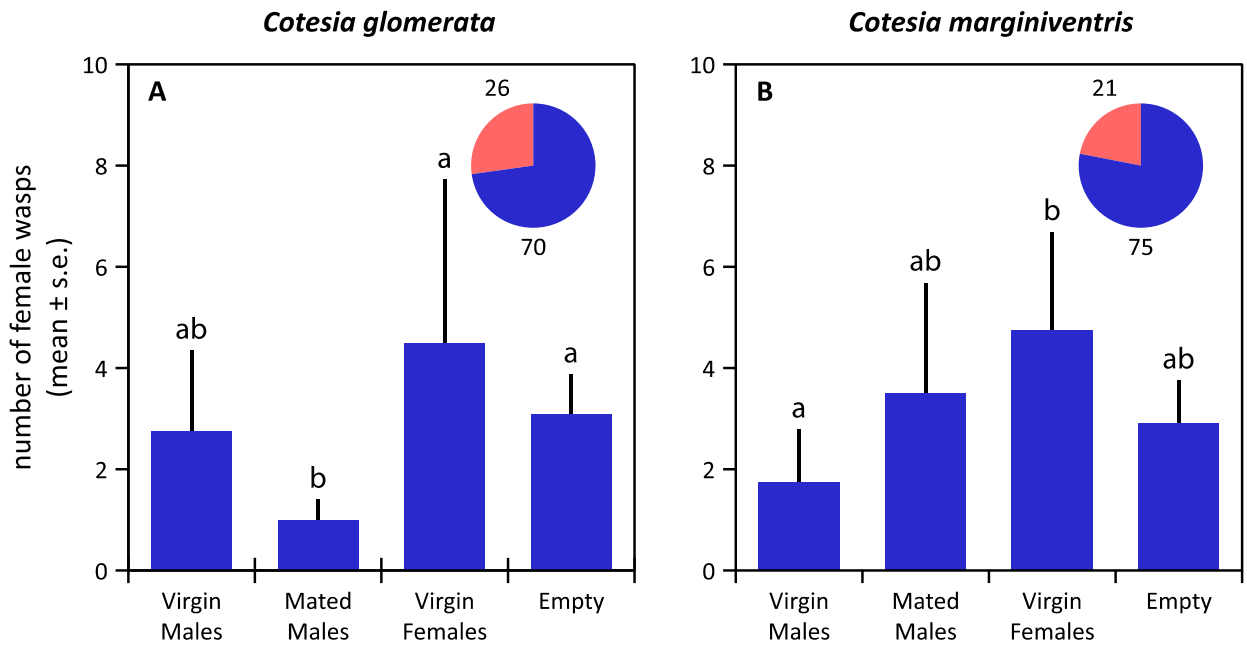
Figure 3. Responses of virgin females to conspecific virgin males, mated males and virgin females in *C. glomerata* (**A**) and *C. marginiventris* (**B**) in a six-arm olfactometer. The proportion of wasps that showed no response, *i.e.*, that did not choose an arm, is given in red in the pie chart, supplemented with the absolute numbers of wasps. Statistical differences ($p <$ 0.05) are indicated with different letters above the bars.
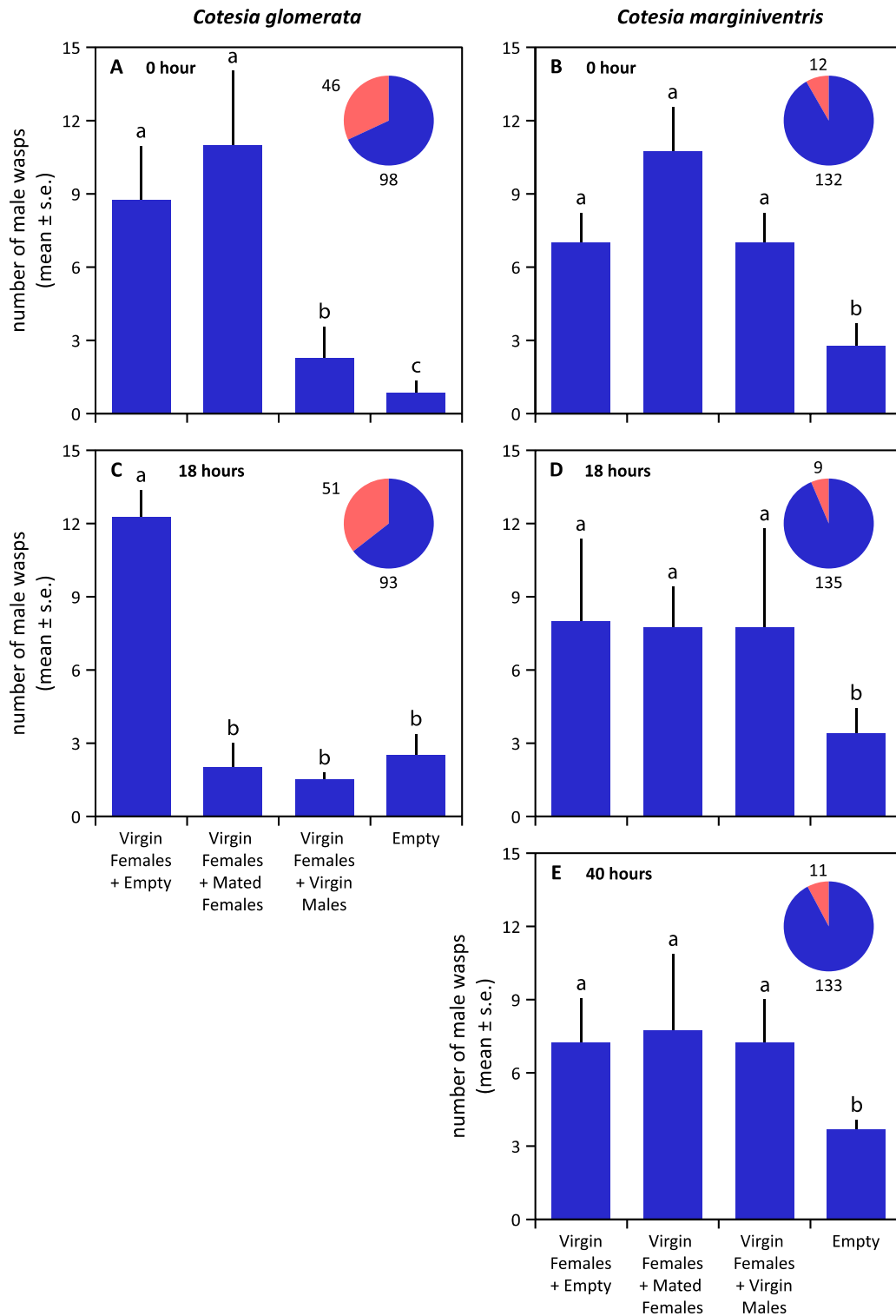
Figure 4. The six-arm olfactometer test for the repellency of mated females and of unmated males in the two parasitoid species, *C. glomerata* (**A**,**C**) and *C. marginiventris* (**B**,**D**,**E**), immediately (**A**,**B**), about 18 hours (**C**,**D**) and about 40 hours (**E**) after females had mated, respectively. The proportion of wasps that showed no response, *i.e.*, that did not choose an arm, is given in red in the pie chart, supplemented with the absolute numbers of wasps. Statistical differences ($p < 0.05$) are indicated with different letters above the bars.

Figure 5. Cross-attractant responses of male wasps, *C. glomerata* (**A,B**) and *C. marginiventris* (**C**, **D**), to con- and inter-specific female cues in a six-arm olfactometer. Interspecific cues were either tested in combination with conspecific cues (**A,C**) or alone (**B,D**). The proportion of wasps that showed no response, *i.e.*, that did not choose an arm, is given in red in the pie chart, supplemented with the absolute numbers of wasps. Statistical differences ($p < 0.05$) are indicated with different letters above the bars.
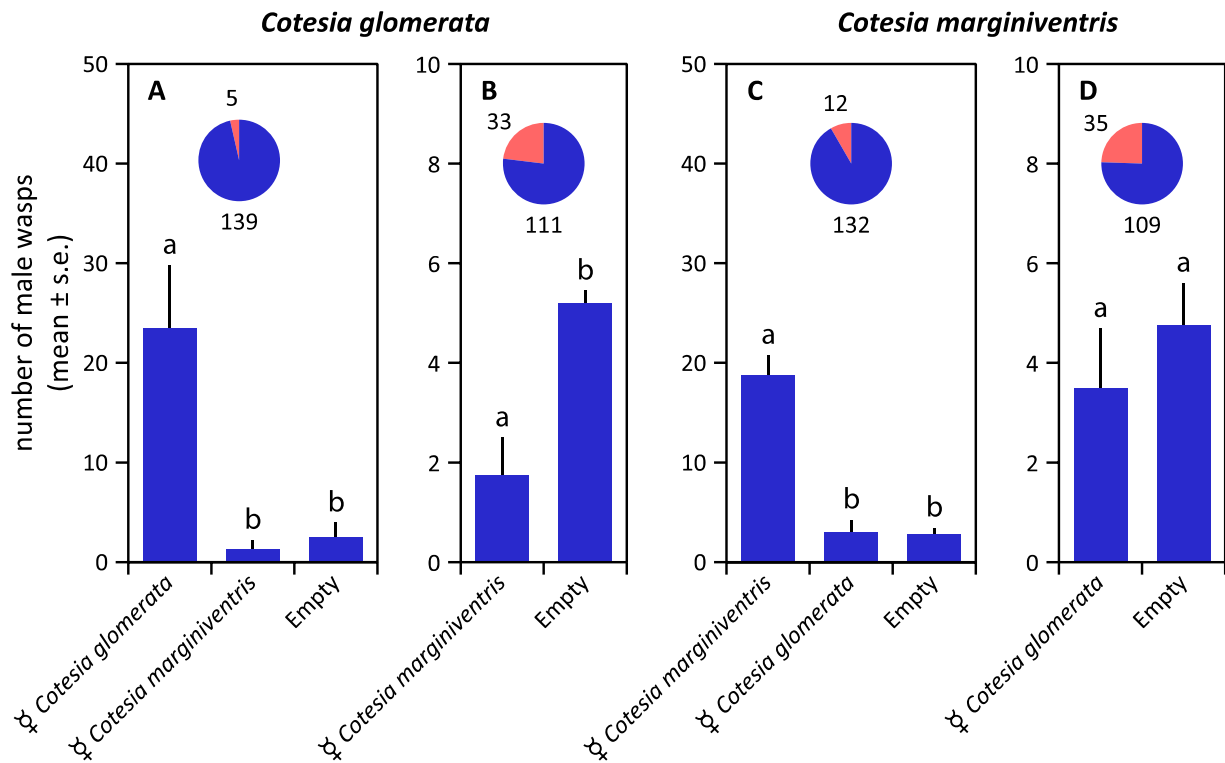
4. Discussion

4.1. Mate Finding Strategy of Cotesia

Our study reveals that virgin *Cotesia* females attract conspecific males by volatizing pheromones. In the two studied species, virgin males do not attract females from a distance (about 15 cm in our test, Figure 1A). As a gregarious parasitoid, it should be easier for males of *C. glomerata* to find females to mate as compared to the solitary sister species. We frequently observed that males of *C. glomerata* emerged earlier and stayed on or near the cocoon cluster, waiting for the females to emerge. The attractant has been shown to be emitted already at the late developmental "black-eye" stage of the cocoons [21]. Mating normally takes place soon after female emergence within 10 cm from the natal patch, which is within the pheromonal functional range [22]. In our tests, the odor cues were attractive at a distance of at least 18 cm (Figure 1B). Not all individuals mate on the natal patch: the percentage of *C. glomerata* wasps mating on the natal patch has been estimated at about 60%

[22]. Since there are very few females that parasitize their hosts without mating in the field [1], outbreeding must be considered an important part of the mating strategy of *C. glomerata*. However, the pheromones are apparently active only over relatively short distances. We found that the attraction over a distance of 18 cm (Figure 4A,C) was lower compared to a 13 cm distance (Figure 2A), as indicated by the response rate. Response rates are further reduced at a greater distance [26]. Over longer distances, the mating strategies of these and other parasitoids may also involve host-induced plant volatiles (HIPVs). *Cotesia plutellae*, for instance, probably uses both sex pheromones and plant volatiles for mate finding at a long distance [27], and a few studies suggest that HIPVs aid in the mate finding in other genera and families of hymenopteran parasitoids [28,29], but direct evidence is still lacking. *C. glomerata* is also able to use visual cues to locate mates [30].

Compared with the gregarious parasitoids, for solitary wasps, the challenge to find a mate would logically require detection over longer distances. Yet, certain solitary wasps may mate in their natal patch, because the hosts are aggregated, as is the case for aphids and scale insects [31]. This is not the case for caterpillars that serve as hosts for *C. marginiventris*, which generally disperse after hatching [32-34]. Therefore the offspring hatching from these hosts has to engage in a longer-range mate search. Our results imply that males actively search for females and that they use a pheromone released by females.

Cotesia females mate only once [16,19]. After mating, the female parasitoids switch their foraging behavior to host finding. Indeed, we found that mated females of the two *Cotesia* species lost their attractiveness rapidly after mating (Figure 2), and mated females of *C. glomerata* even became repellent to males (Figure 4). Something similar has been reported for the braconid aphid parasitoids, *Aphidius nigripes* and *Aphidius ervi*: in a field study, mated females attracted significantly fewer males than did virgin females [5,7]. Secretions transferred during male ejaculation may inhibit pheromone production in females. This phenomenon is known as pheromonostasis [35,36] and is known to occur in dipterans and lepidopterans [11,13,37]. For certain Lepidoptera, including *Pieris* species that serve as hosts of *C. glomerata*, it has even been found that males transfer so-called "anti-aphrodisiacs" to females, resulting in reduced attraction and even repellency [38,39]. This in turn may attract egg parasitoids in search of a ride to oviposition sites [40,41]. An analogous phenomenon has been reported for *Drosophila melanogaster*, where cuticular hydrocarbons that are characteristic for males have anti-aphrodisiac properties and are transferred to females during mating [42,43]. Several hours later, mated females even appear to mimic males by synthesizing this same compound to deter courting males [42]. It is possible that the transfer

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of "anti-aphrodisiacs" also occurs in the studied *Cotesia* wasps. In *C. glomerata*, it could explain why mated females, as well as males are repellent to conspecific males, but the fact that the females are not yet repellent right after mating (Figure 4) suggests a change that occurs in the females. Alternatively, the males might deposit a repellent pheromone during copulation that needs some time to be released or activated.

4.2. Possible Differences in Mating Behaviors between Gregarious and Solitary Parasitoids

Due to their distinct distribution within a colony, gregarious and solitary parasitoids can be expected to differ in their pheromone communication system. For *C. glomerata*, mated females were found repellent to males. This allows males to optimize their efforts to find unmated females and minimizes disturbance of females by males while they forage for hosts. In contrast, mated females of *C. marginiventris* exert no repellent effect upon males (Figure 4).

Parasitoids are expected to choose the lower-cost option between actively searching for mates and the biosynthesis of pheromones in sexual communication [10]. It is not likely for both sexes to release the pheromones at the same range. When the individuals hatch close to each other, as is the case with gregarious parasitoids, it could be expected that the wasps do not invest in costly pheromone release. This is clearly not the case for *C. glomerata*, since the females emit pheromones in spite of the large proportion of females that mate in the natal patch [1,22]. The attractiveness of the pheromones was at least as strong in *C. glomerata* as in *C. marginiventris*, suggesting that at this level, there is no difference in the investment in pheromones between the solitary and gregarious parasitoid.

Cotesia females mate only once, but the males are able to mate several times during their lifetime [16,19]. Interestingly, females of *C. glomerata* avoided mated males in the six-arm olfactometer (Figure 3A). Apparently, females give a priority to unmated males for mating, possibly because they would transfer more sperm. In addition, males of *C. glomerata* are repelled by conspecific males. Males emerge earlier than females, and then, most of them stay around the clustered cocoons, fanning their wings or waiting motionless on an unemerged cocoon [26]. Therefore, the observed mutual repellency may promote male dispersal and reduce competition. Considering that most males in a patch will be 100% related, this competition avoidance will be highly adaptive. The male-male repellency was not observed in *C. marginiventris*, where competition among brothers is much less evident, as their cocoons are much more widely distributed.

4.3. Species Specificity of Sex Pheromones

In several parasitoids, some cross-attraction between related species has been observed [15-17]. In contrast, we found the specificity of pheromonal responses in the two *Cotesia* species to be very high. *C. glomerata* males even avoided the odor of *C. marginiventris* females (Figure 5B). To our knowledge, no evidence of repellency between different parasitoid species has previously been reported. The apparent recognition between the two *Cotesia* species indicates a close evolutionary relationship and enforces prezygotic reproductive isolation. The genus, *Cotesia*, is exceedingly diverse [18], and pheromone-mediated avoidance among different genotypes may have contributed to this diversity. The identification of the actual pheromones that are involved in the interactions is necessary to further analyze the relationships among *Cotesia* species.

5. Conclusion

The sexual communication system in the two *Cotesia* species was found to rely on pheromones released by the females. There was no indication that females use male pheromones for mate finding, but it is not excluded that, upon contact, male-produced pheromones play a role in mate choice. Males of the two tested parasitoids showed very similar and highly specific pheromonal responses. The only difference was that males of the gregarious parasitoid were repelled by mated females and males, whereas the solitary parasitoid showed no response to these sources. The observed repellencies in the gregarious parasitoid may be linked to the initial high aggregation of the offspring around the natal patch. Once they have mated, aggregation is no longer adaptive, and repellency may facilitate proper dispersal and help to optimize foraging for a host.

Acknowledgments

Author Contributions

Hao Xu, Nathalie Veyrat and Ted Turlings designed the experiments. Hao Xu and Nathalie Veyrat performed the experiments. Hao Xu and Thomas Degen analyzed the data and made the figures. Hao Xu, Ted Turlings and Thomas Degen wrote the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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CHAPTER 3 The use of attractive and repellent sex pheromones in a gregarious parasitoid

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Abstract

Mating sites of insects are found mainly at female emergence sites, oviposition sites, female feeding sites or landmark sites (such as a swarming flight). The favoured mating sites are shaped by the ecological pressures peculiar to each species, in which pheromone controls are little understood. The parasitic wasps are key model organisms to study mating systems and sex allocation theories of animals. New-emerged wasps of the gregarious braconid *Cotesia glomerata* (L.) (Hymenoptera: Braconidae) mate at emergence site or disperse from their natal patch to find mating in five minutes. The two choices are thought to be mediated by sex ratio of offspring which results in variable levels of male-male physical competition. Here we report a pheromone-controlled mate system of *C. glomerata*. Virgin females were found to release a combination of an attractive pheromones and an anti-aphrodisiac compound which was identified as heptanal. The combination of these compounds arrested males on natal patches, but after mating the females appear to cease the production of the attractant and the males were repelled. The production of heptanal was not a sex-specific constituent of cuticular lipids (CLs) and was also released by males, probably to reduce male-male competition on the natal patch. Depending on the quality of a host patch, the sex ratio of the wasps emerging from host can range from 25-67% males. The use of pheromones by *C. glomerata* may help to maximize mating success. In case of a female-biased sex ratio emergence from a host, sibling males may perceive enough of the attractive pheromone to be encouraged to stay at their natal patches until most females have been inseminated. In contrast, male-biased offspring, which is typically produced when females parasitizes a host patch of relatively poor quality, may lead to male dispersal from natal patches because of the dominant presence of heptanal. Altogether, we firstly, to our knowledge, demonstrated that the choice of mating site possibly adjusted with pheromone controls at emergence sites, instead of simply with male-male physical fighting in a parasitoid.

Key-words: parasitoid wasps; mate location; sex allocation; sibling mating; natal mating; inbreeding; outbreeding; sex ratio.

Introduction

Mate-seeking males tend to gather in that part of their environment where receptive females are most likely concentrated. Mating at emergence site is adaptive when the new-emerged females are receptive to mate shortly after emergence and they will use the sperms of the first mating male to fertilize their eggs (such as copulating only once in their lives) [1]. Mating at emergence site is very common among gregarious and quasi-gregarious parasitoids, the latter being solitary parasitoids parasitizing on clumped hosts [2,3]. Natal mate system was interpreted as male-male physical competition ranging from mildly to lethally in parasitic wasps [2,3]. For example, newly emerged males of some fig wasps or *Melittobia* wasps fight among themselves until just a single individual remains, and the winner will mate with all females in the emergence area [4-6]. More frequently, the competition among male siblings presents as mild fighting and does not result in killing of the losers [7,8]. For instance, *Nasonia vitripennis* males jostle each other to be near the exit where the females gets out from the host [2]. Some males of fig wasp species have evolved special abilities to mate with pre-emerged females [5]. Other males of some fig wasp species mate with speed competitions: when the fig fruits become mature and open, males rush into the fruit and mate with the females inside within a few minutes [2]. So far, the studies of mating behaviours of gregarious or quasi-gregarious parasitoids at natal patches have mainly focused on male-male physical competition, while pheromone-mediated interactions are largely neglected.

Sex allocation of both plants and animals vary dramatically in different environmental conditions [9,10]. Animals are able to adjust the sex ratio (i.e. ratio of males) in response to the level of competition for mating opportunities and to the availability of resources for the development of their offspring [10,11]. Parasitic wasps with their haplodiploid genetic system are the model organisms for studying sex allocation strategies [3,12,13]. For example, exclusively sibling mating on a natal patch will lead to a female-biased sex ratio in the offspring, which is predicted by local mate competition (LMC) theory [7] and confirmed in several gregarious parasitic wasp species [13]. The foundresses tend to produce a controlled number of male offspring which are merely enough to mate with the sibling females on an emergence site [13]. A female-biased sex ratio of offspring guarantees that the sons mates with sibling females successfully with little male-male competition and further favours males to stay at natal sites [2,8]. Alternatively, in some parasitoid species, a proportion of wasps with well-developed wings disperse from the natal patches without mating with siblings and thereby involve in out-breeding (namely partial LMC) [14-17]. The sex ratio was predicted by

partial LMC to be relatively higher than that of the species which rely completely on sibling mating, and the sex ratio would tend to be approaching 50% when more and more males and females involve in a panmictic mating system [13,15,18,19]. The partial LMC theory was confirmed in fig wasps: the species with winged males had a less female-biased sex ratio than species with wingless males under similar conditions [14].

Sex allocation is not only affected by male-male competition in natal patches but also by competition among foundresses for host resources, which is known as local resource competition (LRC) [3,13,15]. In the LRC theory, sex ratio will bias towards the sex (normally the male in insects) with relatively higher fitness under poor conditions [20,21]. The LRC leads to produce a male-biased offspring in hymenopteran insects. For example, female beewolves produce a biased sex ratio towards males under restrictive conditions [22]. Similarly, in some ant species, male-biased offspring is also observed at a poor condition [23,24]. In parasitoid wasps, the impact of competition for resources on sex allocation is little known [3] and only a few examples is published. For example, in the hyperparasitoid *Gelis acororum,* wingless females produce a male-biased offspring possibly due to fierce female-female competition for host resources [25].

The gregarious parasitoid *C. glomerata*, acting as a model of studying mating system and sex allocation theories, involves in a partial LMC, and about 40% of new emerged wasps leave the emergence site without mating with siblings in field investigations [16,17,26-32]. In average, it has a female-biased offspring (about 50%), but male-biased clutches are also found commonly (about 10%) in field observations [16,17,33]. Superparasitism (multiple oviposition on a host by different foundresses) happens frequently in this species, especially when the hosts are present in low density, and that superparasitism does not apparently affect the mortality of mature parasitoids [31,34,35], but instead leads to a higher sex ratio when the clutch size is getting bigger [16,28,34]. In a previous study [30], we found that only virgin females *C. glomerata* released sex pheromones to attract males. Interestingly, males were repelled by both males and mated females, which indicated that males and mated females produce anti-aphrodisiacs [30]. In this study, with chemical analyses and bioassays, we further studied the pheromone-controlled mating system of this species and its possible contributions in maintaining sex allocation strategy.

Materials and Method

Insects

The parasitoid wasp *C. glomerata* was reared in our laboratory at the University of Neuchatel following the protocol described in a previous publication [30]. *C. glomerata* and its host *Pieris brassicae* were originally collected in gardens, Neuchatel, Switzerland. The offspring of *C. glomerata* was reared on *P. brassicae* caterpillars (first–second instar), which were fed on Chinese cabbage plants. The Chinese cabbages were planted in a pot (ID = 30 cm) and had grown in green house (25 °C, LD 16:8 h) for about one month before used for food of *P. brassicae* caterpillars. To obtain virgin adult wasps, each parasitoid cocoon was placed in a 1.5 mL centrifuge tube until a wasp emerged. Then, 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 new-emerged females and 50 new-emerged males of the same parasitoid species were put into the same Bugdorm-1 cage with honey and moist cotton wool [36]. Wasps were about 1-3 days old when they were used for tests.

Headspace extraction of pheromones or standard compounds

Two kinds of filters with absorbents of SuperQ (25 mg, 80-100 mesh) or polydimethylsiloxane (PDMS) were used for collecting volatile pheromones of wasps. The PDMS filters (OD = 6 mm, ID = 4 mm, length = 60 mm, Gerstel GmbH) were originally designed for Gerstel thermal desorption units.

C. glomerata parasitoids (100-200 individuals, either virgin or mated ones) were put in a glass bottle (250 mL). A stream of pure and moist air entered the glass bottle with a speed of 1.1 L/min. A vacuum pump sucked the air from the glass bottle over the volatile traps with a speed of 0.8 L/min for two hours. In order to avoid contaminations from the environments, the air-flow (1.1 L/min) getting into the glass bottle was higher than the one (0.8 L/min) pumped out through the filters, and the extra air went out through one of the openings of the glass bottle. After collections, both SuperQ and PDMS filters were eluted with 150 μL dichloromethane. The samples were then stored at -80°C until bioassays, further fractionations or chromatography analyses.

In order to test extracting efficiencies of both types of absorbents (SuperQ and PDMS), the headspace of 200 virgin males of *C. glomerata* was extracted with each filter type for two hours. In addition, 800 µl of a blend of six volatile compounds (*Z*-3-hexenyl acetate, *β*-caryophyllene, *β*-phenethyl acetate, nerolidol, heneicosane, *Z*-9-tricosene; each 800 ng in 800

µl dichloromethane) were dropped on a piece of filter paper (OD = 9 cm), placed into a bottle of the same kind as used for the wasp and extracted with either type of filter for two hours. The adsorbents were eluted with 150 µl dichloromethane. The collections were repeated three times, respectively. 10 µl of two internal standards (IS) mixes (n-octane and nonyl acetate, each 200 ng in 10 µl dichloromethane) were added to each sample as a reference to quantify compounds by peak area in the chromatograms.

Solvent extractions

Wasps killed by the freezer with -20 °C. Then, six virgin or mated wasps (both males and females) were extracted with dichloromethane (200 µl) for 10 minutes. Virgin males of *C. glomerata* were dissected into heads, thoraxes (including legs and wings), and abdomens by a scalpel. The heads, thoraxes and abdomens of six wasps were extracted with 50 µl, 100 µl and 100 µl dichloromethane respectively for 10 minutes. The supernatant liquid was taken by a syringe and the samples were by stored at -80°C until tests.

Gas Chromatography-Electroantennographic Detection (GC-EAD) analysis

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 column, 30 m, 0.25 mm ID, 0.25 µm film thickness, Phenomenex), and coupled to an EAD setup (Syntech, Hilversum; Netherlands). Aliquots (2 µl) of the solvent extracts of body segments or the headspace samples collected by SuperQ of *C. glomerata* (virgin females, virgin males or mated females) were injected splitless into the GC injector at an initial temperature of 40 °C, and then temperature was increased at the rate of 15 °C per minute to 250 °C. The GC effluent was split (split ratio FID : EAD = 1 : 1) by using a four-arm splitter (GRAPHPACK 3D/2, Gerstel, Mülheim, Germany), and 30 ml/min of make-up gas (nitrogen) was added to carry separated samples to EAD. The outlet of the EAD was placed in a cleaned and humidified constant airflow (100 ml/min) that was directed over the antennal preparation.

To prepare the antennae, *C. glomerata* males were anesthetized with CO_2, and the heads were pulled out from the body with a forceps, and two tips of both antennae were cut and inserted into one glass capillary. To close the electric circuit, the severed head was connected over 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). The headspace extracts and solvent extracts were tested with five replications.

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

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, 30m, 0.25mm ID, 0.25μm film thickness, Alltech), and coupled to a PFC setup (GERSTEL, Germany). Helium at constant flow (1.9 ml/min) was used as carrier gas. Aliquots (2 μl) of the samples collected by SuperQ of *C. glomerata* (100-200 individuals) were injected splitless into the GC injector. The numbers of injections were about 30 times. The oven temperature of the GC was initially 40 °C and was then increased at the rate of 15 °C per minute to 250 °C. The GC effluent was split (split ratio FID : PFC = 1:9) by using 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 installed in the PFC for helping to carry samples into the respective fractionation traps. The time interval for the six fractions is given in Fig. 2a.

Gas chromatography–mass spectrometry (GC-MS) analysis

In order to identify the compounds from the extracts, samples were analysed with an Agilent 6890 gas chromatograph, coupled with Agilent 5973 Network mass selective detector. A 2 μl aliquot of each sample was injected in the splitless mode with an injector temperature of 280 °C onto a polar column (EC-WAX column, 30m, 0.25mm ID, 0.25μm film thickness, Alltech). Helium at constant flow (1.9 ml/min) was used as carrier gas. The temperature program was initially 40 °C, held for 3.5 minutes, and then temperature was increased at the rate of 15 °C per minute to 260 °C. Compounds were preliminarily 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 synthetic compounds bought from Sigma-Aldrich or provided by other researchers.

Quantifications of compounds

In order to quantify compounds, samples were analysed with an Agilent 6850 gas chromatograph coupled with a FID detector. A 2 μl aliquot of each sample was injected in a splitless mode onto a polar column (EC-WAX column, 30m, 0.25mm ID, 0.25μm film thickness, Alltech). Helium at constant flow (1.9 ml/min) was used as carrier gas. Compounds were quantified by comparing their peak areas with the average peak area of two internal standards, n-octane and nonyl acetate (200 ng each) that were originially dissolved in 10 μL dichloromethane.

Bioassays

The bioassays was performed in a four-arm olfactometer, as shown in Fig. 1a and described in a previous publication [37]. Air entered into the central "wasp release chamber" over 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 within 30 minutes. Males were considered having made choice, when they had trapped in a "wasp trapped chamber" at the end of the 30 min period (Fig. 1a). When all males had made a choice, i.e. ended up in a "wasp trapped chamber", 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 release. The number of males that did not make a choice, i.e. stayed in the release chamber, was generally low and was excluded from the analysis. The assays were repeated six times, and each replication included four releases of a group of six males. So, in total 144 virgin males were tested for each assay. The positions of the treatments were systemically changed (rotated) between releases in such a way as to eliminate positions effects (each treatment once on each position within a replicate).

A volume of 5 µl of the extracts or of pure solvent control (dichloromethane) was dropped on a piece of filter paper (1 × 3 cm), which was rolled up to fit into the "connector glass tube" (Fig. 1a). If a treatment was a combination of two samples or more, then samples would be mixed before dropped on filter papers. 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 [38]. When the olfactometer was running, the air flow would pass through the four pieces of filter paper rolled in "connector glass tube" of each arm and carry 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 mix against a solvent control, two pieces of filter paper treated with a same extract were placed in two opposite arms of the olfactometer, with solvent controls in between. If there were two 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 and make choosing males more sensitive to odour discriminations. If there were three treatments and one solvent control, they were placed randomly into the arms of the olfactometer.

Parasitism tests

To avoid a high proportion of diploid males caused by long-term laboratory rearing [17,26], a new colony of *C. glomerata* was collected from the field in Neuchatel, Switzerland

in the September of 2016. Its second generation were used for parasitism tests which were carried out in insect rearing tents ($40 \times 40 \times 60$ cm) in the laboratory. Different numbers of foundresses (1, 3, 5, 7 and 10 individuals) were released respectively to parasitize on *P. brassicae* caterpillars (50 individuals, second instar) which were feeding on a Chinese cabbage plant. The parasitizing time was 24 hrs with a L : D =16 : 8 hrs at room temperature (about 25 °C), and released female parasitoids were able to access a food source. After the 24 hrs, the wasps were removed from tents, and the caterpillars were kept to feed on cabbage plants until wasp cocoons pupated. The clutches of cocoons and survived *P. brassicae* pupa were harvested and counted, and cocoons number of each clutch was also counted. The cocoons from a test (i.e. a tent) were put in a Bugdorm-1 cage ($30 \times 30 \times 30$ cm, Mega View Science Education Services Co. Ltd, Taiwan), provided with honey and moist cotton wool in a room temperature. After the wasp emerged from the cocoons, the males and the females were recorded respectively, and a sex ratio was calculated. Each test was repeated six times.

Statistics

For olfactometer data, statistical analyses were performed in R 3.0.2 with the package of Lme 4 [39]. To test whether the differences among the responses of the parasitoids to the treatments were significant, we used generalised linear mixed models (GLMMs) with poisson distribution of error. The replicates were treated as the 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 other's responses [40]. 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 (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Results

In the parasitoid *C. glomerata*, only virgin females produced sex pheromones to attract males [30]. We used two types of filters, SuperQ and PDMS, to extract the headspace of parasitoids. Only the extract of virgin females obtained with PDMS was strongly attractive to males (Suppl. fig. 1). Surprisingly, the SuperQ filter generally trapped compounds more efficiently than the PDMS filter from the headspace of both parasitoids and synthetic standards (Suppl. fig. 2). Those differences were more significant for compounds with relatively lower molecular weights (Suppl. fig. 2). The data indicated that the SuperQ filter also extracted repellent compound(s) which decreased the attractiveness of the virgin female extracts.
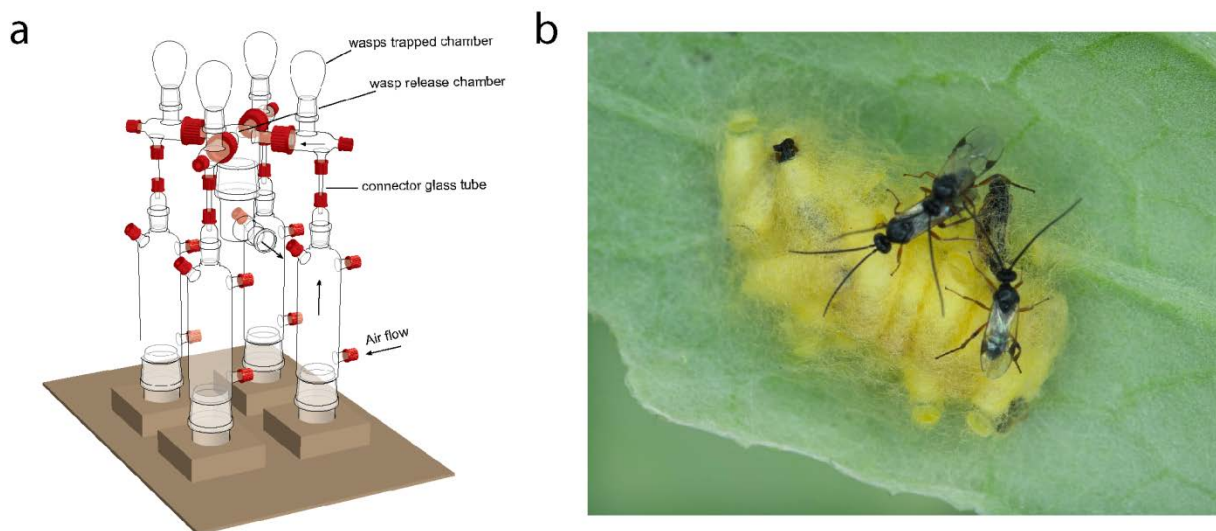
Fig. 1. (a) A 4-arm olfactometer was used for bioassays. A stream of purified and moist air flowed through each arm and entered the "wasp release chamber". The liquid samples were dropped on a piece of filter paper (1 × 3 cm) respectively which had been rolled and inserted in the "connector glass tube". A group of six virgin males of parasitoids was released in the "wasp release chamber" where they were allowed to make a choice among different arms within 30 minutes. After wasps had made a choice, they were normally blocked in "wasps trapped chamber" by the light from a lamp that was placed in the centre of olfactometer. (b) The gregarious parasitoid *C. glomerata* laid many eggs in each host caterpillar, and the last instar of the parasitoid larvae pupated into a patch of cocoons at the same time. The wasp adults showed synchronized emergence in a few minutes. Calling males mate with new-emerged females on the natal patch with little competition with other males.

Antennae of virgin males responded to a number of the compounds that were non-sex-specific, i.e. released by both sexes, and did not depend on the mating status of females

(virgin vs. mated) (Fig. 2a, b and c). We identified the following compounds of the headspace extracts which were confirmed with synthetic compounds (Fig. 2b): heptanal, nonanal, nonadecane, icosane, heneicosane, (Z)-10-heneicosene, ticosane; (Z)-9-tricosene and 1-octadecanol. A further compound, (Z)-12-pentacosene, was only tentatively identified by its mass spectre (NIST mass spectral library), and some compounds still remained unidentified (e.g. P3, P8, P11 in Fig. 2b). The time intervals of fractions (referred as "Fr" in figures and texts below) were presented in Fig. 2a. Only extracts of virgin females, but not virgin males or mated females, elicited a strong electroantennographic (EAG) response (marked with an arrow in Fig. 2a) of male antennae in retention period of Fr6 (Fig. 2a, b and c), but the compound was emitted with a tiny amount and failed to be identified. The compounds, heptanal, nonanal and 1-octadencanol, were released by virgin females, virgin males and mated females and elicited EAG responses of male antennae (Fig. 2a, b and c), which was confirmed with synthetic compounds (10 ng each) in Fig. 2d. The three compounds were produced by head, thorax and abdomen of virgin males (Suppl. fig. 3), indicating the compounds were components of cuticular lipids.

The combination of Fr5 and Fr6 were more attractive to conspecific males than the combinations of Fr1 and Fr2, or of Fr3 and Fr4 (Fig. 2e). Fr6 was more attractive to males than Fr5 (Fig. 2f). And Fr6 was as attractive as the combination of Fr5 and Fr6 to conspecific males (Fig. 2f). Fr6 of virgin females was more attractive to males than Fr6 of virgin males and mated females (Fig. 2g), supporting the key attractive compound (its EAG response marked as an arrow in Fig. 2a) of sex pheromones was only released by virgin females.
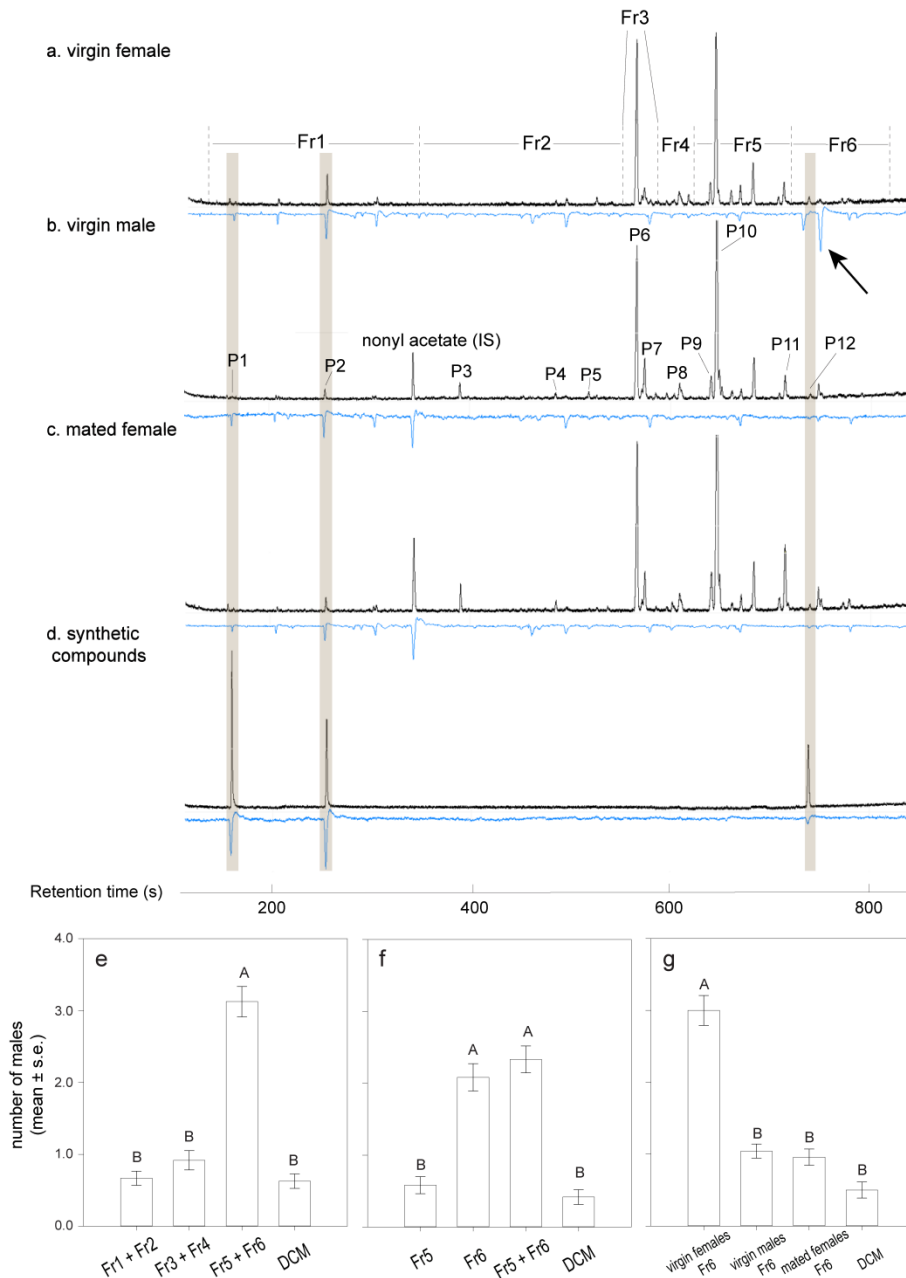
Fig. 2. Electrophysiological response of male antennae to the headspace extracts of virgin females (a), virgin males (b), mated females (c) and synthetic compound mix (d). The headspace extracts were fractionated with GC-PFC. The fractionation included six fractions (Fr1-6) and the time intervals were presented (a). The identification of headspace extracts was shown (b): P1, heptanal; P2, nonanal; P3, unknown; P4, nonadecane; P5, icosane; P6, heneicosane; P7, (*Z*)-10-heneicosene; P8, unknown; P9, tricosane; P10, (*Z*)-9-tricosene; P11, (*Z*)-12-pentacosene; P12, 1-octadecanol. The internal standard (IS), nonyl acetate, was used as the boundary between Fr1 and Fr2 (a and b). The synthetic compound mix was heptanal, nonanal and 1-octandecanal (10 ng each) (d). Fr6 of virgin female extracts was most attractive

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to males compared with other fractions (e and f). Only Fr6 of virgin female extracts, but not mated female or virgin male ones, was strongly attractive to males (g). The letters on the bar indicated the statistic differences (P < 0.05).

The combination of virgin female fractions, Fr1 and Fr6, was strongly attractive to males compared with solvent control (Fig. 3a), but the combination was less attractive to males than the 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 combined respectively with Fr6 of virgin females were more attractive to males than Fr6 of virgin females alone (Fig. 3c, d, e, h, i and j). Fr5 of virgin females or virgin males did not increase the attractiveness of Fr6 of virgin females to conspecific males (Fig. 3f and k).
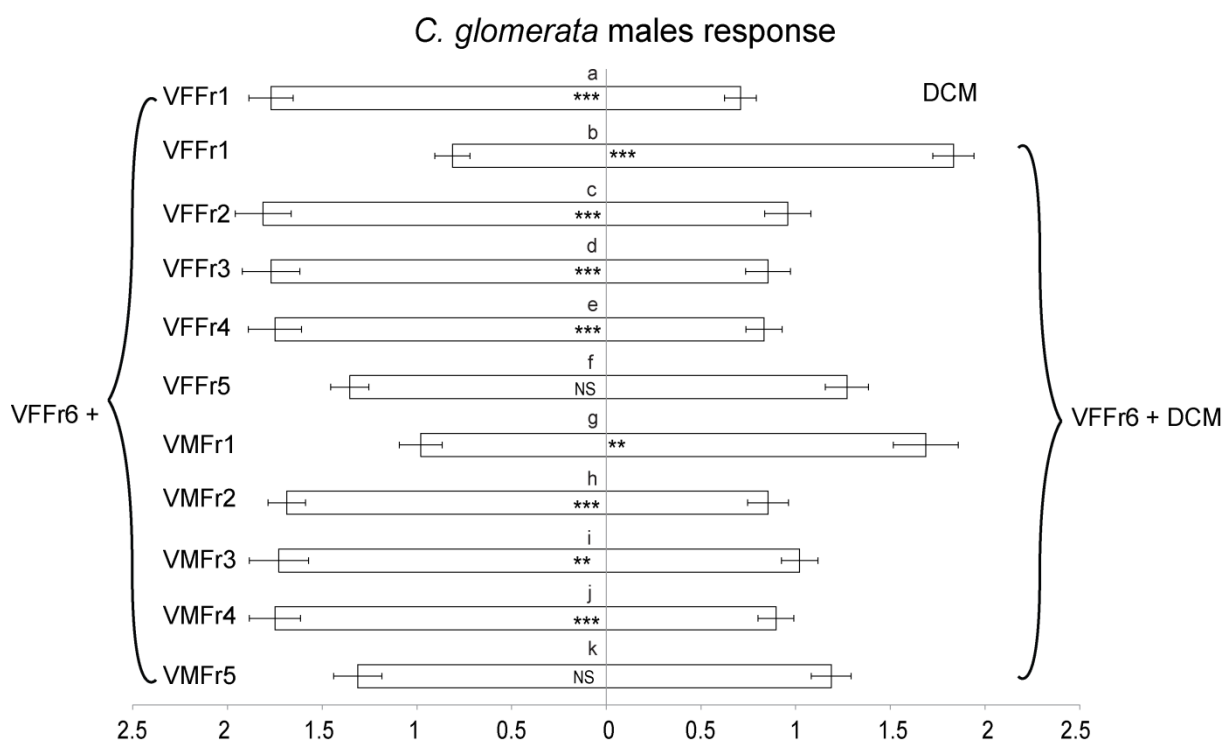

Fig. 3. The attractiveness or repellence of fraction combinations to virgin males. VF: virgin females; VM: virgin males; DCM: dichloromethane (solvent control). NS, no statistical difference; "*" P < 0.05, "**" P < 0.01, "***" P < 0.001.

Heptanal (3 ng, equivalent quantity of about 6 individuals, Suppl. fig. 3a) was strongly repellent to males and the repellence was not counteracted by Fr6 of virgin females and other cuticular lipids, nonanal and 1-octadecanal (40 ng and 6 ng respectively, equivalent quantity of about 6 individuals, Suppl. fig. 3b and c) (Fig. 4a, d, e, f, g and l). Nonanal did not attract males alone (Fig. 4c), but when mixed with Fr6, the attractiveness of the combination to males was stronger than Fr6 of virgin females alone (Fig. 4b). Another cuticular lipid 1-

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octadecanol (6 ng, equivalent amount of about 6 individuals) did not significantly attract more males than solvent control (Fig. 4h). But the combination of 1-octadecanol and nonanal was more attractive to males than one component alone or a solvent control (Fig. 4i, j and k).
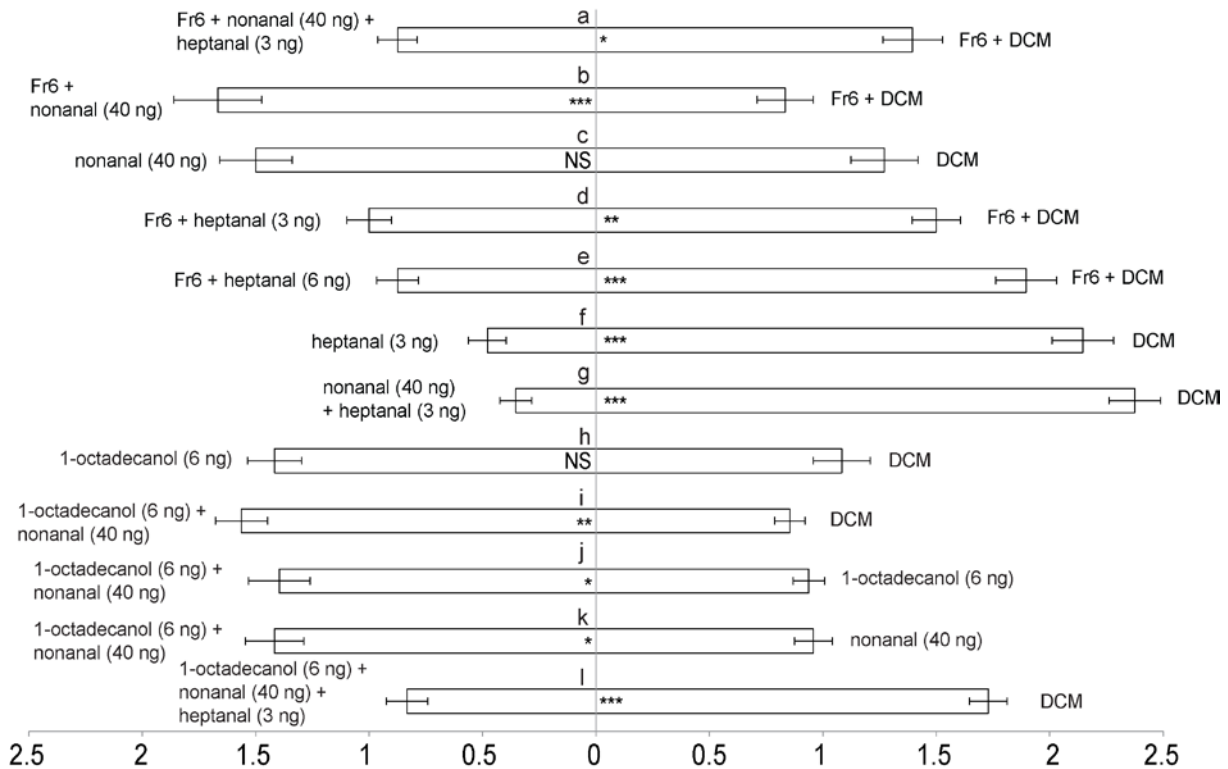

C. glomerata males response

Fig. 4. The attractiveness or repellence of synthetic compounds. The attractiveness or repellence to virgin males of cuticular lipids heptanal (3 ng), nonanal (40 ng) and 1-octadecanol (6 ng), equivalent to six individuals, was tested alone (c, f, and h) or in a combination (g, i, j, k and l). Heptanal and nonanal were mixed with the Fr6 of virgin females to test the synergistic effect in attracting virgin males (a, b, d and e). DCM: dichloromethane (solvent control). NS, no statistical difference; "*" P < 0.05, "**" P < 0.01, "***" P < 0.001.

The antennae of males responded differently to solvent extracts of heads, thoraxes and abdomens of virgin females: only abdomen extracts evoked the EAG response at retention time of the key component of sex pheromones, indicating that the unknown constituent was only produced by the abdomens (Fig. 5a, b and c). The attractiveness of abdomen extracts was much stronger than extracts of heads, thoraxes or a solvent control (Fig. 5d). A thorax extract of virgin females was repellent to males compared with a solvent control or a head extract (Fig. 5e).
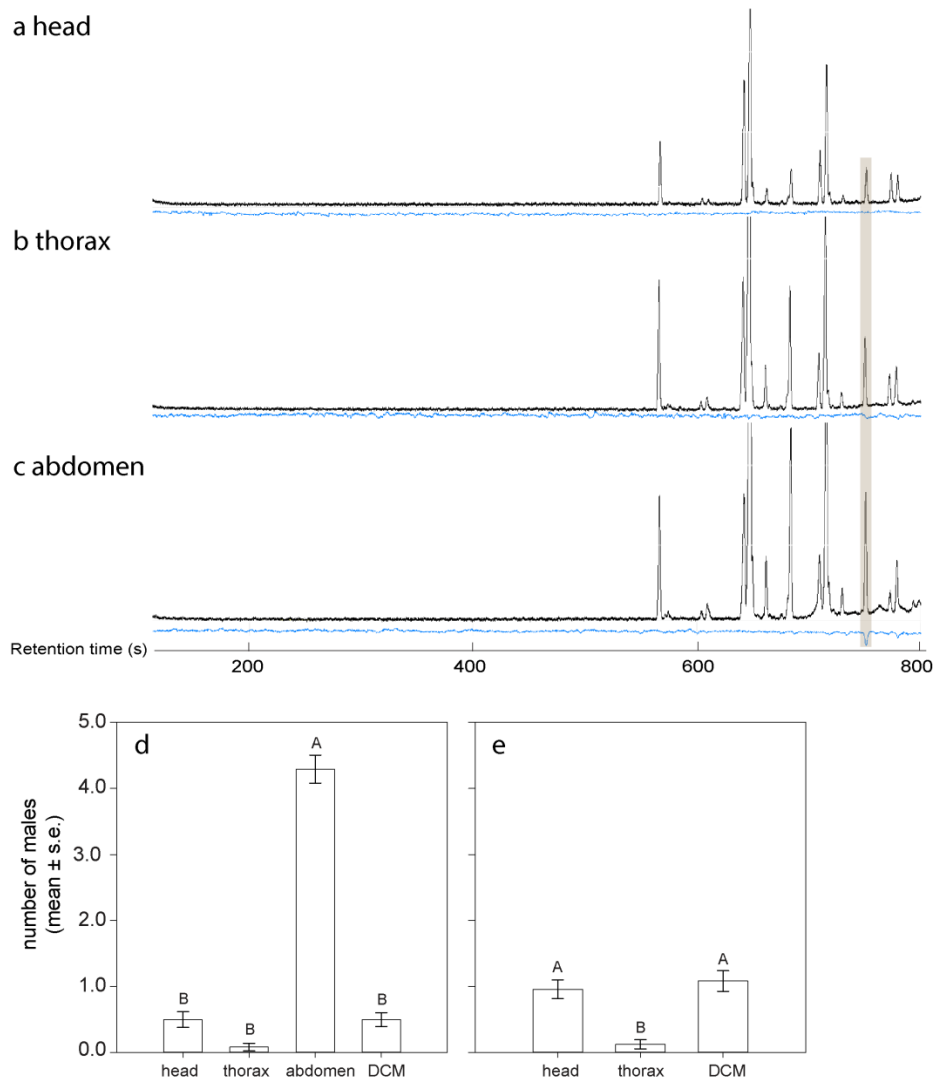
Fig. 5. Electrophysiological response of male antennae to solvent extracts of head, thorax and abdomen sections of virgin females (a, b and c). Only solvent extracts of virgin female abdomens is attractive to males (d). The extracts of thoraxes were repellent to males compared with head extracts or solvent controls (e). The letters on the bar indicated the statistic differences (P < 0.05)

The number of the clutches of cocoons generally increased positively when more foundresses were applied to parasitize on 50 hosts (Fig. 6a). Correspondingly, the number of survived pupa of *P. brassicae* decreased as more foundresses were applied (Suppl. fig 4). The number of cocoons per clutch also increased positively according to the number of foundresses (Fig. 6b). Importantly, a higher proportion of males was produced when more foundresses were applied (increased from a female-biased ratio 25% to a male-biased ratio 67%) (Fig. 6c).
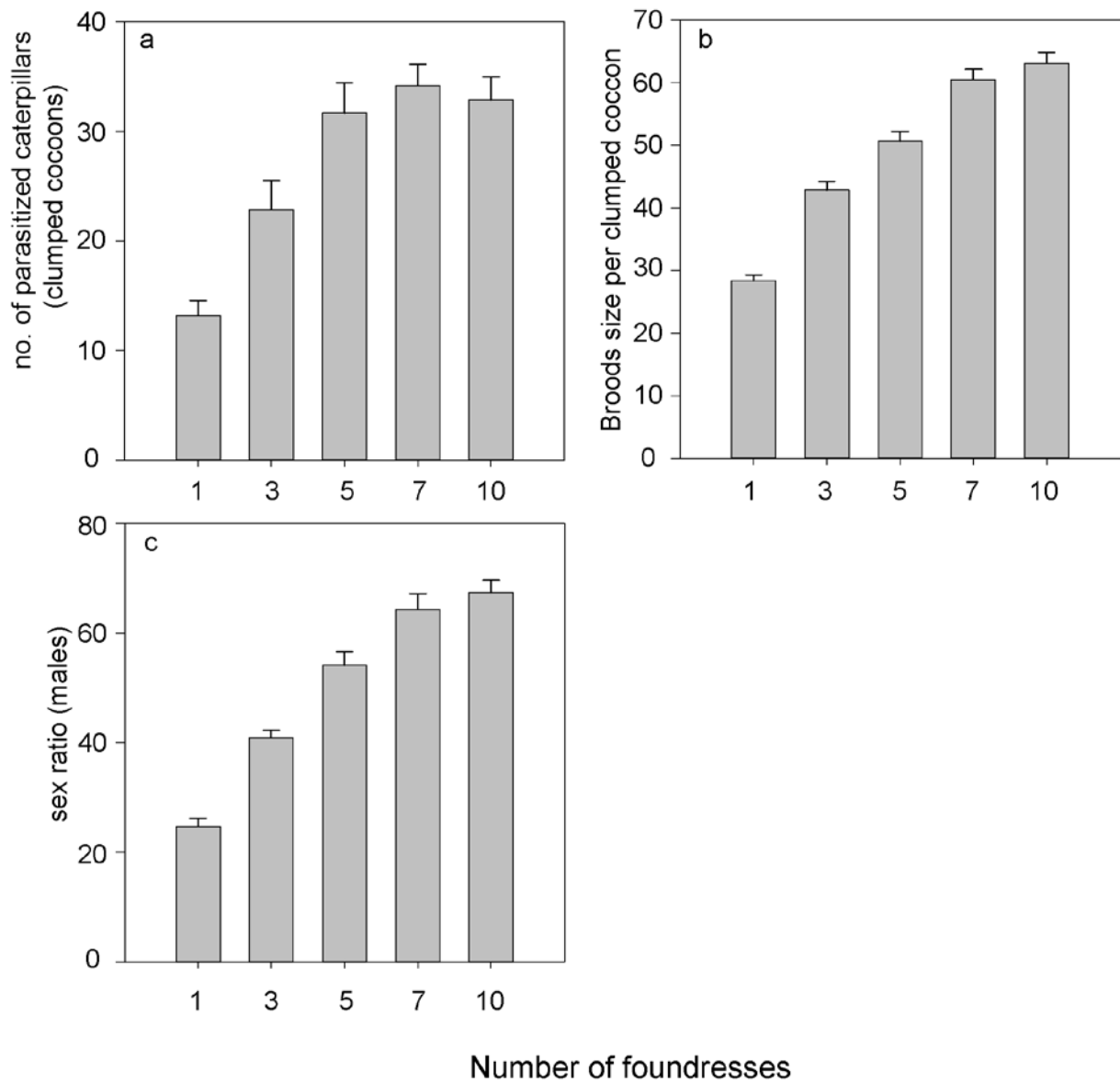
Fig.6. The sex allocation of *C. glomerata*. The number of clutches of coccoons (a), cocoons per clutch (b), and sex ratio (c) increased positively according to the number of foundresses was applied.

Discussion

Mating at emergence sites and leaving natal patches for mating are two antagonistic mate systems in animals [1]. Pheromone-driven mating is little known in these processes. The endoparasitoid *C. glomerata* is a model organism in studying mate systems, sex allocations and complementary sex determination [16,17,26,29,30,34]. The wasp is partially mating on the natal patch, in which about 50-60% of siblings involve in inbreeding [17,32]. The percentage of sibling mates is possibly influenced by the sex ratio on host patches and a higher sex ratio on a natal patch possibly trigger a greater proportion of male dispersal and female stays [32,34]. But the mechanism is largely unknown.

Here we report a pheromone-controlled mating strategy at natal patches in the gregarious parasitoid *C. glomerata*: males emerge a few minutes before females and the cocoon stage of females already start to emit sex pheromones to arrest newly emerged males (attractive effect) [41]. Arrested males mate with females in a short time after their emergences (Fig. 1b). The male, already arrested by an emerging female, is not disturbed by other males due to the release of anti-aphrodisiac compound, heptanal (repellent effect). When the sex ratio is relatively high (e.g. more than 50%, and Fig. 6c), high concentration of heptanal may drive males to leave the natal patch. In contrast, if the sex ratio is relatively low (e.g. <30%, and Fig. 6c), new-emerged females might disperse before mating on natal patches, which was observed in the filed [17]. After dispersal, both virgin males and virgin females are strongly attracted by herbivore-induced plant volatiles (HIPVs) and they probably locate mating partners on a host damaged plant [30,36].

Sex allocation strategy is regulated by both the choice of mate sites and the availability of host resources in parasitoids [3,15]. First of all, mating at the emergence site and involving in sibling mate, for example, tend to result in a female-biased sex ratio [7]. With the proportion of non-sibling mating increasing, the sex ratio will go up accordingly [13]. When parasitoids change from sibling mate towards panmixis, the sex ratio would have a tendency to 50% [13]. In addition, the richness of host resources also affect sex allocation, which is probably due to different nutrients are needed for development of females and males [3,13]. A poor host resource only supports the growth of a male-biased offspring. In *C. glomerata*, when there is a fierce competition for host resources, a male-biased offspring is produced instead of a female-biased one that is only raised in rich host patches (Fig. 6c). This probably results from limited resources for the development of offspring, in which only males are able to survive. Superparasitism in *C. glomerata* is possibly a main way of competing host

resources when unparasitized hosts are in a low density [34]. The superparasitism does not lead to a significant higher mortality of next generation [31], and resulting in a male-biased offspring [34]. A shift from optimal natal mate (a female-biased offspring) to competing host resources (a male-biased offspring) under a poor resource condition theoretically promotes male-male competition in gregarious parasitoids. In this circumstance, it is critical that males are capable to judge the situation to disperse from or stay at natal patches for mating. Given the well-developed olfactory system in insects, one most likely solution is based on chemical cues. In this study, we find that with the pheromones (both attractive and repellent) and possibly other cues like HIPVs [30,36], *C. glomerata* is able to survive on both rich and poor host conditions with producing a female-biased or a male-biased offspring and still locate mates successfully not only on natal patched but also away from natal patched (e.g. on a host-feeding plant).

Anti-aphrodisiacs are the important pheromone controls of mated females to resist other males in insects [42,43]. The published examples on anti-aphrodisiacs are relatively scarce compared with a diversity of attractive pheromones [43]. In hymenopteran insects, the anti-aphrodisiacs are largely unknown or not commonly exiting [44]. The production of anti-aphrodisiacs was normally by glands [45-48], and anti-aphrodisiacs were transferred from males to females during ejaculations [46,47,49]. Some cuticular hydrocarbons with big molecular sizes could be transferred from males to females during mating, and acting as anti-aphrodisiacs in *Drosophila* [50,51]. Here, we report a volatile anti-aphrodisiac compound heptanal which is a polar lipid of cuticle and continuingly released by both males and females independent on mate status (Fig. 2 and Suppl. fig. 3a and d). Importantly, the anti-aphrodisiac does not completely counteract the attractiveness of sex pheromones released by a gland located at virgin female abdomen (Fig. 3a) [30,41], and the virgin females still arrested males on natal patch (Fig. 1b). By using the heptanal, *C. glomerata* males are able to avoid physical competition among each other and probably encourage dispersal when males are with high density on natal patches.

In conclusion, pheromones are possibly acting as essential cues for new-emerged gregarious parasitoids to either stay at or disperse from natal patches to locate mate, which is largely overlooked by the previous studies. If the host patch is rich, the foundresses are able to produce female-biased offspring, and the female offspring attracts male siblings to stay on the patch with sex pheromones. By contract, if the host patch is poor, the foundresses have to produce a higher sex ratio offspring or even a male-biased offspring, and then anti-aphrodisiacs may encourage males to leave the natal patch and locate mates in other places.

Altogether, our study reported one of mechanisms of mating strategy at emergence site in gregarious animals in which the proportion of inbreeding and outbreeding were adjusted with the help of pheromones.

Acknowledgements

Author contributions

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

## Supporting information

Supplementary figures.

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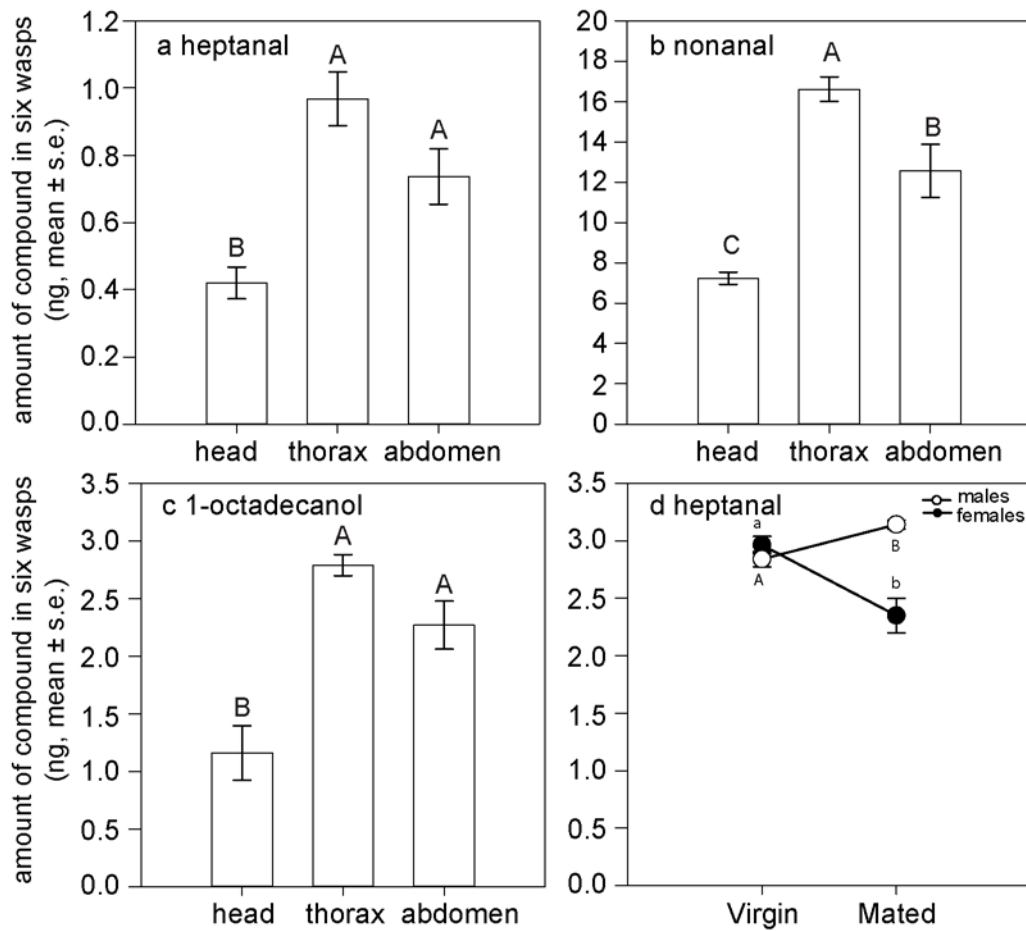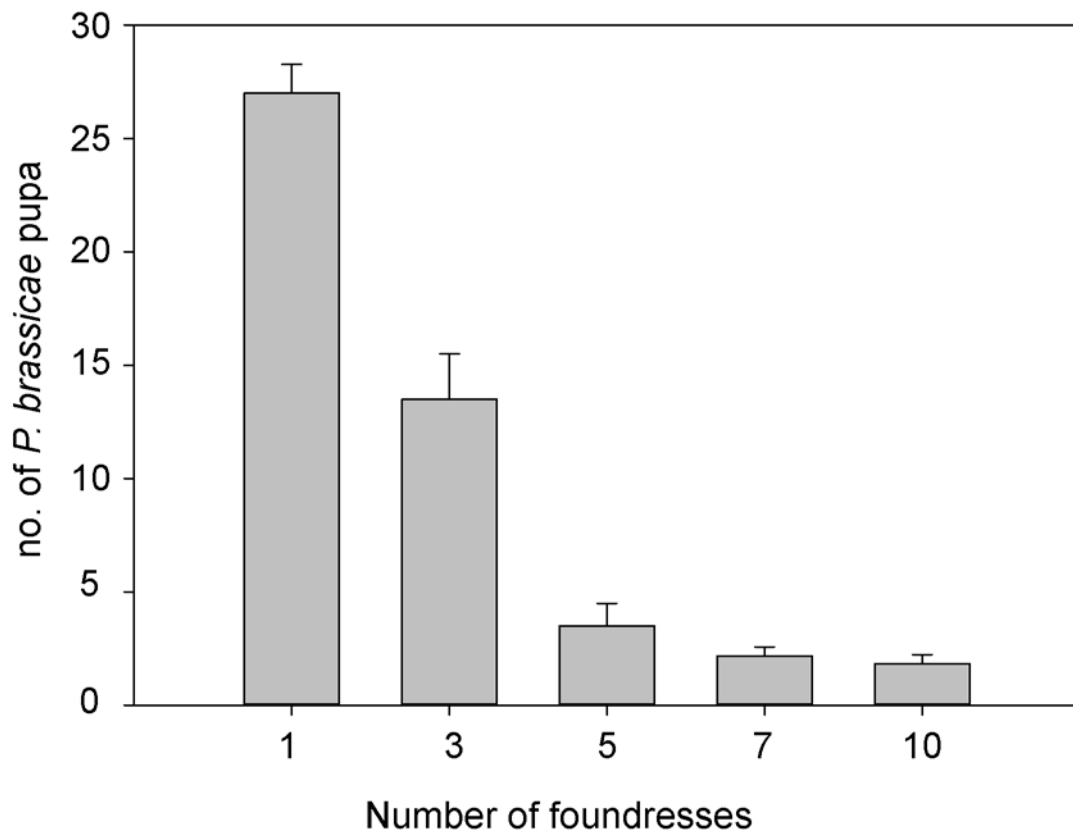
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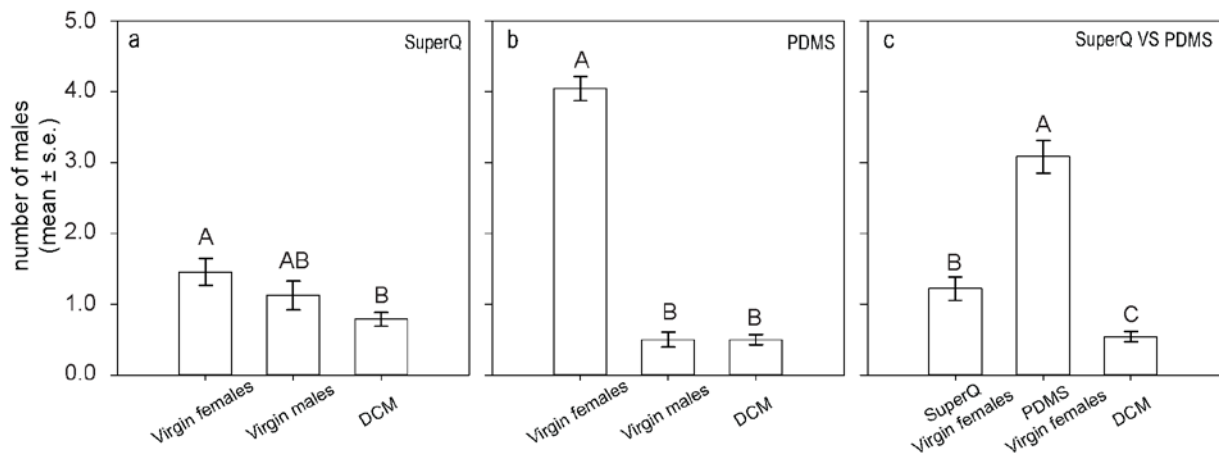
Suppl. fig. 1. The attractiveness of the headspace extracts of virgin females and virgin males to virgin males when samples were collected with different absorbents. Virgin males were not strongly attracted by the SuperQ extracts (a). But PDMS extracted samples of virgin females was strongly attractive to males (b), and the samples were more attractive to males than the SuperQ extracted ones (c).
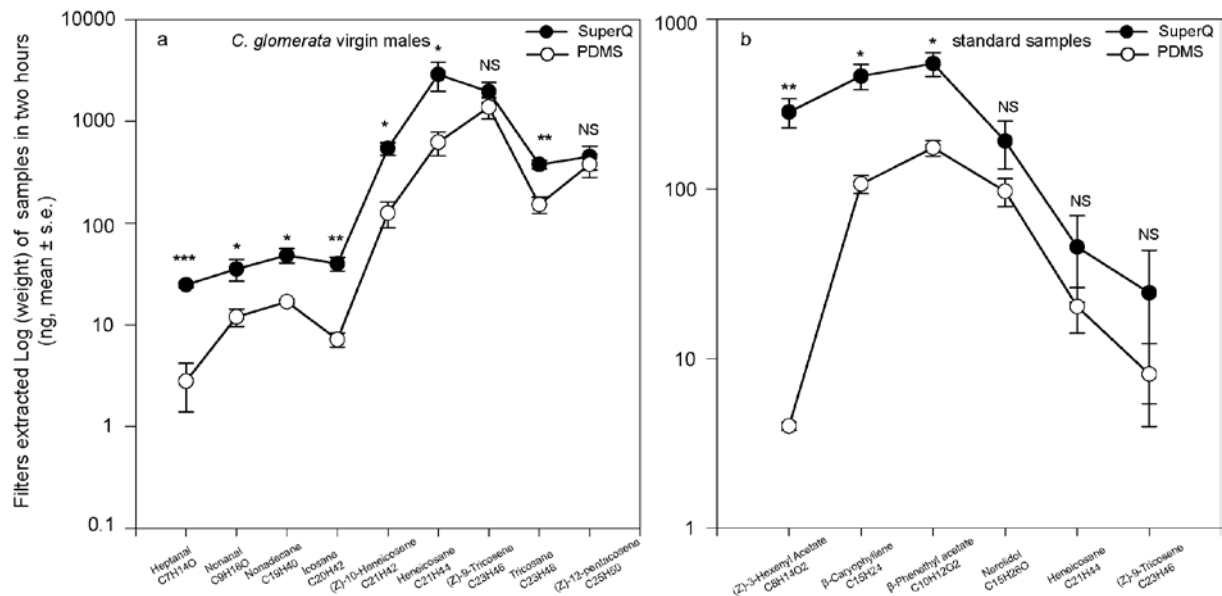
Suppl. fig. 2. Efficiency in collecting volatiles with the two types of filters. The headspace of wasps (200 individuals) (a) and synthetic compound mix (800 ng each) (b) were extracted with the two types of filters for two hours. NS, no statistical difference; "*" $P < 0.05$, "**" $P < 0.01$, "***" $P < 0.001$.

Suppl. fig. 3. The quantifications of heptanal (a), nonanal (b) and 1-octadecanol (c) in three different sections (head, thorax and abdomen) of six virgin males. The amount of anti-aphrodisiac, heptanal, was produced by both males and females independent on difference mate status (d). The letters on the bar indicated the statistic differences ($P < 0.05$)

Suppl. fig. 4. The number of survived *P. brassicae* pupa after the parasitism of different number of foundresses on 50 host caterpillars.

CHAPTER 4 The dynamic use of cuticular lipids as pheromonal cues in two *Cotesia* parasitoids

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Abstract

Cuticular lipids (CLs) are almost found in every insect species and are believed to work primarily as barriers to avoid desiccation or the infection by entomopathogens. Studying on evolved signalling functions of CLs is possibly a good way to study the evolution of chemical communications. In a previous paper, the gregarious parasitoid, *Cotesia glomerata* (L.), used a cuticular aldehyde heptanal as an anti-aphrodisiac to avoid natal mate competition among sibling males, and another cuticular aldehyde nonanal is a component of female sex pheromones which is not significantly attractive to males alone, but synergistically increased the attractiveness of other components. Interestingly, in this study, we found that heptanal had evolved differently into pheromonal functions in congeneric parasitoid species: in the solitary species *Cotesia marginiventris* (Cresson), heptanal synergistically increased the attractiveness of other components of sex pheromones. Another cuticular aldehyde nonanal did not increase the attractiveness of the main components of the sex pheromones. For both species, some nonspecific compounds synergistically increased the attractiveness of the main constituents of the sex pheromones of each species. The specificity of sex pheromones was probably retained by two specific compounds which evoked biggest electroantennographic (EAG) responses of male antennae of each species. To our knowledge, our results firstly reported that certain components of CLs had a strong flexibility to evolve into distinct pheromonal functions according to the specific habitat of each species.

Keywords: braconid wasps; cuticular hydrocarbons; semiochemicals; pheromonal evolution; insect pheromones; profile; volatiles.

Introduction

Chemical communication is one of oldest and most common signalling ways in nature environment, used by many organisms ranging from microbes to animals [1]. For example, animals use chemical cues to locate food and hosts, avoid predators, and communicate with the same species [1]. Plants release volatiles to defence herbivores and pathogens directly or indirectly, and warn neighbour plants about impending attacks [2]. Bacteria possibly release bioactive compounds to affect the community of nearby plants, fungi, animals and bacteria [3]. How these signals have evolved could be a challenge for ecologists [4]. Some theories predicted that chemical communications of insects had evolved from pre-existing compounds which were originally used as other purposes [1,4,5]. However, evidences are relatively scarce.

Cuticular lipids (CLs) are almost found in every insect species and are originally used to protect insects from desiccation or entomopathogens [6-8]. However, CLs have evolved into extremely diverse roles in different insect groups [9]. For example, long-chain cuticular hydrocarbons (CHCs) have evolved into sterility-inducing queen pheromones on three social insect lineages: wasp, bumblebee and ant species [10]. CLs were important hints for many social insects to recognize nestmates and defeat conspecific or interspecifc insects from other colonies [7,9]. Parasitoid species qualitatively mimic the CHCs profile of their host species to have a higher success of parasitism [11,12].

CLs profiles, evolved into pheromones, could be strongly a species and a sex specific [9,13]. For example, CLs profiles of sympatric *Drosophila* species were different, which is essential for mate recognition and probably leads to speciation in natural selection [14]. Closely related species normally use the same pathways with their common ancestor to biosynthesize CLs and thereby share a similar composition of CLs [9]. Therefore, the profiles of CLs sometimes showed a gradual process of evolution in some groups of insects (e.g. ants) [15]. In contrast, CLs profiles frequently did not correspond with phylogenetic relationships and showed some independent evolutions (e.g. bark beetles) [12-14,16], probably for fulfilment of ecological functions, like successful mate [14] or easily detecting parasitoids [12]. Interestingly, the field population of *Drosophila serrata* had a distinct CHCs profile with the sympatric species *Drosophila birchii*. When exposed field sympatric and allopatric populations of *D. serrata* to experimental sympatry with *D. birchii*, the allopatric *D. serrata* changed its CHCs profile to the sympatric population of the same species only after nine generations, which indicated the CHCs profiles, important for mate and species recognitions,

possibly significantly changed in a short time in insects [14]. Profiles of CLs or CHCs in different insect species were frequently reported to be important as pheromonal cues, but how certain components of CLs have evolved differently into pheromonal communications was not well understood.

Evolution of pheromones is possibly a gradual process, in which phylogenetically related species share some components of pheromones [4]. For example, the pteromalid pupal parasitoid *Trichomalopsis sarcophagae* shared the same pheromone components with members of the sister genus, *Nasonia*, [17], and non-selective inter-species attractions were tested to be significant in *Nasonia* spp. in laboratory assays [13,17]. The larval parasitoids of *Drosophila* spp., *Leptopilina heterotoma* and *Leptopilina boulardi* shared a main component of female sex pheromones, (-)-iridomyrmecin, which was originally used as a defensive compound to repel insect predators [18]. These gradual changes of pheromonal constituents were probably resulted in phylogenetic conservatism [4]. However, pheromones normally need to be highly species-specific to maintain the reproductive isolations among sympatric species, which is unlikely to bring out a gradual change of pheromones in evolutionary processes [4]. Then, major shifts possibly explained the diversity of pheromones [19], which were reported, for example, in moth and beetle species [16,20].

In a previous study [21], we found that the virgin females of the both gregarious parasitoid *Cotesia glomerata* and solitary parasitoid *Cotesia marginiventris* were strongly attractive to conspecific males, but not to interspecific males. Interestingly, *C. glomerata* males were repelled not only by conspecific males and mated females with a cuticular aldehyde heptanal (Chapter 3), but they were also repelled by virgin females of *C. marginiventris* [21]. The results indicated that the *C. glomerata* anti-aphrodisiac heptanal was possibly nonspecific which was possibly produced by *C. marginiventris* as well. Importantly, the compound possibly had evolved into different functions in the two congeneric species because there was no anti-aphrodisiac found in *C. marginiventris* [21]. In this paper, with fractionations, electrophysiological methods, and behavioural assays, we studied on how CLs had evolved into pheromonal cues of two congeneric species which had distinct habitats.

Materials and Method

Insects

The two endoparasitoid species were reared on their respective hosts in our laboratory at the University of Neuchatel following the protocol described in a previous publication [21]. The gregarious parasitoid *C. glomerata* and its host, *Pieris brassicae*, were originally collected in gardens, Neuchatel, Switzerland. The offspring of *C. glomerata* was reared on *P. brassicae* caterpillars (first–second instars) which were fed on Chinese cabbage plants. The solitary wasp *C. marginiventris*, originally collected from Mexico, was reared on *Spodoptera littoralis* caterpillars (first–second instars) which were fed on a piece of wheat germ-based artificial diet [22]. The eggs of *S. littoralis* were provided by Syngenta (Stein, Switzerland). To obtain virgin adult wasps, each parasitoid cocoon was placed in a 1.5 mL centrifuge tube until a wasp emerged [21]. Then, 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).

Headspace extraction of sex pheromones

Two kinds of filters, SuperQ (25 mg, 80-100 mesh) and polydimethylsiloxane (PDMS), were used for collecting volatile sex pheromones of wasps. The PDMS filters (OD = 6 mm, ID = 4 mm, length = 60 mm, Gerstel GmbH) were originally designed for heating desorption on Gerstel thermal desorption unit.

100-200 of *C. glomerata* virgin females or 50-100 of *C. marginiventris* virgin females (about three-day old, the number of sampled wasps depends on level of difficulty of rearing) were put in a glass bottle (250 mL). A stream of pure and moist air entered the glass bottle with a speed of 1.1 L/min. The vacuum pump connected with a filter and sucked the air from the glass bottle with the speed of 0.8 L/min for two hours. In order to get fewer contaminations from the environments, the air-flow (1.1 L/min) getting into the glass bottle was faster than the air-flow (0.8 L/min) pumped out through filters, and the extra air will go through one of openings of glass bottle. After collections, filters were eluted by 150 μL dichloromethane. The samples were then stored at -80°C until doing bioassays, further fractionations or chromatography analyses.

Gas Chromatography-Electroantennographic Detection (GC-EAD) analysis

The GC-EAD system was consisted of a HP 6890 gas chromatograph (Agilent Technologies, Germany) and an EAD setup (Syntech, Hilversum; Netherlands). The GC was equipped with a flame ionization detector (FID) and a polar column (ZB-WAX column, 30 m,

0.25 mm ID, 0.25 µm film thickness, Phenomenex). Aliquots (2 µl) of the headspace samples collected by SuperQ of virgin females of *C. glomerata* (~200 individuals) and *C. marginiventris* (~50 individuals) were injected splitless into the GC. The oven temperature of the GC was initially 40 °C and then increased at the rate of 15 °C per minute to 260 °C. The GC effluent was split (split ratio FID:EAD = 1:1) by using a four-arm splitter (GRAPHPACK 3D/2, Gerstel, Mülheim, Germany) and 30 ml/min of make-up gas (nitrogen) was added. The outlet of the EAD was placed in a cleaned and humidified constant airflow (100 ml/min) that carried compounds to the antennal preparation.

To prepare the antennae, the head of *Cotesia* males were pulled out from neck with a forceps, and two tips of both antennae were cut and inserted into one glass capillary. Meanwhile, the broken neck on the head was connected to another glass capillary to close the electric circuit. 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). The headspace extracts of two parasitoids were tested with five replications.

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

The GC-PFC system was consisted of a HP 6850 Series gas chromatograph (Agilent Technologies, Germany) which was equipped with a FID detector and a polar column (EC-WAX column, 30m, 0.25mm ID, 0.25µm film thickness, Alltech) and an PFC setup (GERSTEL, Germany). Helium flow (1.9 ml/min) was used as carrier gas. Aliquots (2 µl) of the headspace samples collected by SuperQ of *C. glomerata* (100-200 individuals) and *C. marginiventris* (50-100 individuals) were injected splitless into the GC. The injection times were 20 for *C. glomerata*, and 40 for *C. marginiventris*, under consideration of different numbers of individuals were used for collecting samples. The oven temperature of the GC was initially 40 °C and then increased at the rate of 15 °C per minute to 260 °C. The GC effluent was split (split ratio FID:PFC = 1:9) by using a four-arm splitter (GRAPHPACK 3D/2, Gerstel, Mülheim, Germany). The temperature of transfer line (between GC and PFC) and PFC chamber 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 added in PFC for helping to carry samples into respective fractionation traps. The fractionation was carried out with the same program of the same GC-PFC in Chapter 3 and the intervals of six fractions were illustrated in Fig. 2a.

Quantifications of compounds

Virgin females of *C. marginiventris*, killed by a minus 20 °C freezer, were dissected into heads, thoraxes (including legs and wings), and abdomens by a scalpel. The heads, thoraxes and abdomens of six wasps were extracted with 50 µl, 100 µl and 100 µl dichloromethane respectively for 10 minutes. The supernatant liquid was taken by a syringe and the samples were by stored at -80°C until tests. Samples were analysed with an Agilent 6850 gas chromatograph coupled with a FID detector. A 2 µL aliquot of each sample was injected in a splitless mode onto a non-polar column (HP-1ms, 30m, 0.25mm ID, 0.25µm film thickness, Agilent J&W Scientific) in the GC. Helium flow (1.9 ml/min) was used as carrier gas. Two aldehydes heptanal and nonanal were confirmed by retention times of synthetic compounds. A mixture of two internal standards (n-octane and nonyl acetate, each 200 ng in 10 µL dichloromethane) was added to each sample to quantify compounds by peaks area in chromatographies.

Bioassays

The majority bioassays were performed in a four-arm olfactometer, as described by previous publications (Chapter 3) [23]. The assays were repeated six times, and each replication included four releases of a group of six males. After all six males had made a choice, or choosing time was up to 30 min, the number of chose wasps was recorded and the old group of wasps was removed from olfactometer and a new group of six wasps was released again. In total, 144 virgin males were tested for each assay. The position of treatments in olfactometer was described in Chapter 3. Each extract or solvent control was dropped on a piece of filter paper (1 × 3 cm). Before testing, the filter papers already applied with samples or solvent controls were put into a running fume hood for 15 min to allow solvent to evaporate[24].

Statistics

For olfactometer data, statistical analyses were performed in R 3.0.2 with the package of Lme 4 [25]. To test whether the differences among the responses of the parasitoids to the treatments were significant, we used generalised linear mixed models (GLMMs) with poisson distribution of error. The replicates were treated as the 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 other's responses [26]. Each model was fitted by maximum quasilikelihood estimation in the software package R. For the quantification of the cuticular compounds, the

data were analysed with paired t-test, performed by SigmaPlot 12.5. Statistical differences ($p < 0.05$) were indicated with different letters in the bar figures or with different number of the mark "*", presenting different levels of significant differences in graphs ("*", $P < 0.05$; "**", $P < 0.01$; "***", $P < 0.001$).

Results

For both *Cotesia* parasitoids, only virgin females produced sex pheromones to attract males [21]. Extracts of virgin females with both filters (SuperQ and PDMS) were strongly attractive to conspecific males in *C. marginiventris* (Fig. 1a and b). The extract of virgin females with SuperQ was more attractive to males than an extract obtained with PDMS in *C. marginiventris* (Fig. 1c).
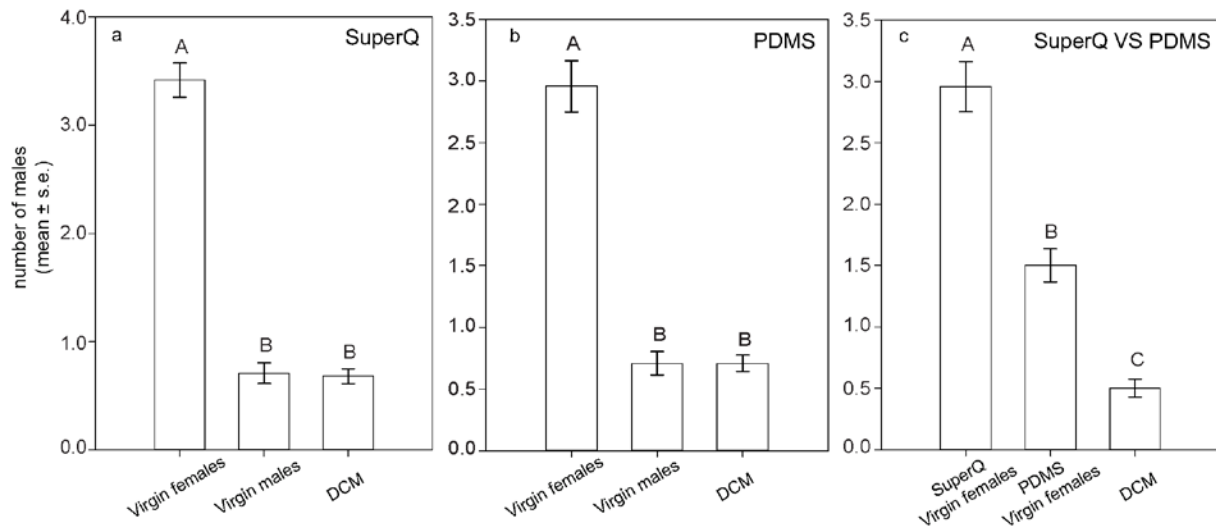

Fig. 1. Virgin males of *C. marginiventris* responded to headspace extracts of conspecific wasps obtained with SuperQ (a), or with PDMS (b), or with both SuperQ and PDMS (c).

Virgin male antennae of two *Cotesia* parasitoids had some similar electroantennographic (EAG) responses to SuperQ extracts of conspecific virgin females and interspecific females (indicated with "*" in Fig. 2a and b). A specific compound of each species evoked a strongest EAD response only of conspecific male antennae, but not of interspecific male antennae (the black arrow marking for *C. glomerata* in Fig. 2a and the grey one marking for *C. marginiventris* in Fig. 2b). The nonspecific cuticular aldehydes heptanal and nonanal were identified in a previous paper (Fig. 2a and b, Chapter 3).
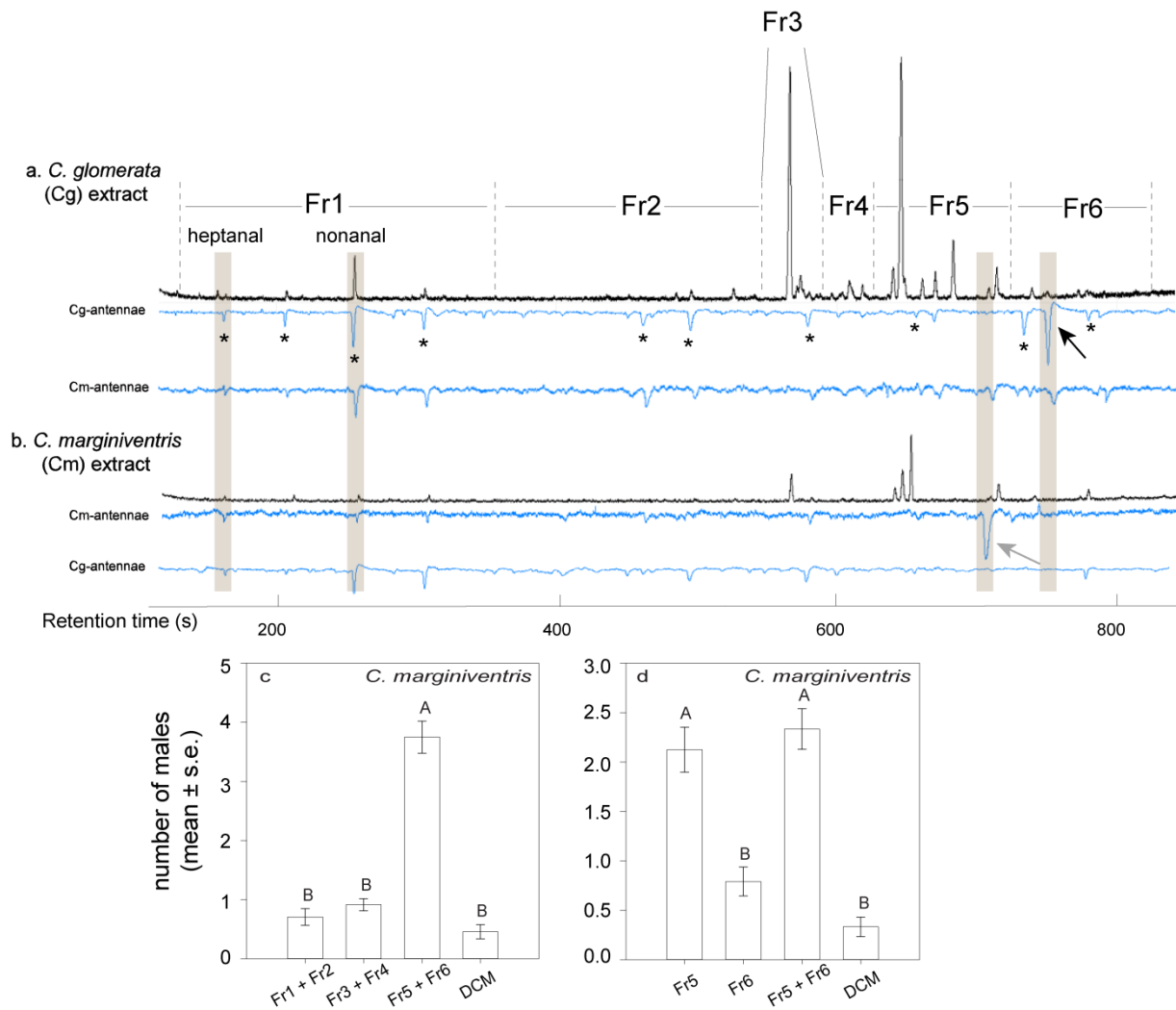
Fig. 2. Electrophysiological responses of male antennae to SuperQ-extracted virgin female samples of *C. glomerata* (a) and *C. marginiventris* (b). The intervals of fractionation, carried out with GC-PFC, were illustrated in (a) for both species (Fr1-6: Fraction 1-6). Attractiveness of fractions to conspecific males was tested for *C. marginiventris* (c and d), and for *C. glomerata* in Chapter 3. Cg: *C. glomerata*; Cm: *C. marginiventris*.

SuperQ extracts of virgin females of both *Cotesia* parasitoids were fractionated with GC-PFC and the intervals of fractions were shown in Fig. 2a and b. In *C. marginiventris*, the combination of Fr5 and Fr6 were more attractive to males than the combination of Fr1 and Fr2 or of Fr3 and Fr4 (Fig. 2c). Fr5 was more attractive to males than Fr6 (Fig. 2d). In *C. glomerata*, Fr6 was the most attractive fraction to conspecific males (Chapter 3). When the Fr1, Fr2 or Fr3 of each species respectively combined with Fr5 of *C. marginiventris*, the combinations were more attractive to *C. marginiventris* males than the Fr5 alone (Fig. 3a). But the attractiveness of the combinations did not increase when the Fr1, Fr2 or Fr3 was replaced by Fr4 or Fr6 of each species (Fig. 3a). Fr1 of *C. marginiventris* decreased the attractiveness of Fr6 of *C. glomerata* to *C. glomerata* males, whereas the Fr2, Fr3 and Fr4 of *C. marginiventris* increased the attractiveness of the Fr6 of *C. glomerata* to *C. glomerata* males (Fig. 3b). The Fr5 of *C. marginiventris* did not significantly increase the attractiveness of Fr6 of *C. glomerata* (Fig. 3b). In a previous paper (Chapter 3), when Fr6 of *C. glomerata* respectively combined with Fr1-5 of *C. glomerata*, instead of *C. marginiventris*, the attractiveness of each combination to *C. glomerata* males were similar: Fr1 decreased its attractiveness, Fr2-4 increased its attractiveness, and the combination of Fr5 and Fr6 was as attractive as the Fr6 alone.

Heptanal and nonanal were produced by three sections (heads, thoraxes and abdomens) of virgin females of *C. marginiventris*, which indicates they were components of CLs (the amounts of two compounds produced by each section were shown in Suppl. fig. 1). Heptanal and nonanal alone did not significantly attract the *C. marginiventris* males, but when mixed with Fr5 of *C. marginiventris*, the heptanal, but not nonanal, increased the attractiveness of Fr5 to conspecific males (Fig. 4). The solvent extracts of heads, thoraxes and abdomens evoked the EAG responses at the retention time of the candidate of pheromone (grey arrow in Fig. 2b, Fig. 5a, b and c). The extracts of three sections were more attractive to conspecific males than solvent controls (Fig. 5d), indicating the sex pheromones were cuticular compounds.
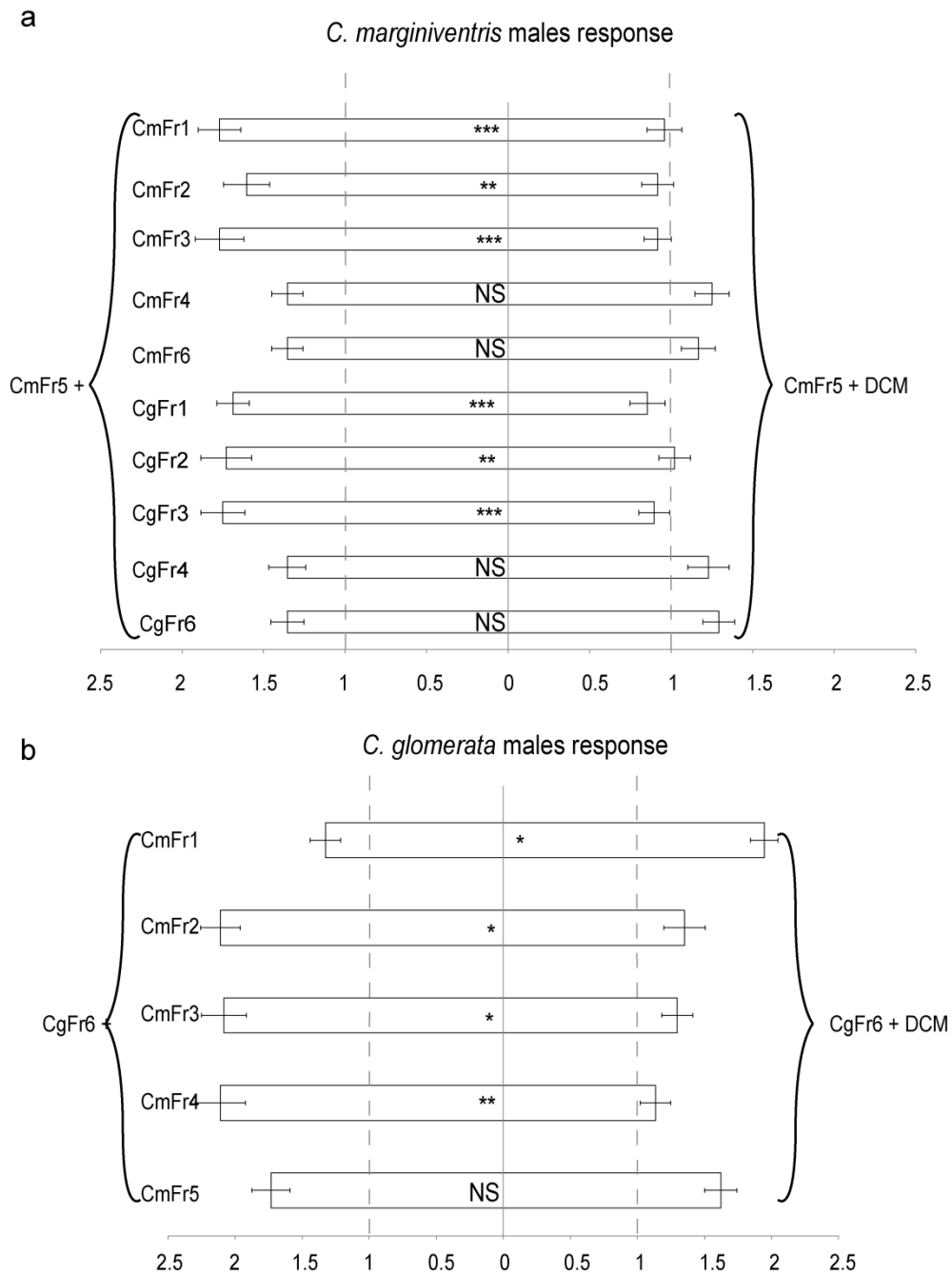
Fig. 3. Attractiveness of fraction combinations to conspecific or interspecific males. (a) *C. marginiventris* males responded to combinations of fractions of *C. marginiventris* (CmFr5 mixed respectively with CmFr1-4, 6) or of both *C. marginiventris* (CmFr5) and *C. glomerata* (CgFr1-4, 6). (b) *C. glomerata* males responded to the combinations of fractions of *C. glomerata* (CgFr6) and *C. marginiventris* (CmFr1-5). *C. glomerata* males responded to the combinations of fractions only of *C. glomerata* (CgFr6 mixed respectively with CgFr1-5) were tested in Chapter 3. Cg: *C. glomerata*; Cm: *C. marginiventris*.
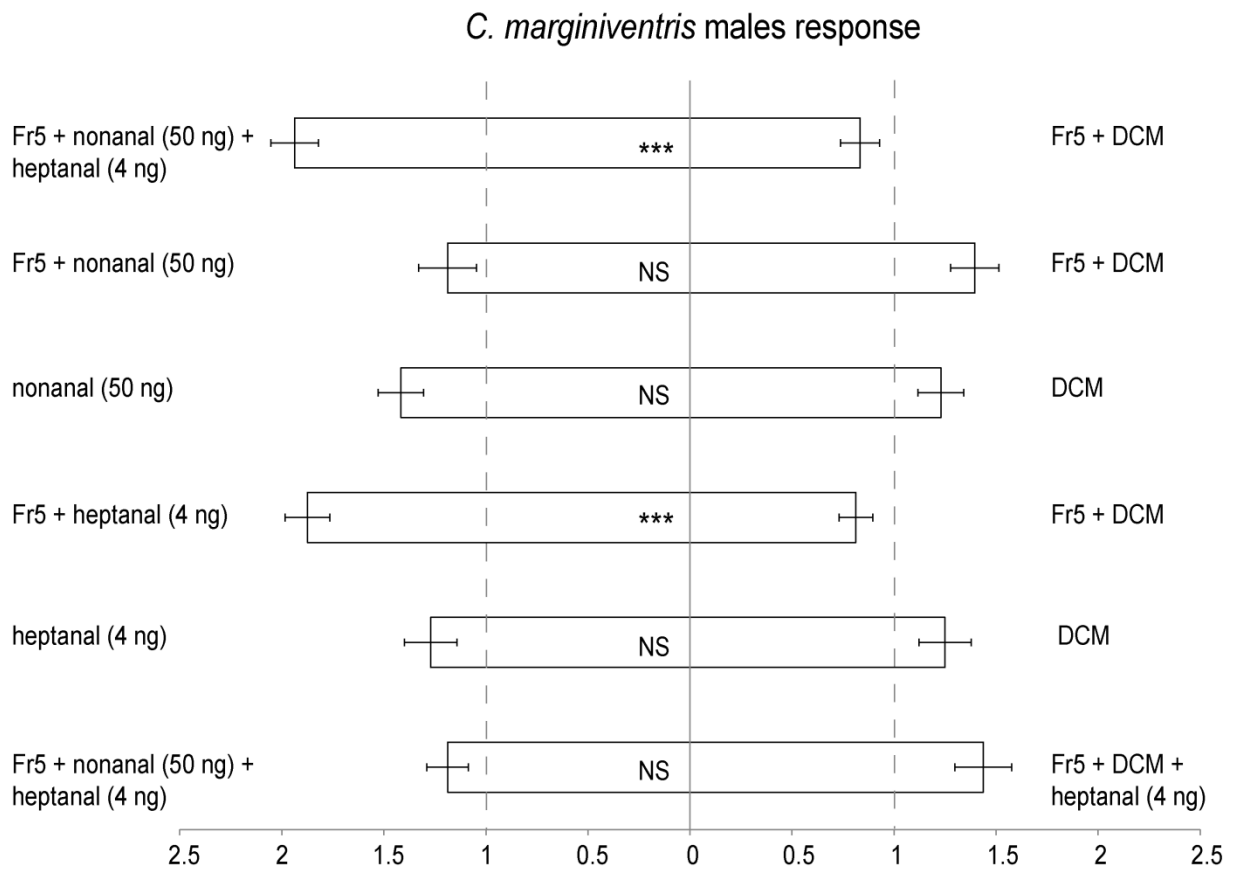
Fig. 4. Attractiveness of heptanal and nonanal to *C. marginiventris* males. Heptanal, but not nonanal, increased the attractiveness of Fr5. The two aldehydes alone did not significantly attract or repel *C. marginiventris* males. The attractiveness or repellence of heptanal and nonanal to *C. glomerata* males was tested in Chapter 3.
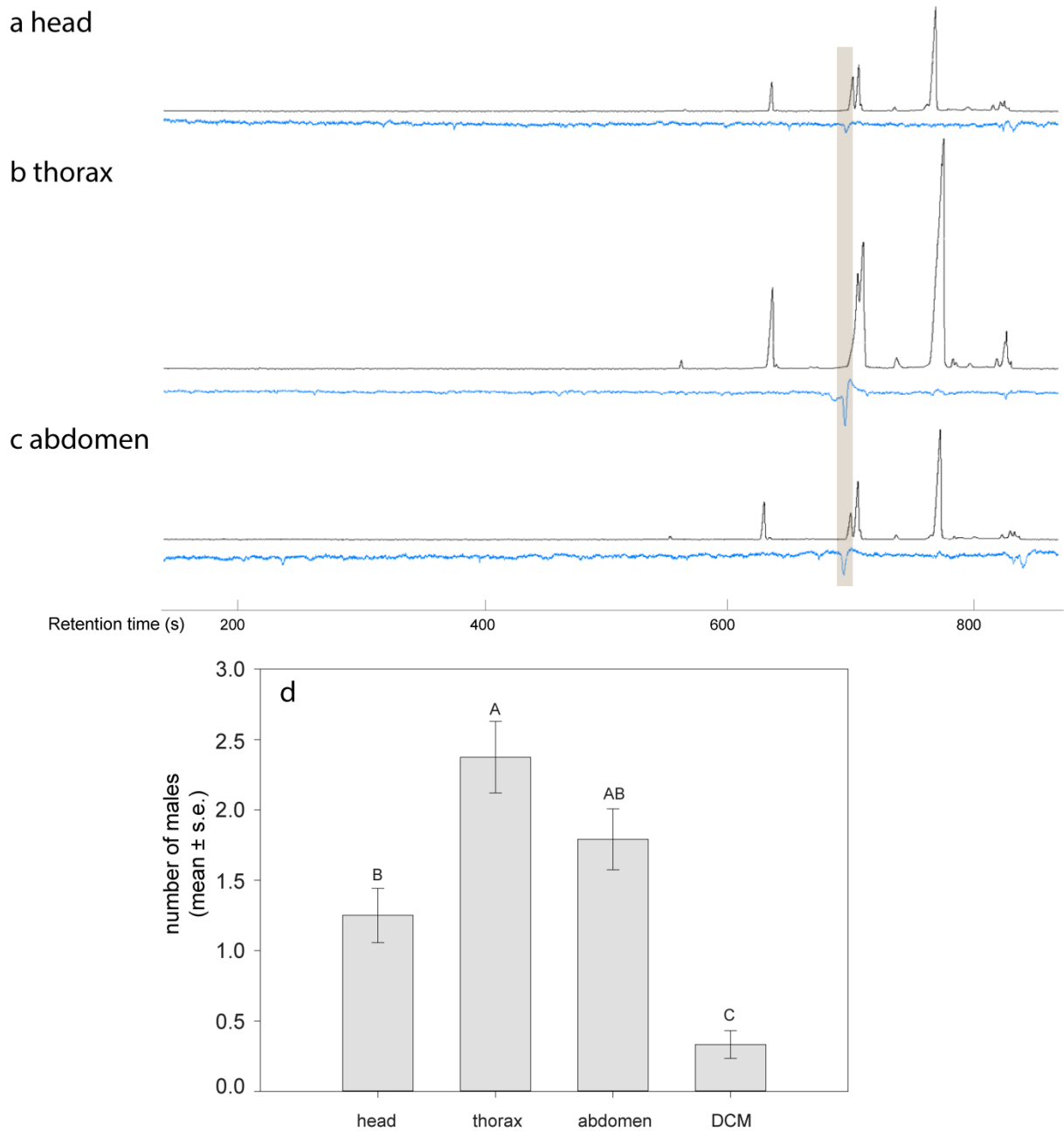
a head

b thorax

c abdomen

Retention time (s)   200   400   600   800

d

Fig. 5. Electrophysiological responses of male antennae to solvent extracts of head, thorax and abdomen sections of six virgin females in *C. marginiventris* (a, b and c). The attractiveness of solvent extracts of heads, thoraxes and abdomens of virgin females to males was tested in C. *marginiventris* (d). The similar tests on *C. glomerata* were presented in Chapter 3.

Discussion

It is well aware that CLs play essential roles in pheromonal communications in insects. Compared with gland-produced pheromones which mainly released at a certain occasion, for example, ready to mate (sex pheromones) or under attacks (alarm pheromones), CLs seem to be released continuously with a relatively smaller amount and thus mostly working in a shorter distance or by touch. CLs profiles were studied to be important for mate recognition and speciation in many different species [9], such as fruit flies [14] and bark beetles [16]. In parasitoid species, some cuticular components synergistically increased the attractiveness of other constituents of sex pheromones [27]. In general, previous studies revealed that the whole composition of CLs involved into pheromonal communications with strong species-specific differences, but how certain components of CLs had evolved into different pheromonal roles was largely unknown. By studying phylogenetically related species which have distinct mate finding strategies, we possibly get a better understanding on how flexibility of CLs acts as pheromones.

The two aldehydes heptanal and nonanal were non-specific components of CLs of both *Cotesia* parasitoid species, but they acted as distinct pheromone cues in each species. In *C. marginiventris*, heptanal was a component of sex pheromones and increased the attractiveness of Fr5 of virgin females (Fig. 4), whereas in *C. glomerata*, heptanal was an anti-aphrodisiac which is strongly repellent to males (Chapter 3). In addition, nonanal was a component of sex pheromones and increased the attractiveness of Fr6 of *C. glomerata* virgin females (Chapter 3), but nonanal did not increase the attractiveness of Fr5 of *C. marginiventris* to conspecific males (Fig. 4).

The gregarious parasitoid species in *Cotesia* genus had evolved from their solitary ancestor [28], and by using cuticular anti-aphrodisiac compounds (e.g. heptanal), the evolutionary divergence of the gregarious parasitoid species may easily form because the pheromonal controls on the natal patches reduced the deadly competitions among sibling males (Chapter 3). Meanwhile, how the nervous system has changed when anti-aphrodisiac compounds evolve from a sex pheromone (e.g. heptanal) is necessary to understand the evolutionary shift.

Notably, there were a couple of non-specific compounds in the headspace extracts of two species and they were possibly components of sex pheromones of a species or both species (Fig. 3). Since some non-specific compounds evoked the EAG responses of male antennae of both species (positions marked as "*" in Fig. 2a), the capability of detecting those

compounds possibly inherited from their common ancestor. These compounds were probably constituents of CLs, because phylogenetically related species normally shared a similar composition of CLs and used the same pathways with their common ancestor to biosynthesize CLs [9]. Closely related species with similar nervous systems capably sense the same compounds, and when these compounds transmit beneficial signals continually, the pheromonal cues possibly develop for both species [29,30], even for different purposes in different species like heptanal in the two *Cotesia* species.

Two modes were proposed in which pheromone blends can evolve [4]: a gradual process by activating or deactivating some components of pheromones [17,18], or changing the proportion of components [31]; and major shifts of sex pheromone components [16,20]. In our study, although the main composition of CLs might remain unchanged in two *Cotesia* species, the specificity of pheromones was possibly retained by two specific compounds (Fig. 2a and b): an unknown cuticular compound for *C. marginiventris* (Fig. 5) and a specific compound probably produced by an abdomen gland in *C. glomerata* (Chaper 3).

When studied on the pheromonal functions of CLs in insects, long-chain CHCs were often found to be important to work especially by contact [10,32]. In contrast, CLs with higher volatility, oxygenation or more polarity were rarely found to be involving in pheromonal communications or other signalling roles. That was possibly due to when analyzing CLs samples, long-chain CLs compounds were produced in extremely higher quantities and much easier to be identified than small CLs compounds (for example in Fig. 5a-c). Our results suggested volatile CLs also played essential roles in pheromonal communications. The pheromone blends of the both *Cotesia* species were made of both a couple of nonspecific compounds and some specific constituents. In insects, a complex composition of pheromones is sometimes necessary, because pheromonal components with different physical and chemical characters are more efficient to be detected by different sensory modalities in different ranges and circumstances [33], and the pheromone blends possibly need to transmit a complex information such as the distance to a target, receptivity to mate, and appropriate steps of courtship to the recievers [33,34].

Chromatographic separations of CLs and the compounds with similar characters are sometimes challenges, because those compounds are consisted with a large number of compounds with similar polarity [35]. The key components of female pheromones of two *Cotesia* parasitoids were failed to be identified. Those compounds seemed to be released in small quantities and the retention times of them overlapped with the ones of other compounds in our analyses. Sex pheromones of the two parasitoid species seemed to work in a relatively

short range [21]. Then, it would be difficult to find mate only with sex pheromones in nature for the solitary species *C. marginiventris* because their host (i.e. new-emerged wasp) locations are widely distributed in the field, and also for the individuals of the gregarious parasitoid *C. glomerata* who leave the natal patch and try to mate with the ones from anther colony [21,36-38]. Interestingly, virgin males and virgin females of both *Cotesia* species were strongly attracted by host damaged plants which probably serve as rendezvous sites for mate-seeking parasitoids [39].

In conclusion, components of CLs have a great potential to evolve into pheromonal functions. Normally, CLs, especially long-chain CHCs, are reported as contact cues, but some CLs with smaller molecular sizes probably work in a distance, which is important for some species such as the gregarious parasitoid *C. glomerata*, because male-male competition is possibly lethal when touch happens (Chapter 3). Closely related species, with similar nervous systems and composition of CLs, use the non-specific components of CLs for pheromonal cues, but each component possibly has evolved into a distinct function depending on the specific habitat of each species.

Acknowledgements

Author contributions

H.X., G.Z., and T.T. designed experiments and H.X. and G.Z. preformed bioassays, and I.S., S.D., G.Z. and H.X. preformed the electrophysiological analyses. H.X. and G.Z. did fractionation and chemical analyses. H.X. analysed data and made the figures. H.X. and T.T. wrote the manuscript. All authors commented on the manuscript.

## Additional information

Supplementary fig. 1

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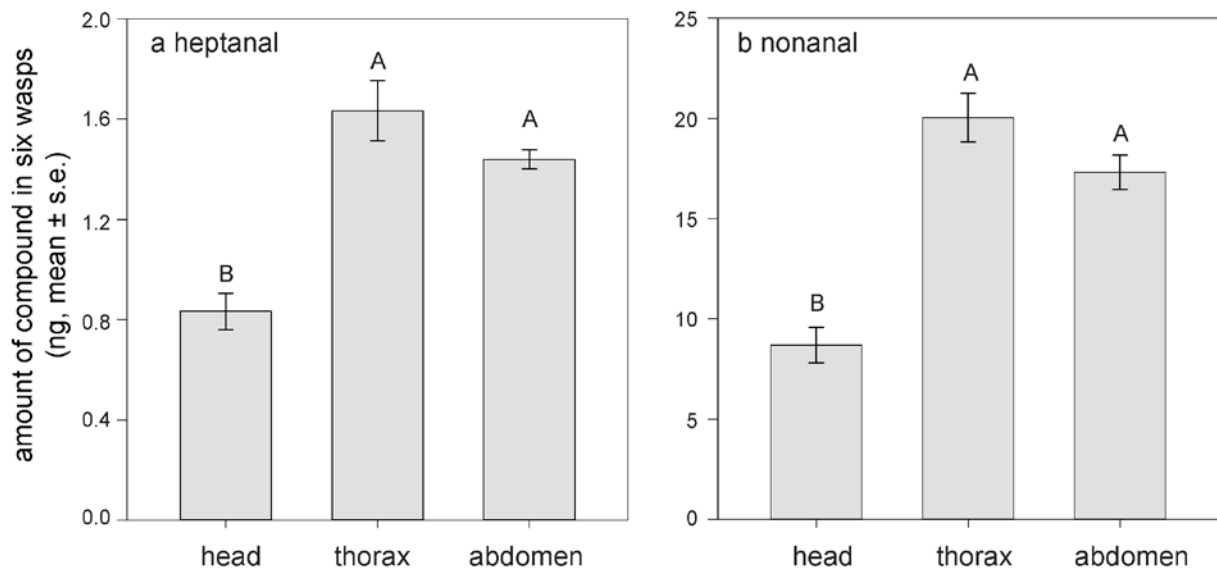
Suppl. fig. 1. Quantification of heptanal (a) and nonanal (b) in three sections (heads, thoraxes and abdomens) of six virgin females of *C. marginiventris*.

CHAPTER 5 Combined use of herbivore-induced plant volatiles and sex pheromones for mate location in braconid parasitoids

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Abstract

Herbivore-induced plant volatiles (HIPVs) are important cues for female parasitic wasps to find hosts. Here we investigated the possibility that HIPVs may also serve parasitoids as cues to locate mates. To test this, the odour preferences of four braconid wasps, the gregarious parasitoid *Cotesia glomerata* (L.) and the solitary parasitoids *Cotesia marginiventris* (Cresson), *Microplitis rufiventris* Kokujev, and *Microplitis mediator* (Haliday), were studied in olfactometers. Each species showed attraction to pheromones, but in somewhat different ways. Males of the two *Cotesia* species were attracted to virgin females, whereas females of *M. rufiventris* were attracted to virgin males. Males and females of *M. mediator* exhibited attraction to both sexes. Importantly, females and males of all four species were strongly attracted by HIPVs, independent of mating status. In most cases, males were also attracted to intact plants. The wasps preferred the combination of HIPVs and pheromones over plant odours alone, except *M. mediator*, which appears to mainly use HIPVs for mate location. We discuss the ecological contexts in which the combined use of pheromones and HIPVs by parasitoids can be expected. To our knowledge, this is the first study to show that braconid parasitoids use HIPVs and pheromones in combination to locate mates.

Keywords: Plant-insect interactions; caterpillar-induced plant volatiles; leaf volatiles; mate finding strategy; tritrophic interactions.

Introduction

Mate location strategies in parasitoids are mainly based on the use of sex pheromones [1,2]. Some parasitoids are able to locate mates at a relatively long distance with the help of highly volatile pheromones, but in some cases less-volatile pheromones are used at a short range [2], and in other cases no evidence has been found for the involvement of sex pheromones in mate location [3]. Therefore, other cues like host-associated volatiles or host-induced plant volatiles may possibly also aid parasitoids to find mates. In some phytophagous insects, host-plant semiochemicals play a significant role in the biosynthesis or release of sex pheromones, or even directly guide herbivores to mating sites [4,5], but the use of such signals by parasitoids is unknown.

Newly emerged female parasitoids seem to have two options: either they search for hosts right away without mating, at the risk of only producing haploid male offspring due to the haplo-diploid sex determination; or they invest time and energy searching for mates before looking for hosts, to ensure that they can produce both male and female offspring [6-8]. Previous studies generally advocate the idea that the two foraging strategies are mutually exclusive and the latter is better supported. For example, virgin females of the parasitoid *Cotesia vestalis* are not attracted by host-induced plant volatiles until they mate [9]. The males of the aphid parasitoid, *Lysiphlebus testaceipes*, are only attracted by conspecific females, and not by aphid-induced plants [10]. In the gregarious parasitoid *Cotesia glomerata,* more than half of newly-emerged females leave their natal patch without mating with siblings, but nevertheless most females find mates and produced both female and male offspring in the field [11]. Generally, once they have mated, female wasps search for hosts rather than for additional mates [7,9,12]. Mate finding strategies may differ between solitary and gregarious parasitoids. Gregarious parasitoids usually clump their cocoons together on the hosts, and both males and females show synchronized emergence, making mate finding relatively easy [1]. For solitary wasps, however, it must be a considerable challenge to find suitable mates due to the more widely dispersed emergence sites. Based on these differences in life history, a possible prediction would be that the quantity and volatility of the sex pheromones may be different between solitary and gregarious parasitoids. However, the situation is even more complex in the gregarious parasitoid *C. glomerata*, as inbreeding and outbreeding both occur frequently in this species. It is know that a part of females leave the natal patch without mating with sibling males, and those females probably mate with males from other cocoon

clusters [11,13]. Therefore, they too should rely on relatively long range cues to find each other. The hosts and host-damaged plants might provide such cues.

Several studies have shown that volatiles originating from the host or the host frass can act as sex kairomones (benefiting the parasitoids, but harmful to the hosts in this case), favouring mate location [14-16]. Herbivore-induced plant volatiles (HIPVs) have almost exclusively been studied in the context of host location, and their potential role in mate finding strategies have rarely been discussed. Yet, a number of studies have found that plant volatiles (either from host-induced plants, from fruits or even from intact plants) are also attractive to parasitoid males [17-21]. It has been suggested that this attraction can help the males in their efforts to find females. For example, *Cotesia plutellae* males more easily locate females when females are presented together with an intact cabbage leaf or a host-damaged cabbage plant, but, as yet, the relative importance of plant-volatiles and of sex pheromones in this attraction have not been tested separately [17]. Also, *Campoletis sonorensis* males have been shown to locate females more efficiently when a leaf of the host's food plant is presented together with the females [21]. Host-damaged plants are more attractive than intact plants to females and males of *Apoanagyrus lopezi*, a parasitoid of mealybugs [22]. In several *Microplitis* species, males and females are both responsive to HIPVs in behavioural or electrophysiological tests [19,23-25]. For *Cotesia marginiventris,* both males and females were attracted to certain green leaf volatiles (GLVs) and HIPVs [19]. None of these previous studies tested for potential additive or synergistic effects of the combined use of both sex pheromones and HIPVs in mate location strategies of parasitoids.

In the current study, we used four braconid wasps, the gregarious parasitoid *C. glomerata*, the solitary parasitoids *C. marginiventris, Microplitis rufiventris*, and *Microplitis mediator*, to test the respective importance of sex pheromones and HIPVs for mate finding. Parasitoid olfactory preferences were assessed in six-arm and four-arm olfactometer bioassays. The four parasitoids are common natural enemies of important lepidopteran pests and a good understanding of their foraging behaviour may help to optimize their use as biological control agents. The plant and insect species involved in each of the four tritrophic systems studied are listed in Table 1.

Materials and methods

Insects

Four parasitoids and their respective hosts were used to test the role of HIPVs and pheromones in parasitoid attraction (Table 1). The four endoparasitoids were reared in our laboratory at the University of Neuchatel, Switzerland. The two *Cotesia* species were reared following the protocol described by Xu, Veyrat, Degen and Turlings [12]. The parasitoid *M. mediator* was reared on 1st instar caterpillars of cabbage moth *Mamestra brassicae*, which were fed on Chinese cabbage. *M. brassicae* eggs were provided by Forschungsinstitut für biologischen Landbau (FiBL), Frick, Switzerland. The parasitoid *M. rufiventris* was reared on *Spodoptera littoralis* caterpillars (1st instar) kept in square plastic boxes (15 × 13 × 5 cm) and fed with a wheat germ-based artificial diet. The eggs of *S. littoralis* were provided by Syngenta, Stein, Switzerland. In order to get virgin wasps, each parasitoid cocoon was placed in a 1.5 ml centrifuge tube until the wasp emerged. Then, virgin females and virgin males were kept separately in two Bugdorm-1 cages (30 × 30 × 30 cm, Mega View Science Education Services Co. Ltd, Taiwan). In order to obtain mated wasps, about 50 females and 50 males of the same parasitoid species were put into the same Bugdorm-1 cage. The wasps were provided with honey and moist cotton wool, and the cages were stored in an incubator at 25°C (LD 16 : 8 h) for about three days before each test.

Table 1. The four tritrophic systems used in this study

Trophic level	System 1	System 2	System 3	System 4
3rd	*C. glomerata*	*C. marginiventris*	*M. rufiventris*	*M. mediator*
2nd	*P. brassicae*	*S. littoralis*	*S. littoralis*	*M. brassicae*
1st	*B.rapa*	*Zea mays* (variety Delprim)	*Zea mays* (variety Delprim)	*B. rapa*

Bioassays

The bioassays were carried out in four-arm and six-arm olfactometers described in previous studies [26,27]. Each olfactometer arm had an airflow of 0.6 litre per minute that entered the central release chamber (Figure 1). The wasps were released in groups of six into

an olfactometer. They were allowed to make a choice among treatments for a maximum of 30 min [12]. At the end of this period or as soon as all six wasps had made a choice, the result was recorded, the tested wasps were removed, and a new group of six naive wasps was released. Each replicate included four releases of six wasps. In most cases, we did two replicates on a given day. Each experiment consisted of six replicates (144 wasps in total). Glassware was cleaned and the positions of the treatments in the olfactometer were changed randomly for each replicate.

Plants used for the bioassays were 3-4 week old seedlings of wild cabbage *Brassica rapa* and 2-week old maize *Zea mays* (variety Delprim) grown in plastic tubes (OD = 4 cm, L = 11 cm) kept in a phytotron (25°C, LD 16 : 8 h). These tubes fitted the size of the glass vessels in the olfactometer systems (Figure 1A). To induce the release of HIPVs, 20 caterpillars (1st instar) were put on their respective host plant seedlings which had been placed into the olfactometer vessels and left feeding overnight (about 14 hours, LD 16 : 8 h) before the start of the bioassay. Intact plants were used as the control treatments. Species used in assays were listed in Table 1.

Presence of sex pheromones.

To test whether sex pheromones are involved in attraction of the opposite sex of the two *Microplitis* wasps, we used the same six-arm olfactometers as for *Cotesia* parasitoids in a previous study [28]. Six virgin females, six mated females and six virgin males were placed as the three treatments (odour sources) in the olfactometer to test their relative attractiveness to virgin males. Similarly, six virgin males, six mated males and six virgin females were presented as the three treatments in the olfactometer to test their relative attractiveness to virgin females. The groups of six wasps were placed in the three of the arms of the olfactometer as shown in Figure 1B, and the arms between the three treatments were left empty [12].

Attractiveness of plant volatiles.

To test the attractiveness of plant volatiles, two treatments, a herbivore-damaged plant and an intact plant, were placed in the vessels (Figure 1A) attached to the opposite arms of a four-arm olfactometer [27], and the two other vessels were left empty.

Attractiveness of plant volatiles and sex pheromones in combination.

These tests were carried out in a six-arm olfactometer [26]. Two groups of six virgin males (or mated males) were combined with an herbivore-damaged plant and an intact plant, respectively, to test their attractiveness to virgin females. An herbivore-damaged plant and an intact plant without wasps served as two alternative odour sources, and the two remaining

arms were left empty. The wasps (virgin or mated) were placed in the respective arms as shown in Figure 1B, whereas the plant treatments (herbivore-damaged or intact one) were placed in the odour source vessels (Figure 1A). In an analogous way, two groups of six virgin (or mated females) were also combined with a herbivore-damaged plant and an intact plant respectively to test their attractiveness to virgin males. Two additional treatments were a herbivore-damaged plant and an intact plant, both without wasps, and the two remaining arms were empty. In both experiments, the two empty arms were at opposite positions, and the treatments including the wasps and the treatments including plants treated in the same way were never placed in adjacent arms.

Statistics

Statistical analyses were performed in R 3.0.2 with the package of Lme 4 [29]. To test whether the differences among the responses of the parasitoids to the treatments were significant, we used generalised linear mixed models (GLMMs) with poisson distribution of error. The replicates were treated as the random factor. Tukey's post-hoc test was performed for multiple comparisons. 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 other's responses [30]. Each model was fitted by maximum quasilikelihood estimation in the software package R. In the figures, the number of wasps choosing empty arms was divided by the number of empty arms present in the setup to make it comparable to the other treatments. Statistical differences ($p < 0.05$) are indicated with different letters in the bar figures.
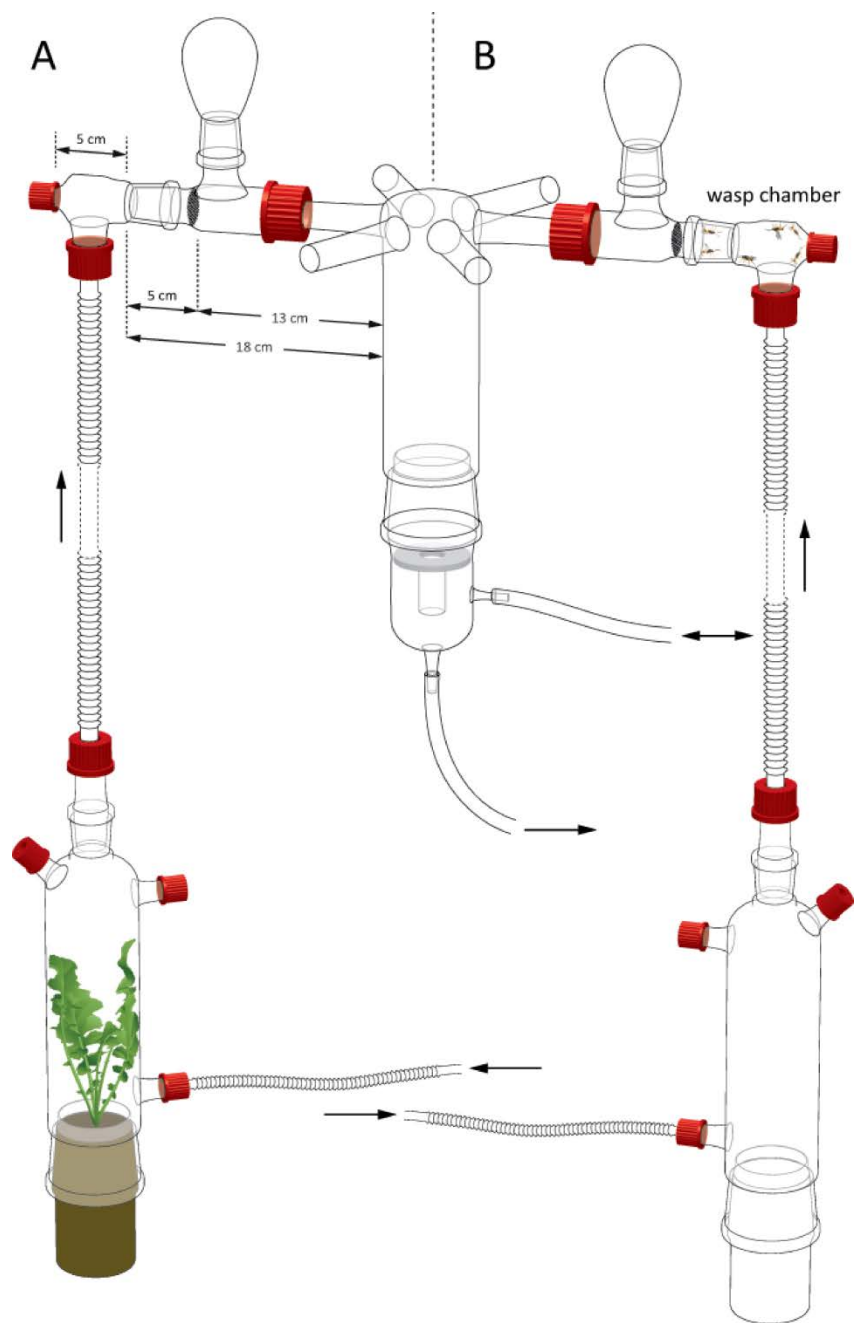
Figure 1. The six-arm olfactometer that was used to test for the attractiveness of plant volatiles (A) and/or sex pheromones (B). To serve as odours sources, plants were placed in glass vessels as indicated and wasps were placed in "wasp chamber".

Results

For *M. rufiventris*, virgin males were equally attracted to virgin or mated females, as well as to virgin males themselves, but, surprisingly, virgin females were strongly attracted to virgin males only (Figures 2A and B). For *M. mediator*, we did not find any clear evidence for the use of sex pheromones, but we observed a consistent pattern that virgin females and males were more attracted to the odours of conspecific wasps than to the clean air from the empty arms independent of their sex and mating status (Figures 2C and D). For both *Cotesia* wasps it is known that males are strongly attracted to virgin females, but not to mated females [12].
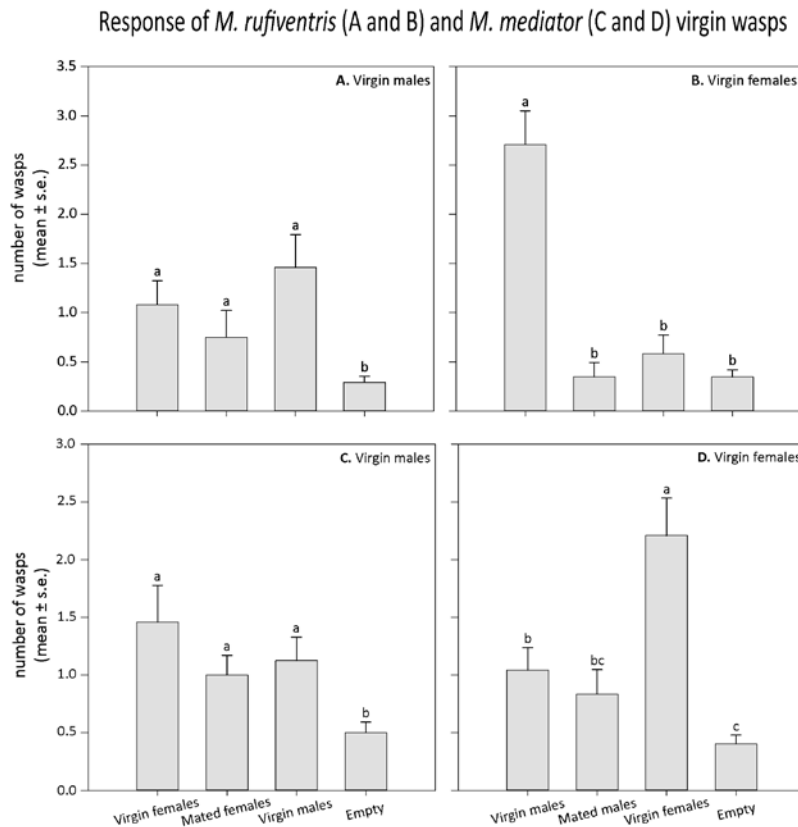

Response of *M. rufiventris* (A and B) and *M. mediator* (C and D) virgin wasps

Figure 2. Attraction of virgin and mated *M. rufiventirs* (A and B) and *M. mediator* (C and D) to conspecific wasps (virgin or mated).

The four braconid wasps, irrespective of sex and mating status, were strongly attracted by the HIPVs (Figures 3 and 4). Herbivore-damaged plants were generally much more attractive than intact plants for all parasitoids (Figures 3 and 4), with the exception of mated males of *C. glomerata*, *C. marginiventris* and *M. rufiventris,* which did not discriminate between damaged and intact plants (Figures 3D, H and 4D). Generally, significantly more wasps ended up in arms connected to vessels with intact plants than to arms with empty vessels (Figures 3 and 4).

For the two *Cotesia* species, virgin females were predominantly attracted by herbivore-damaged plant, independently of the presence of virgin or mated males (Figures 5A, B, E, and F). By contrast, virgin males were only strongly attracted by treatments including virgin females, independently of whether they were combined with herbivore-damaged or intact plants (Figures 5C and G). Virgin males of *C. glomerata* were more attracted by herbivore-damaged plant than the combined odours of herbivore-damaged plant and mated females (Figure 5D), which confirms the intriguing repelled effect of mated females on males [12]. Virgin males of *C. marginiventris* were attracted equally by HIPVs and the combined odours of herbivore-damaged plant and mated females (Figure 5H).

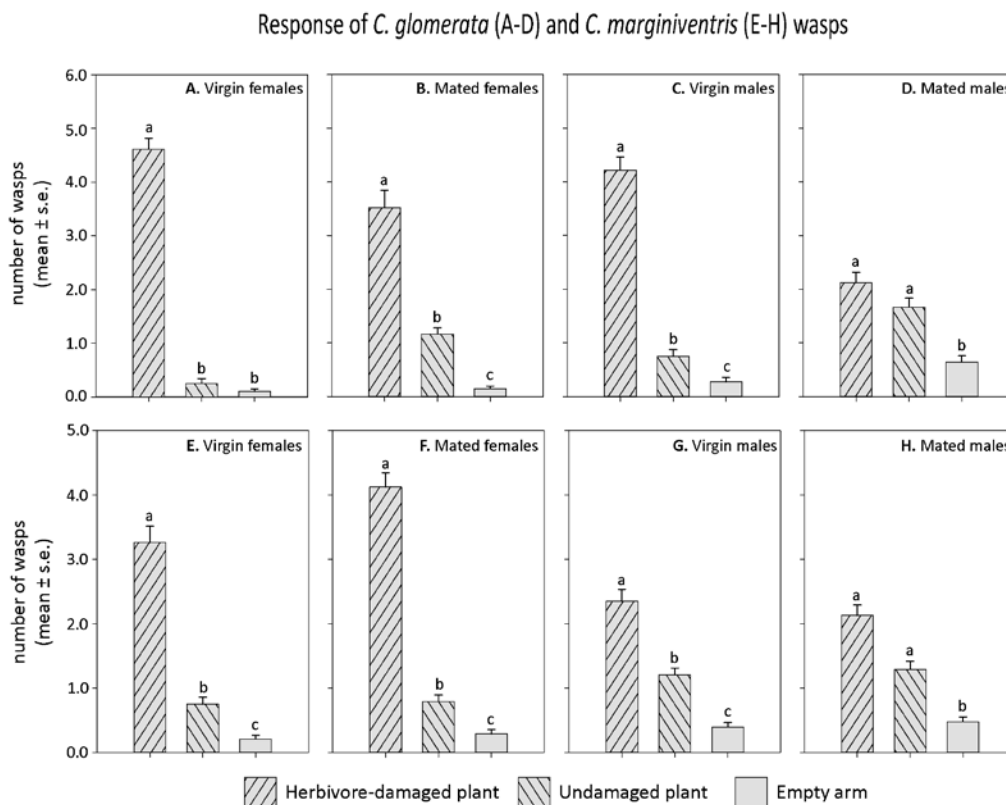Response of *C. glomerata* (A-D) and *C. marginiventris* (E-H) wasps

Figure 3. Responses of females (virgin, A and E; or mated, B and F) and males (virgin, C and G; or mated, D and H) of *C. glomerata* (A - D) and *C. marginiventris* (E - H) to the odours of herbivore-damaged plants and undamaged plants.

For *M. rufiventris*, the combination of plant odours (either from herbivore-damaged plants or intact plants) and the odour of virgin males was more attractive to the virgin females than respective plant volatiles alone (Figure 6A). The combination of virgin males and an intact plant was as attractive to the females as the herbivore-damaged plant (Figure 6A). When exposed to mated males, virgin females predominantly chose the treatments that included the herbivore-damaged plants (Figure 6B), which suggests that sex pheromones are no longer released by *M. rufiventris* males after mating. Virgin males were strongly attracted

by the treatments including HIPVs, no matter whether the females (virgin or mated) were present or not (Figure 6C and D).

For *M. mediator*, both virgin females and virgin males were strongly attracted by the herbivore-damaged plants independent of the presence or absence of conspecific pheromones (Figure 6E - H). The virgin males chose the combined odours of intact plant combined with virgin females more than intact plants alone (Figure 6G). All results are summarized in the Table 2.

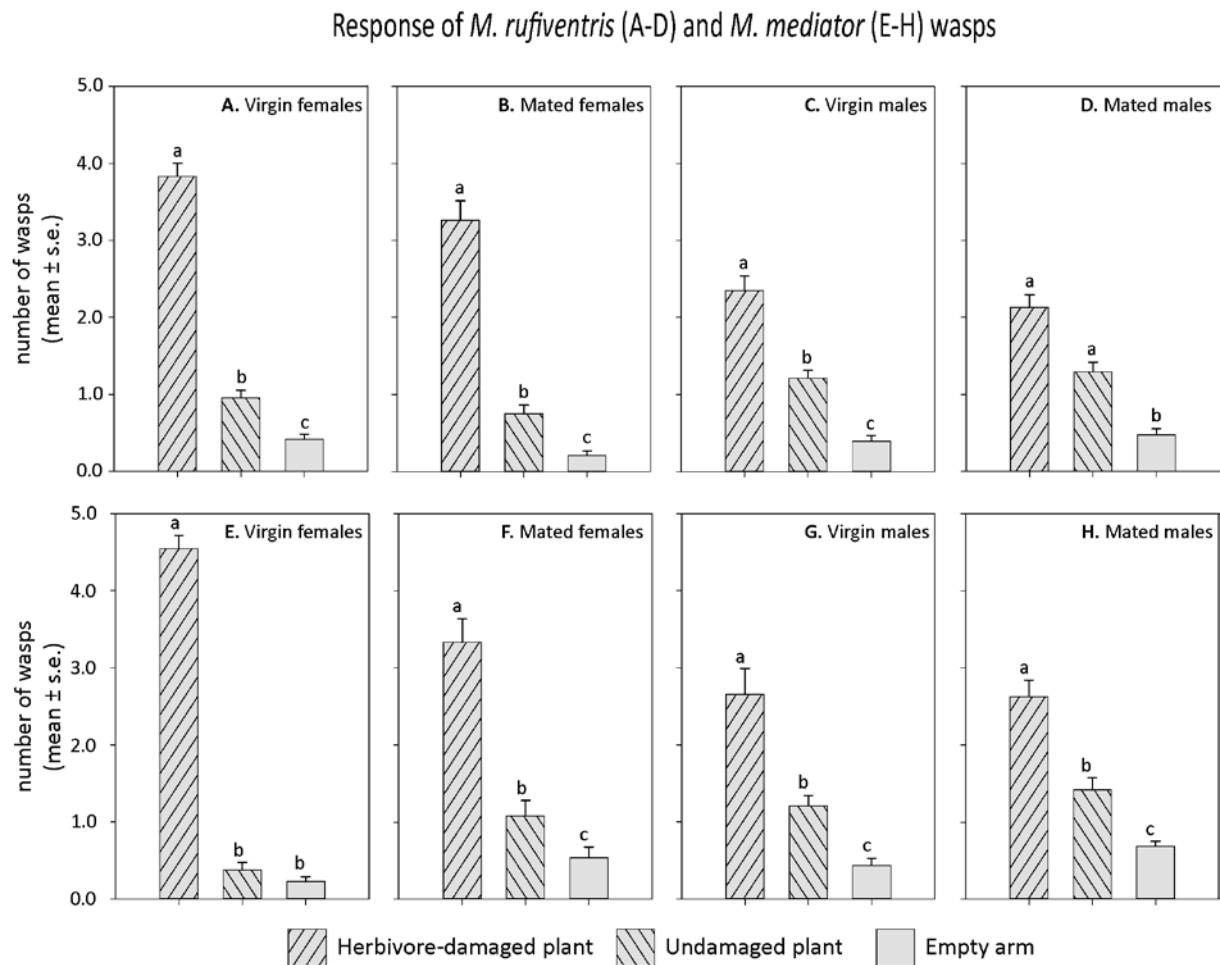Response of *M. rufiventris* (A-D) and *M. mediator* (E-H) wasps

Figure 4. Responses of females (virgin, A and E; or mated, B and F) and males (virgin, C and G; or mated, D and H) of *M. rufiventris* (A - D) and *M. mediator* (E - H) to the odours of herbivore-damaged plants and undamaged plants.

Figure 5. Responses of virgin females (A, B and E, F) and virgin males (C, D and G, H) to plant volatiles or combined odours of plants (damaged or not) and potential pheromonal sources (virgin or mated wasps of the opposite sex) by *C. glomerata* (A - D) and *C. marginiventris* (E - H). HDP: herbivore-damaged plant; UDP: undamaged plant.
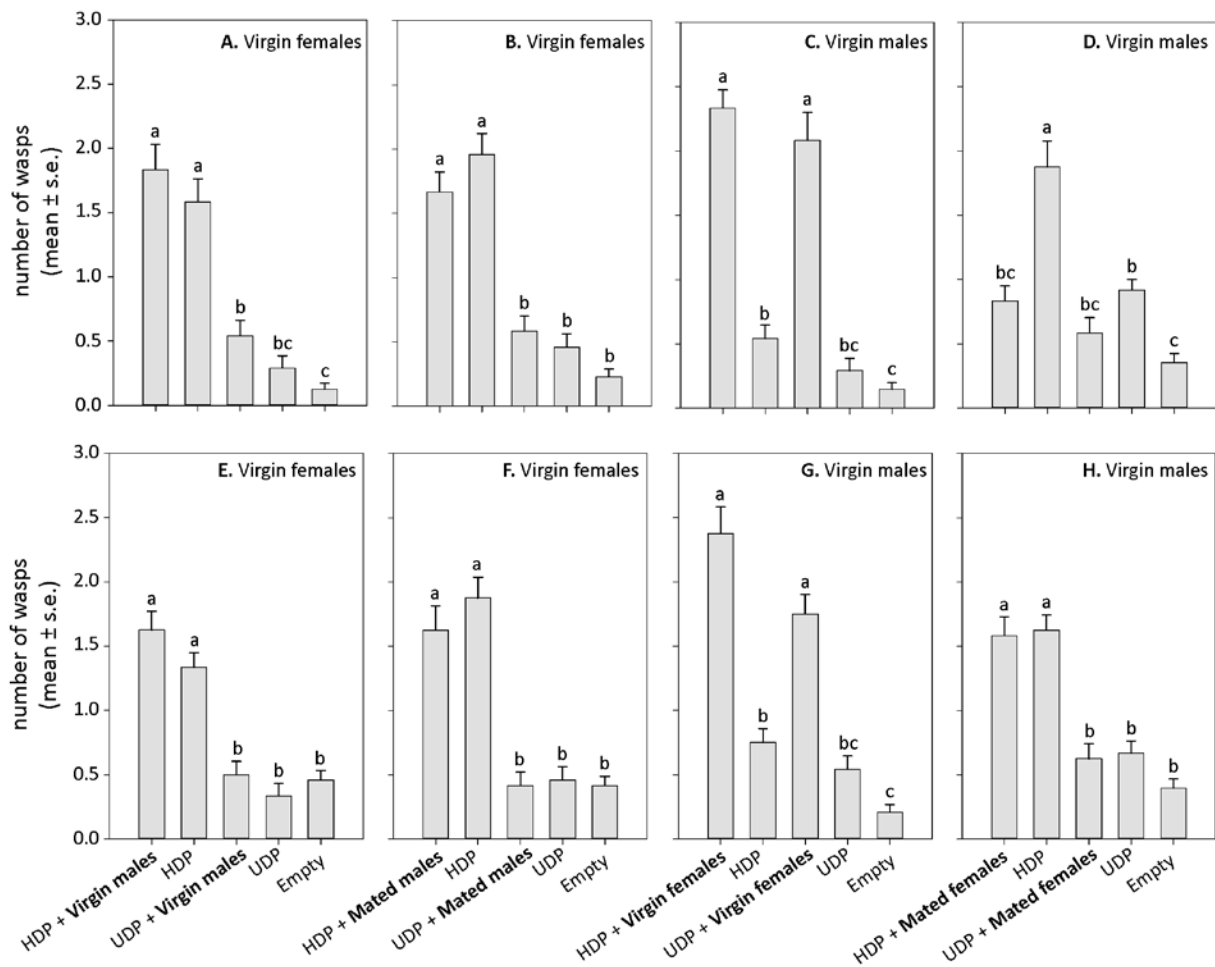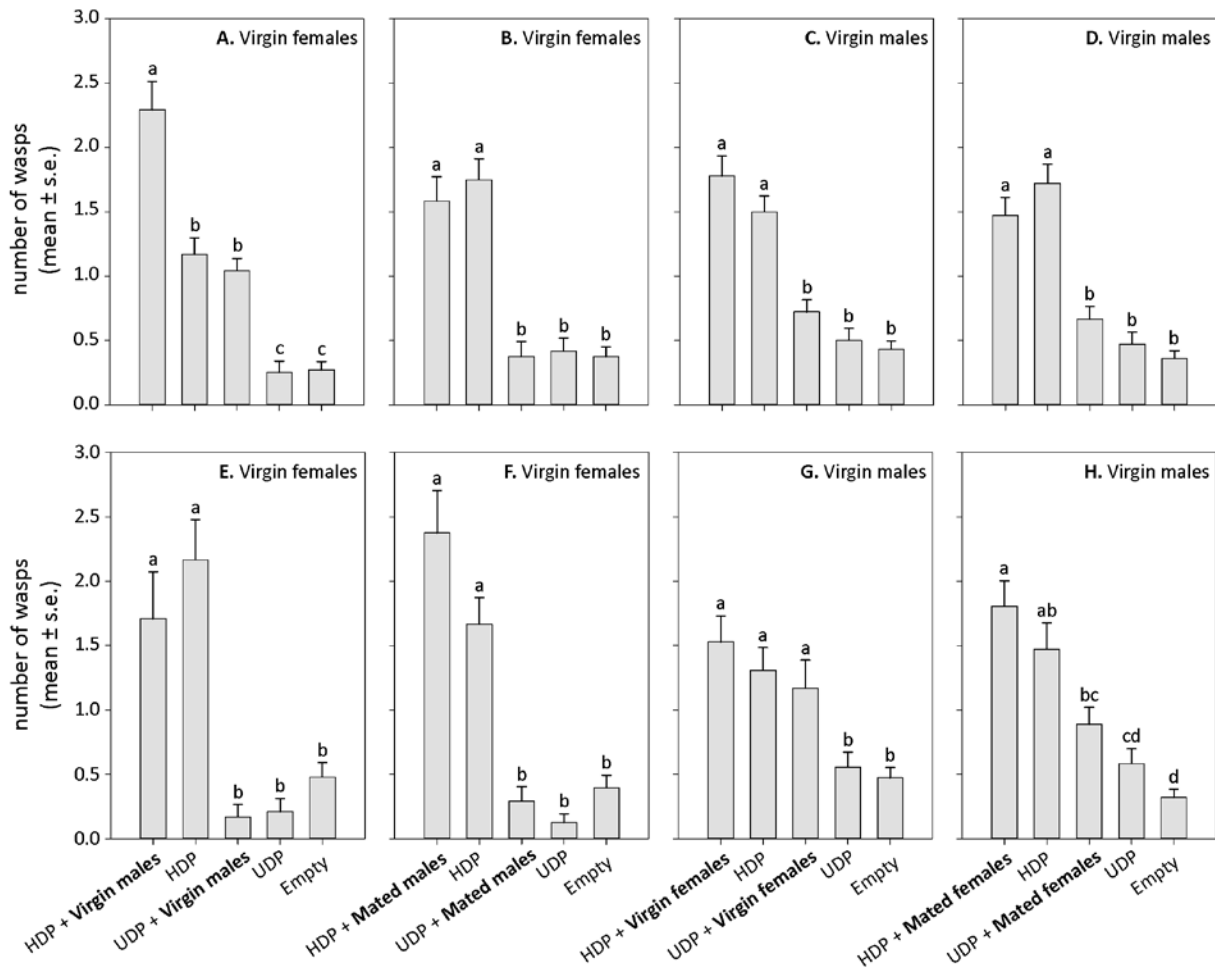
Figure 6. Responses of virgin females (A, B and E, F) and virgin males (C, D and G, H) to plant volatiles or combined odours of plants (damaged or not) and potential pheromonal sources (virgin or mated wasps of the opposite sex) in *M. rufiventris* (A - D) and *M. mediator* (E - H). HDP: herbivore-damaged plant; UDP: undamaged plant.

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Discussion

The diverse strategies of mate location in braconid wasps

Mating strategies in parasitoids have received considerable attention, but the respective importance of sex pheromones and plant-produced volatile for mate location has rarely been examined. In our study, involving four braconid parasitoid species of leaf-feeding caterpillars, we found that virgin females of the two *Cotesia* species [12] and virgin males of *M. rufiventris* release sex pheromones that attract conspecific wasps of the opposite sex. Virgin females and virgin males of all four parasitoids were also strongly attracted by herbivore-damaged plants. When sex pheromones and HIPVs were present at the same time, the males of *Cotesia* species were predominantly attracted to sex pheromones, irrespective of combining them with odours of herbivore-damaged plants or intact plants (Figures 5C and G). For *M. rufiventris*, the combination of virgin males and herbivore-damaged or intact plants was more attractive to females than herbivore-damaged or intact plants alone (Figure 6A), illustrating an additive effect of these two types of cues for this species. This changed when the virgin females were offered odour sources with mated males, in which case they switched their preferences to treatments including herbivore-damaged plants. This suggests that the mated males stopped releasing their sex pheromone (Figure 6B). The results also suggest that for *M. mediator* pheromones are of lesser importance: both virgin females and males largely chose the treatments including herbivore-damaged plants, irrespective of the additional presence of conspecific wasps (Figure 6E - H). Thus, HIPVs are likely to be the predominant cues for mate location in this solitary wasp, which was found to not just attract the opposite sex, but instead appears to release an aggregation pheromone attractive to both sexes.

The male-produced sex pheromone of *M. rufiventris* was not as powerful an attractant as the female sex pheromones in the two *Cotesia* species, whose sex pheromones were more attractive to males than HIPVs alone (Figures 5C, and G). In contrast, the attractiveness of virgin males of *M. rufiventris* increased dramatically when combined with HIPVs, while herbivore-damaged plants alone or the combination of virgin males and intact plants attracted significantly fewer virgin females (Figure 6A). This implies that *M. rufiventris* relies on both HIPVs and sex pheromones for mate location. Something similar is known for males of the parasitoid *Venturia canescens*, which are considerably more strongly attracted to the combined odours of hosts and females than to the odours of hosts or females alone [15].

Males of parasitoids are usually able to mate several times during their lifetime [1,31]. However, copulating with already mated males can result in male-biased sex ratios of the

offspring, possibly as a result of sperm depletion in the males [32]. In a previous study we found that mated males of *Cotesia* species are less attractive to females (see Table. 2) [28]. In the current study, the males no longer discriminated between herbivore-damaged and intact plants once they had mated (Figures 3D, H and 4D). This remains unexplained, but could mean that they switch to food foraging in order to restore resources for sperm production. In contrast to males, females generally mate only once during their lifetime [31], and they cease to release their sex pheromone after mating [12,33,34]. Therefore, the combined use of HIPVs and pheromones (switched on or off by virgin or mated wasps) is probably a reliable strategy for mate location in braconid parasitoids.

Table 2. The responses of four parasitoids of different sexes or mating status to the odours of conspecific wasps, plant volatiles, or their combined odours.

Parasitoids / Treatments	*C. glomerata* #				*C. marginiventris* #				*M. rufiventris*				*M. mediator*			
	VF	MF	VM	MM	VF	MF	VM	MM	VF	MF	VM	MM	VF	MF	VM	MM
VM	NS		NS		NS		NS		+++		+++		++		++	
MM	-				NS				NS				NS			
VF	NS		+++		NS		+++		NS		+++		+++		+++	
MF			NS				NS				+				+	
HDP	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
UDP	NS	+++	+	+++	++	+	+++	+++	++	+	+++	+++	NS	+	++	++
HDP + VM	+++				+++				+++				+++			
HDP + MM	+++				+++				+++				+++			
HDP + VF			+++				+++				+++				+++	
HDP + MF			NS				+++				+++				+++	
UDP + VM	+				NS				+++				NS			
UDP + MM	NS				NS				NS				NS			
UDP + VF			+++				+++				NS				+++	
UDP + MF			NS				NS				NS				++	

The VF, MF, VM, and MM refer to virgin females, mated females, virgin males, and mated males of each parasitoid species. The HDP and UDP refer to the herbivore-damaged and undamaged plants. The NS indicates that there is no statistical difference between treatments

and empty arms. The symbol "+" or "-" indicates that the responses to the treatments are significantly higher or lower than to empty arms in respective tests (GLMMs with Tukey's post-hoc test, taking "+" as an example, "+" $P < 0.05$, "++" $P < 0.01$, "+++" $P < 0.001$). The symbol "#": the data showing attraction of *C. glomerata* and *C. marginiventris* to pheromones have been published in a previous study [12].

Mate and host locations are not two distinct processes

As a consequence of their haplo-diploid sex determination, newly emerged wasps face the dilemma to either search for hosts as soon as possible and producing only haploid male offspring or first search for a mate, after which they would be able to produce both female and male progeny. The latter option has generally been observed to be favoured [6-8,11]. However, we found that both virgin males and females of all species were strongly attracted by HIPVs. Therefore, mating is not a prerequisite for attraction to HIPVs, and HIPVs may not only be indicative of where to find hosts, but may also help guiding wasps to potential mates. Attraction toward herbivore-infested plants seemed more pronounced in virgin females of *C. glomerata*, *M. rufiventris* and *M. mediator* than in mated individuals (Figures 3A, B and 4A, B, E, F). This implies that herbivore-damaged plants are important mating sites for the wasps. This is also evident from the study on a parasitoid of leaf-miner pests, where both males and females are frequently found together on their host food plant, even if they both emerged at a distance away [31]. Fauvergue, Lo Genco and Lo Pinto [6] suggest, based on field observations, that virgin females release their sex pheromone while foraging for hosts. They propose therefore that searching for hosts and for mates are not two exclusive activities, as supported by our results.

HIPVs may serve as long-range cues in mate location by braconid wasps

The synthesis and release of sex pheromones, as well as searching for mates can be costly for parasitoids [2]. Moreover, it has been proposed that the use of sex pheromones by parasitic wasps generally comprises two steps: one attracts the conspecific wasps from a distance and the others involves the subsequent stages of courtship [1,34]. In this context, the use of HIPVs may benefit parasitoids by alleviating the costs of producing a volatile pheromone for long range attraction. HIPVs are released in large amounts as a bouquet of semiochemicals with relatively low molecular weight, highly suited for detection at relatively long distances (up to several meters or more) [35]. Once they are in host-damaged plants, short-range visual, wing vibrations and chemical cues may be effective to locate and court the opposite sex [21,31,36,37]. We have evidence that the sex pheromone of *C. glomerata* females is composed of relatively large molecules of low volatility working at a relatively short range (unpublished data). Indeed, their attractiveness to males decreased considerably when virgin females were placed at a longer distance in the bioassay system [12]. The use of HIPVs may therefore be critical to find mates, even for this gregarious species because some individuals leave the natal patch without mating with siblings, and thus favour the maintenance of outbreeding in this species [11,13].

In conclusion, the four braconid wasp species were found to release pheromones, but each in a different way, and virgin individuals of all species were attracted to HIPVs. This implies that gregarious and solitary braconid wasps may rely on pheromones in combination with HIPVs to locate suitable mates. By switching on or off pheromones release, depending on the mating status, parasitoids may optimize their foraging efficiency. It will allow female wasps to concentrate their efforts on host location after mating without further harassment by males. We also hypothesize that the HIPVs work at a relatively long range due to their high volatility, while the pheromones are possibly working at a relatively close range, and can be expected to be less volatile. The parasitoid *M. rufiventris* was somewhat different in that it responded always better to a combination of pheromones and plant volatiles. Based on our results, we hypothesize that for many parasitoids host and mate locations are not exclusive processes, and plant odours are important cues for mate location, as has been reported for several phytophagous insects [4,5].

Acknowledgement

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CHAPTER 6 General discussion: Plant volatiles as mate finding cues for insects

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Abstract

Plant volatiles are not only used by herbivorous insects to find their host plants, but also by the natural enemies of the herbivores, such as predators and parasitoids, to find their prey or hosts. Increasing evidence suggests that plant volatiles are also used by these insects to find mating partners. As yet, the role of plant volatiles in mate finding has only been considered in a few cases, as most studies on the interactions between the sexes generally focus on the importance of species-specific pheromones. Here, we reviewed the current evidence that volatiles of different plant structures may significantly contribute to mate-finding of various insect species. We propose that plant volatiles and pheromones contribute differently to the insect mate-seeking process, in particular in the distance at which they operate. We show that the average boiling point (volatility) of relevant plant volatiles is lower than that for typical sex pheromones and we address recent theoretical and experimental advances on how these differences affect odor plumes. We postulate that in many insects the use of plant volatiles to find food as well mate has evolved as an efficient foraging strategy.

Keywords: plant-insect interactions; plant volatiles; mate finding; sex pheromones; odor plumes; insect foraging strategies.

1. The underestimated role of plant volatiles in mate location

Plants provide many insects with food and shelters and are key players in orchestrating multitrophic interactions. The interactions between plants and insects mostly result from the detection of a diversity of plant volatiles by the well-developed olfactory system of insects. Plants normally emit a bouquet of volatile compounds of low molecular weight, lipophilic character and high volatility, and these compounds easily meander a few meters away from the source in natural environments [1-4]. As such, plant volatiles contribute importantly to the habitat (background) odor that insects have to deal with while searching for resources and mates [3-5]. Mate location in insects almost always involves species-specific pheromones, which are commonly considered to be the principle cues leading to successful mate finding [6], but increasing evidence suggests plant volatiles play an additional role in the mate location process. Their role has been mainly reported for phytophagous insects, as summarized in two previous reviews [7,8], but they are also used as mate finding cues by other insect groups, such as parasitoids and insects that forage for pollen and nectar (Fig. 1 and Table 1). Here we review a number of key published examples and we address recent theoretical and experimental advances on how the chemical properties of plant volatiles and insect pheromones may result in different kinds of odor plumes that may synergistically or additionally help insects to find mates in a timely and effective way.

2. Widespread use of plant volatiles in mate location by insects

Leaf-produced volatiles

Leaf-emitted volatiles are important cues for phytophagous insects to locate host plants not only as food sources [9], but also to find conspecifics of the opposite sex, as host plants are opportune mating sites. There is ample evidence for the combined use of plant volatiles and sex pheromones for mate location. Wind tunnel assays, for instance, have shown that males of both the grapevine moth *Lobesia botrana* and the grape berry moth *Eupoecilia ambiguella* are attracted more by conspecific female pheromones if they are presented in combination with host plant volatiles, such as the sesquiterpene (*E*)-β-caryophyllene [10,11]. Plant volatiles are well-known to increase the attractiveness of aggregation or sex pheromones of beetle species in the field [7,12-18], which has been particularly well studied for bark beetles [7,18]. Males of cockchafer species (Coleoptera: Scarabaeidae) represent another nice example. They show swarming flights at dusk, while conspecific females feed on host plants [19,20]. The freshly damaged leaves emit volatiles that possibly indicate the relatively precise location of feeding females, and the specific sex pheromones emitted by the females help males to identify the right cockchafer species at short range [19-22].

It has also been found that males of certain moth species are caught more often in traps baited with a combination of typical green leaf volatiles (GLVs) and synthetic female pheromone than in traps baited only with the pheromone [23-26]. Similar results have been obtained with aphid traps [27]. Males of two different aphid species were caught much more frequently in traps that included a mixture of conspecific female pheromone and host plant volatiles than in traps with only the pheromone. As with the cockchafer examples, plant volatiles were only attractive to male aphids when combined with the correct conspecific female pheromone [27]. The positive effect of plant volatiles on mate recruitment is probably not only limited to direct attraction. For instance, exposing adult insects to plant volatiles may also stimulate the release of pheromones, as has been suggested for females and males of the cabbage looper moth *Trichoplusia ni* [28,29].

Fruit volatiles

Fruit-feeding insects are generally attracted to fruit volatiles. For these insects, fruits are also ideal mating sites and the fruit volatile blends not only indicate mating opportunities, but may also stimulate calling (pheromone release) and mating behaviors. For example, females of certain tephritid fruit fly species (Diptera: Tephritidae) show increased receptivity to mating after exposure to host fruit volatiles [30-34]. Papaya extracts were found to increase

the attractiveness of the male pheromone to virgin females of the papaya fruit fly *Toxotrypana curvicauda* [35]. In the corn earworm moth *Helicoverpa zea*, volatiles such as ethylene from ripe corns stimulate calling behaviors by females resulting in increased release of sex pheromones [36].
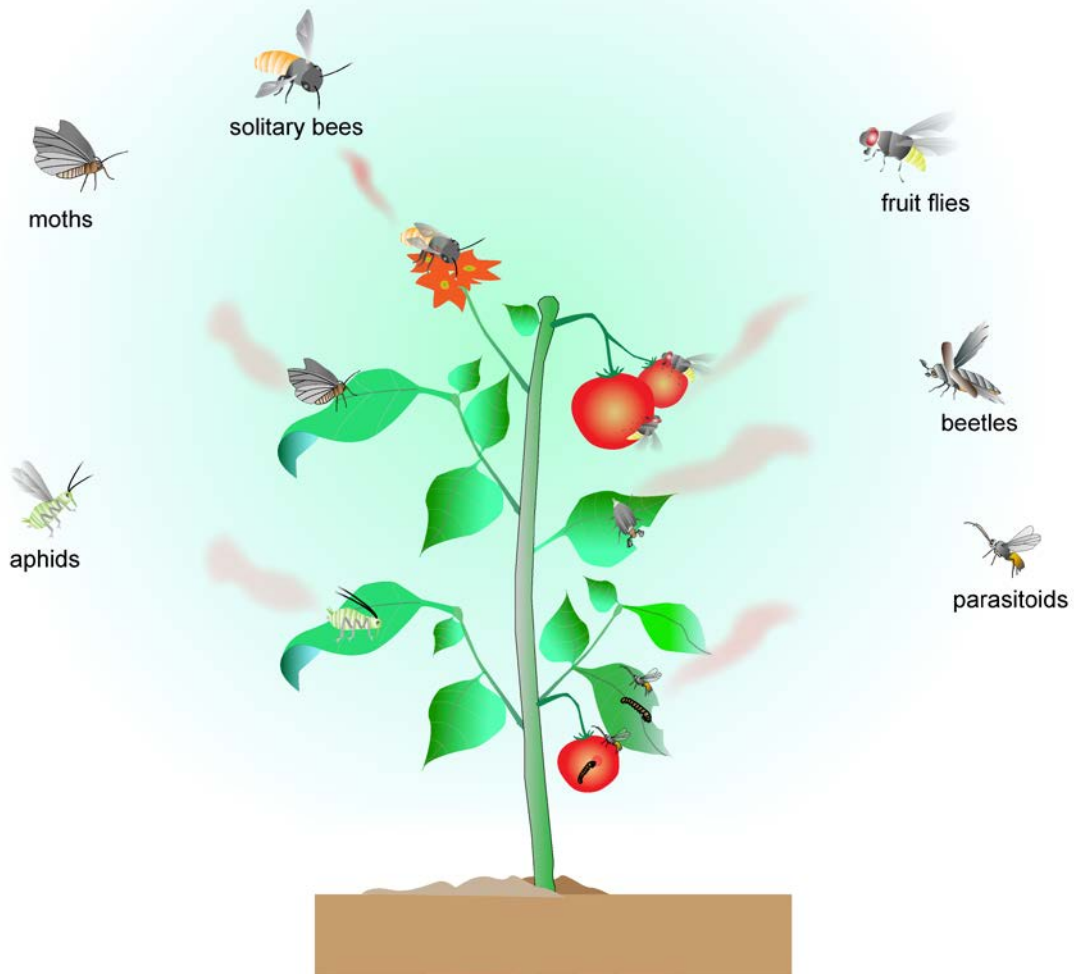

Fig. 1. Diverse insect species seek mate on different plant tissues (e.g. leaves, fruits and flowers). Plant volatiles (green background) of different plant tissues (the rendezvous sites) play important roles in mate location of insects. Insect pheromones are presented as red plumes.

Flower volatiles

Flower scents not only attract pollinators, but also act as kairomones (benefiting receiver, but harmful to emitter) that attract florivores (flower-feeding herbivores) [37]. Mating by these insects often occurs on flowers, possibly because florivores and pollinators are stimulated by flower volatiles to release more pheromones and/or to be more receptive. For example, females of the sunflower moth *Homoeosoma electellum* show increased calling behaviors and produced more mature eggs when they are exposed to the pollen volatiles [38]. Nocturnal plant bugs of the genus *Neella* (Hemiptera: Miridae) located Araceae flower pollens as food with the flower scent, *cis*-jasmone, and mating by these species frequently happened on the flower [39]. The flower volatile 1,4-dimethoxybenzene synergistically increases the attractiveness of the aggregation pheromone of the strawberry blossom weevil *Anthonomus rubi* in the field [40]. A mixture of a flower volatile compound, methyl phenylacetate, and male pheromones caught more females of longhorn beetle *Anaglyptus subfasciatus* than did male pheromones or the flower volatile alone in the field [41]. Virgin males of solitary bee species patrol around the flowers of their host plants, where they are likely to encounter females in the process of collecting pollens. Both flower volatiles and flower color possibly played a role in attracting the males [42,43]. All in all, it seems that phytophagous insects generally do not emit high doses of pheromones or perform their full spectrum of mating behaviors until they have located a suitable host plant, allowing them to emit their pheromone blend in a background of host plant odors, a combination that is optimally attractive to mate-seeking conspecifics of the opposite sex.

Parasitoid wasps and herbivore-induced plant volatiles (HIPVs)

Parasitoid wasps use herbivore-induced plant volatiles (HIPVs) to find hosts [44,45]. Parasitoid mating systems have mainly received attentions in the context of rearing efforts of these important biological control agents. These studies have mainly focused on the use of pheromones in mate finding and acceptance [46]. However, for several parasitoid wasps, it appears that they do not release pheromones or only in very small amounts [47,48]. In other cases, the parasitoids produce pheromones of high molecular weights that work only at relatively short distances [46,49-51]. In these cases, the parasitoids may primarily rely on plant volatiles to first locate their hosts' host-plants as rendezvous places, where they then rely of pheromones for close-range mating behaviors. For example, we reported that both virgin males and virgin females of several braconid wasp species are strongly attracted to HIPVs [47]. Others found that males of *Cotesia plutellae* and *Campoletis sonorensis* more easily located conspecific females when the females were presented on a host-food plant

(damaged or intact) [52,53]. Also, mealybug-infested cassava plant have been shown to be attractive to both male and female *Apoanagyrus lopezi* [54], and both male and female wasps of *Cotesia marginiventris* are attracted to certain GLVs and HIPVs [55]. Both sexes of certain parasitoids may also be attracted to volatiles from fruits (infested or not) that may harbor their hosts. This is the case for the braconids *Psyttalia concolor* [56] and *Diachasma alloeum* [57], both parasitoids of larvae of tephritid. Hence, an increasing number of studies indicate that the use of plant volatiles in mate finding is not restricted to herbivorous insects.

Table 1. Examples of host plant volatiles assisting mate location in insects

order*	species	PVs/plant tissue#	pheromones (M/F/A)◊	st□	sy□	ad□	refs
Lep	*Helicoverpa zea*	ethylene (**fruit**) and linalool; (Z)-3-hexenol; (Z)-3-hexenyl acetate; corn silk volatiles	(Z)-11-hexadecenal; hexadecanal; (Z)-9-hexadecenal (F)	+	+		[23,36, 58-60]
	Cydia pomonella	GLVs; linalool; (E)-β-farnesene	(E,E)-8,10-dodecadien-1-ol (F)		+		[23,61, 62]
	Heliothis virescens	(Z)-3-hexenyl acetate; (E)-2-hexenyl acetate	(Z)-9-tetradecenal; (Z)-11-hexadecenal; (Z)-11-hexadecenol (F)	nt			[26,63]
	Spodoptera exigua	benzaldehyde; phenylacetaldehyde; (Z)-3-hexenyl acetate; linalool	(Z)-9-tetradecenyl acetate; (Z)-9-tetradecen-1-ol; (Z,E)-9,12-tetradecadienyl acetate (F)	nt			[24]
	Spodoptera littoralis	cotton plant	(Z,E)-9,11-tetradecadienyl acetate (F)			+	[64]
	Eupoecilia ambiguella	(Z)-3-hexen-1-ol; (+)-terpinen-4-ol ; (E)-β-caryophyllene; methyl salicylate	(Z)-9-dodecenyl acetate; dodecenyl acetate; octadecyl acetate (F)	nt			[11,65]
	Plutella xylostella	(Z)-3-hexenyl acetate	(Z)-11-hexadecenal; (Z)-11-hexadecenyl acetate (F)		+	+	[25]
	Lobesia botrana	(E)-β-caryophyllene; (Z)-3-hexenyl acetate; 1-hexanol ; 1-octen-3-ol	(E,Z)-7,9-dodecadienyl acetate; (E,Z)-7,9-dodecadien-ol; (Z)-9-dodecenyl acetate (F)	nt			[10,66]
	Homoeosoma electellum	**sunflower** pollen	nd (F)	+			[38]
	Trichoplusia ni	Cabbage and cotton plants	(Z)-7-dodecen-1-ol (F); (S)-(+)-linalool; p-cresol; m-cresol (M)	+		+	[28,29]
	Yponomeuta spp.	host plants	nd (F)	+			[67]
Coleo	*Melolontha hippocastani*	(Z)-3-hexen-1-ol	1,4-benzoquinone (F)			+	[19,21]
	Melolontha melolontha	(Z)-3-hexan-1-ol; (E)-2-hexen-1-ol; 1-hexanol	toluquinone (F)			+	[20,22]
	Anthonomus grandis	(E)-2-hexen-1-ol	Glandlure I, II, and III (A)‡		+		[16,17]
	Anthonomus rubi	1,4-dimethoxybenzene (**flower**)	Grandlure I, II and (±)-lavandulol (A)‡		+		[40]
	Leptinotarsa decemlineata	GLVs	(S)-3,7-dimethyl-2-oxo-6-octene-1,3-diol (A)	nt			[68,69]
	Scolytus multistriatus	hexanal; hexan-1-ol	α-multistriatin; methyl heptanol; α-cubebene (A)	nt			[17,70]
	Conotrachelus	benzaldehyde (**fruit**)	grandisoic acid (A)		+		[71]

	nenuphar						
	Callosobruchus maculatus	cowpea **seeds**	nd (F)	+			[72]
	Anaglyptus subfasciatus	methyl phenylacetate (**flower**)	(R)-3-hydroxy-2-hexanone; (R)-3-hydroxy-2-octanone (M)			+	[41,73]
	Rhynchophorus palmarum	ethyl acetate; isoamyl acetate	(E)-6-methyl-2-hepten-4-ol (A)	+	+		[13,15]
	Rhynchophorus ferrugineus	coconut palm tissue	4-methyl-5-nonanol (A)		+		[12]
	Rhynchophorus cruentatus	ethyl acetate	5-methyl-4-octanol (A)	nt			[14]
	Gnathotrichus spp.	α-pinene	(S)-(+)-sulcatol (A)		+		[74]
Di	*Ceratitis capitata*	(E)-2-hexenal; tea oil; ginger **root** oil	nd (M)	+			[17,75, 76]
	Anastrepha fraterculus	guava (**fruit**)	nd (M)	+			[33,34]
	Toxotrypana curvicauda	papaya **fruit** extract	2-methyl, 6-vinylpyrazine (M)	nt			[35,77]
	Bactrocera oleae	α-pinene (**fruit**)	1,7-dioxaspiro[5.5]undecane; methyl dodecanoate; α-pinene; nonanal (F) and (Z)-9-tricosene (M)	+			[31]
	Bactrocera dorsalis	host flower volatiles (flower)	nd (M)	+			[78]
Hemi	*Rhopalosiphum padi*	benzaldehyde; methyl salicylate	(1R,4aS,7S,7aR)-nepetalactol (F)		+		[27]
	Phorodon humuli	benzaldehyde; methyl salicylate	(1RS,4aR,7S, 7aS)-nepetalactol (F)		+		[27]
	Dysaphis plantaginea	hexyl butyrate; (E)-2-hexenyl butyrate; (Z)-3-hexenyl isovalerate; hexyl 2-methylbutyrate	(1R,4aS,7S,7aR)-nepetalactol; (4aS, 7S,7aR)-nepetalactone (F)		+		[79]
	Neella spp.	(Z)-jasmone (**flower**)	nd	nt			[39]
	Cacopsylla bidens	pear plant	nd (F)	nt			[80]
Hymen	*Nomia triangulifera*	**sunflower**	nd	nt			[42]
	Panurgus banksianus	catsear **flower**	nd	nt			[43]
	Panurgus calcaratus	catsear **flower**	nd	nt			[43]
	Cotesia plutellae	HIPVs	nd (F)	nt			[52]

	Species	#	◊ M/F/A	□			References
	Cotesia marginiventris	HIPVs	nd (F)			+	[47,49,55]
	Cotesia glomerata	HIPVs	nd (F)			+	[47,49]
	Microplitis rufiventris	HIPVs	nd (M)			+	[47]
	Microplitis mediator	HIPVs	nd (A)			+	[47,81]
	Campoletis sonorensis	intact leaf volatiles	nd (F)	nt			[53]
	Psyttalia concolor	host-induced **fruit** volatiles	nd (F)	nt			[56]
	Apoanagyrus lopezi	HPIVs	nd (F)	nt			[54]
	Diachasma alloeum	host **fruit** odours	nd (F)	nt			[57]
	Antistrophus rufus	dry plant **stem**	nd	nt			[82]

"*": different orders of insects: "Lep": Lepidoptera; "Coleo": Coleoptera; "Di": "Diptera"; "Hemi": Hemiptera; "Hymen": Hymenoptera. "#": "PVs", plant volatiles; "GLVs", green leaf volatiles; the sources of plant volatiles are shown in bold characters unless they are emitted by plant leaves. "◊ M/F/A": the pheromonal compounds are emitted as sex pheromones by males (M) or females (F), or to work for aggregations (A). "‡": Glandlure I is racemic grandisol and (Z)-2-isopropenyl-1-methylcyclo-butaneethanol; Glandlure II is (Z)-3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexaneethanol and Glandlure III is 3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde. "□": the working manners of plant volatiles in insect mating location, "st": stimulation; "sy": synergism; "ad": addition. "nd": no data. "nt": not tested.

3. Plant volatiles affecting pheromone production and signaling

Stimulation

Several phytophagous insects only start to release pheromones or show mating behaviours when they perceive the odour of a host plant. For example, males of the palm weevil *Rhynchophorus palmarum* release an aggregation pheromone when they are exposed to the host plant volatile, ethyl acetate [15]. Females of cowpea weevil *Callosobruchus maculatus* increase the release of pheromones in the presence of host plant seeds [72]. Males of some tephritid fly species (Diptera: Tephritidae) are more inclined to mate after they have been exposed to host plant volatiles [30-34,76,78,83]. The same is true for various species of Lepidoptera. Female sunflower moths, *Homoeosoma electellum*, when exposed to sunflower pollen extracts, increase their typical calling behaviors [38]. Both males and females of cabbage looper moth, *Trichoplusia ni,* appear to increase their sex pheromone production when exposed to cabbage or cotton plants [28,29], suggesting that this generalist moth first locates a host plant before engaging in mating behaviors. A similar phenomenon was found for four species of small ermine *Yponomeuta* moths, of which females began to release pheromones when they had settled on their respective host-plant species [67]. Females of the corn earworm *Helicoverpa zea* delay their reproductive behaviors and pheromone release until they have found a ripening corn cob on which to deposit their eggs [36]. These resource-based mating strategies by phytophagous insects effectively combine the search for a suitable host plant for oviposition with a reliable rendezvous site for mating [6].

Synergisms

Those host-plant volatiles synergistically increasing the attractiveness of pheromones is most evident from integrated pest control (IPM) research. It is frequently shown that traps with a combination of pheromones and plant volatiles are more efficient at catching pests than traps with only pheromones, whereas traps with only plant volatiles are not significantly attractive. The phenomenon is known for various agricultural pests, ranging from Hemiptera, Coleoptera, to Lepidoptera, (Table 1) [12,13,16,23,25,27,40,71,74,79,84]. Several of these insects typically emit pheromones when they are perched on host-plant fruits, flowers, or leaves, which will result in a much larger combined odor plume [30,85-87]. As opposed to the broad plume that is produced by an entire plant, the pheromone originates from a point source (a pheromone gland) and this combination may be ideally suited for a stepwise location of a mate, as explained below (Fig. 2b).

Additive effects

Unlike the above synergism examples, for certain insect species plant volatiles by themselves are already strongly attractive to male and/or female herbivores (Fig. 2b), and the increase in attractiveness by combining plant volatiles and pheromones is not more than the combined attractiveness of each element alone. This is expected and regularly found for females of phytophagous insects (e.g. [29,41]), probably because females (even virgin females [29]) use host-plant volatiles to locate oviposition sites [7]. Virgin males of the African cotton leafworm *Spodoptera littoralis* have been found to be strongly attracted to cotton plant volatiles, as well as to virgin females in wind tunnel assays. The attraction of the cotton plant volatiles dramatically decreased after males have mated, which indicated that the initial attraction mainly serves to find females (Fig. 2b) [64]. Male cockchafers are more attracted to GLVs, which typically released upon feeding by female cockchafers, than to female pheromones in the field. It seems that GLVs act as long-range attractants for them, whereas the pheromones are used to locate females at a relative short range (Fig. 1) [19,20].

The responses to plant volatiles may be context dependent and they can have different effects on mate location in different environments. For example, in a wind tunnel assay, males of the diamondback moth *Plutella xylostella* were found to be strongly attracted by the GLV (*Z*)-3-hexenyl acetate. Yet, in a field investigation, traps with only (*Z*)-3-hexenyl acetate did not attract males, but a mixture of this volatile and the female pheromone caught more males than did the female pheromone alone [25]. Since GLVs are ubiquitously released by most plant species and given that the diamondback moth, lays eggs on a diverse range of plant species within the family Brassicaceae, its strategy to combine GLVs and pheromone as foraging cues may be an efficient way to pinpoint the location of females in a background of plant odors. However, it can be expected that only the female pheromone arrests males on a plant (Fig. 2b).

More evidences for an additive effect were reported in the context of parasitoid foraging. Virgin braconid parasitoids of both sexes have been found to be strongly attracted by HIPVs [47], and for some solitary parasitoid species it appears that they even do not release a long-range pheromonal signal (e.g. [47,48]). Given that the HIPVs are generally released in high quantities and with a host-related specificity [2,88], they should provide some parasitoid species with highly reliable information on where to find mates (Fig. 2c) [47,88]. For different types of insects (e.g. parasitoids, herbivores, pollinators etc.), the additive effect of pheromones can be of great importance. The pheromones that typically have larger molecular weights and higher boiling points (Suppl. table 1) are easily absorbed onto plant tissues or may function as contact cues that are deposited on plants by the emitters (e.g.

[50,89-91]). A deposited or absorbed pheromones on a host plant may act as slowly released territorial markers [7], and by first foraging with the use of plant-produced signals, the finding of the pheromonal cues should be greatly facilitated.

Synergism or additive effect possibly depends on the specificity of a plant volatile blend

The specific information that is provided by plant volatiles has received little attention in the context of mate finding. A volatile blend of a host plant (for herbivores) or a host-food plant (for parasitoids) that does not have a strong specific profile is more likely to work synergistically, whereby a plume consisting of both a volatile pheromone and plant volatiles is needed for insects to locate mates at a distance. GLVs may be such general indicators of the location of plant communities. Their release increases in most plant species when they are under biotic or abiotic stresses [4,92,93], and GLVs are known to synergistically act in mate location by some moth species in field investigations [23,25,60]. In contrast, a more specific volatile blends that includes HIPVs that are specifically induced by insect feeding, possibly attract mate-seeking insects such as aggregating herbivores and parasitic wasps much more strongly, without the need of a pheromone [47,88]. The high specificity sometimes also involves GLVs, as herbivory can strongly manipulate the composition of GLVs and therefore can provide specific information to adapted insects [94,95].
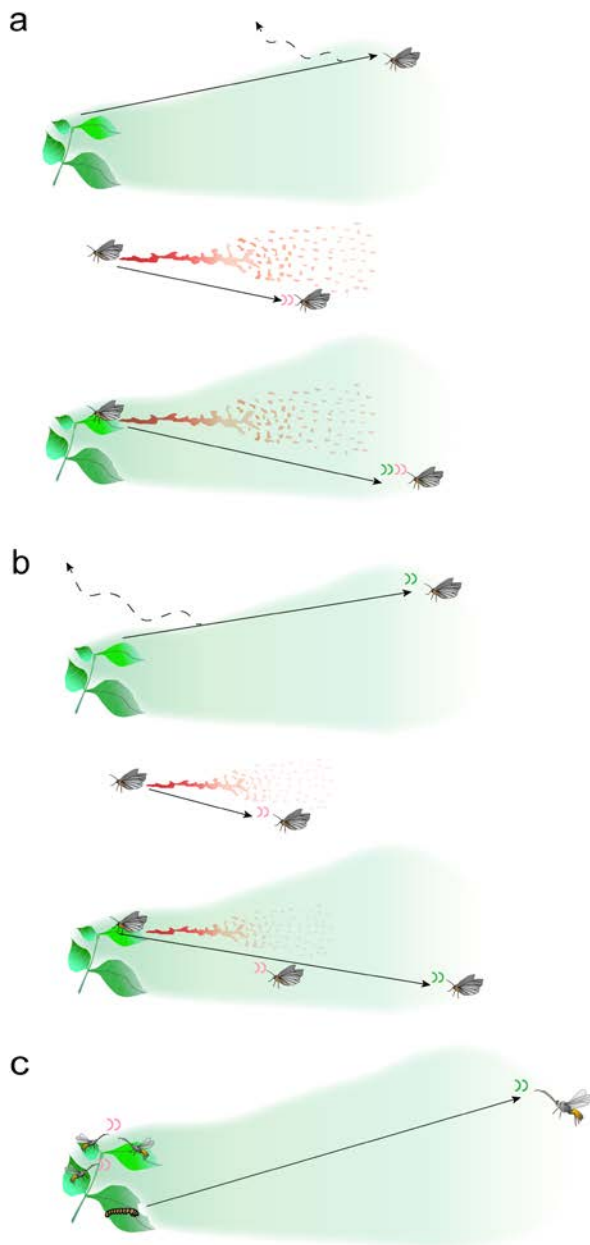
Fig. 2. Synergistic (a) and additional (b and c) effects of plant volatiles in mating location of insects. (a) A mate-seeking insect is not significantly attracted by plant volatiles (green, the same below), but it is strongly attracted by the pheromone (red, the same below). When the pheromone is released in a background of plant volatiles, the attractiveness of the mixed plume increases (e.g. [23,60]). (b) Both plant volatiles and pheromone are strongly attractive to a mate-seeking insect, but in a short range, only conspecific pheromone strongly attracts the insect to land on the plant (e.g. [25]). (c) Some insects (e.g. parasitic wasps) do not release a strong pheromone and thus they probably use plant volatiles (e.g. HIPVs) to locate mate (e.g. [47]). The solid line arrows illustrate the work range of attractants. The dash line arrows indicate the insects are not strongly arrested by the volatiles.

4. Differential roles for plant volatiles and insect pheromones in mate-seeking processes: specificity and functional distance

Specificity

Plant volatiles are mainly non-specific components (e.g. GLVs and terpenes) that are emitted by numerous plant species in a vegetation community, whereas pheromones are typically highly specific. For example, the attraction of male cockchafers to GLVs such as (Z)-3-hexan-1-ol can lead them to plants that are damaged by a conspecific females, but also to plants with a female of another cockchafer species, or an entirely different herbivore [19-22]. The males will have to rely on a female-produced sex pheromone to guide them to the right place and help them to avoid mistakes. Similarly, non-specific volatile compounds of *Prunus* spp. plants synergistically increased attractiveness of the female pheromones of different aphid species to conspecific males in the field, but they alone did not significantly attract male aphids [27]. It is also important to note that different parasitoid species (also closely related ones) may parasitize the same host species and possibly host-damaged plants are attractive to males and females of these species at the same time [47]. To identify conspecifics of the opposite sex on these it is essential that they can use more specific cues, such as pheromones, but perhaps also visual and vibrational cues.

Working distances of odour plumes

We propose that tracking plant volatile plume and a pheromone plume will be quite different. Plant volatiles, especially those from damaged plants or from specific organs such as ripe fruits or flowers, are emitted continuously and in a relatively high quantity. In contrast, insect pheromones were released in low quantities and mostly only at a specific time when the emitter is ready to mate [96,97]. In addition, the two types of signals differ in volatility, the boiling points of the plant compounds are normally lower than the boiling points of typical pheromones (Fig. 3a). This difference also implies that some pheromonal compounds that are deposited or absorbed onto vegetation surfaces mostly work in short-range and as contact cues, and thereby operate in a relatively longer period [7]. Because of these differences, plant volatiles should attract insects over a longer range, and pheromones are more likely to help guide the insects over a relatively short distance.

An odor plume, composed of repeated and spatial distributed filaments, is formed at the moment the volatiles are emanating from a source [96-98]. Insects need to be able to derive useful information from the plumes in a highly dynamic environment [4,96,98]. Different factors, such as turbulence, temperature, light, humidity and vegetation, all affect

the structure of a plume [98]. The common assumption that the ratios among different compounds in a plume (volatile profile) is retained away from the source [98,99] has recently been challenged. For instance, different compounds released from *Datura wrightii* flowers such as aromatics and terpenes travel at different speeds, with aromatics meandering faster than terpenes in a plume (illustrated in Fig. 3b) [97]. Given the great diversity of volatiles from different chemical groups in nature, it is important to consider these novel insights into plume structures if we wish to fully understand how an odor plume carries complex information to insects. For this, it is imperative to study the physical and chemical characters of the signal chemicals (e.g. [84,85]), and to determine how components with different characteristics (e.g. vapor pressures and structures) move away from a source in nature. Plant volatiles of high volatility, such as GLVs and some aromatic components, can be expected to travel more rapidly and over longer distances than most insect pheromone compounds (Fig. 3a). The ratios among other informative volatiles probably remain unchanged in a plume when the compounds, such as GLVs, or isomerically similar pheromones (e.g. [73,100]), have similar structures and characteristics (illustrated in Fig. 3b), which is essential to transmit specific information to insects [99].

The habitat (background) odour theory, used to explain host location of insects [4,96,99], applies to the combined use of plant volatiles and pheromone for mate location. Very common volatiles can contribute to a background that is reliably associated with a particular resource and thus should readily facilitate resource location. For example, CO_2 is a typical indicator of plant roots for soil dwelling insects or of animal hosts for blood-feeding insects [4]. GLVs point out vegetation for herbivores. At closer range, the odor of certain host plant species may help insects to pinpoint the location of such plants [4]. The examples and concepts reviewed in this paper further support and contribute to this theory of the background odor.
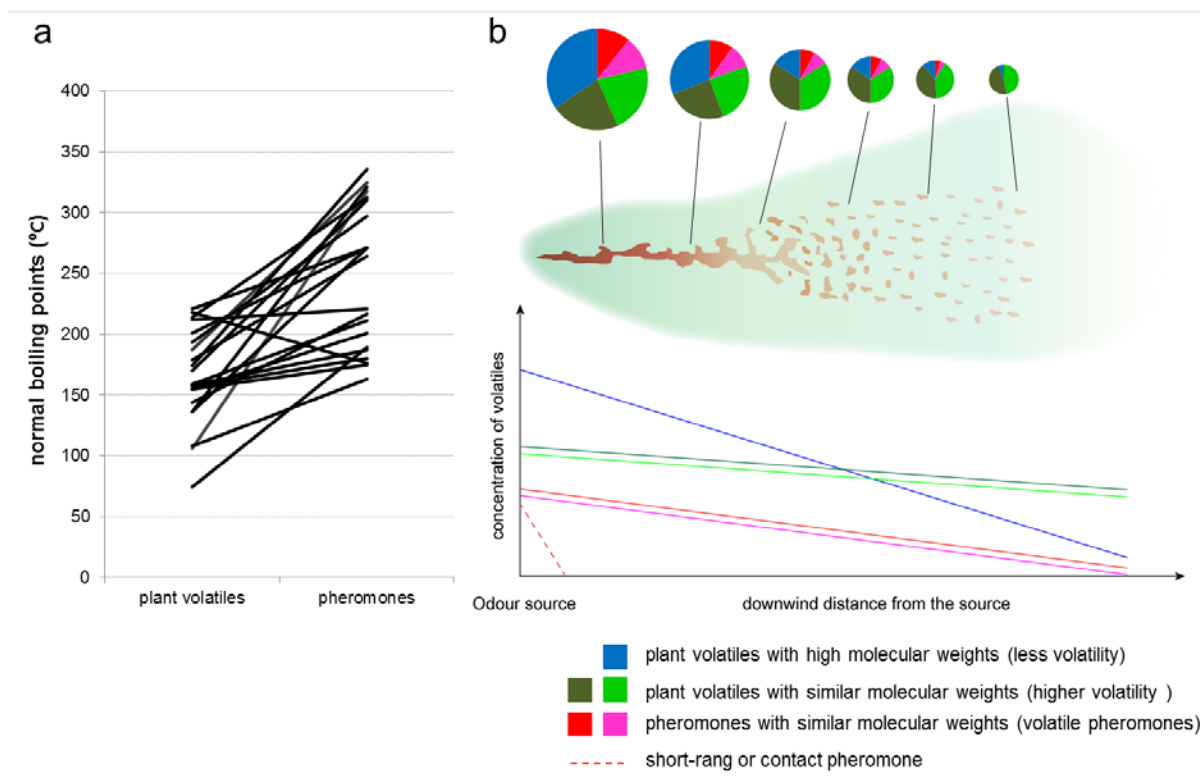
Fig. 3. (a) The boiling points (in average, data in Suppl. table 1) of plant volatiles that help insects find mate and the relevant pheromone of each species. (b) Generally, plant volatiles (the green plume, and blue, dark green, bright green in the pie and line graphs illustrating different components) are emitted in a higher quantity than pheromone (the red plume, and red, pink in pie and line graphs). The concentrations of volatile compounds usually decrease in a plume flowing direction (the size of pie charts and the Y-axis in the line graph) [96]. The illustration on how plumes flow in nature is presented: different volatile components meander with different speeds, and the concentration of blue compound(s) (e.g. terpenes) decreases faster than dark and light green compounds (e.g. aromatics) in a plume flowing direction [97], but the some components of volatile compounds (e.g. some GLVs components) or pheromones (e.g. isomers), which probably retain a specific ratio (1:1 as an example in the pie and line graphs) in a plume due to the similar characters and structures (e.g. boiling points in Suppl. table 1). The ratio is important for insects to detect both pheromone blend and plant volatile blend [99,100]. Some insect pheromones mainly act as contact cues and are not strongly evaporating (red dash line in the line graph, e.g. parasitoid species [47]).

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5. Foraging mate and host plants are closely related

Most studies have treated food (host) and mate location as two exclusive processes (e.g. [96,101,102]). However, it is increasingly evident from various studies on parasitic wasps and phytophygous pests that we should reconsider this distinction. For example, parasitoid females may release sex pheromones to attract males while they forage for hosts [103]. This is further substantiated by the fact that virgin males and virgin females of certain parasitoid species are strongly attracted by HIPVs [47]. and by the fact that males and females of some phytophygous insects are stimulated by host plant volatiles to release more pheromone or be more receptivity to mating [28-36,38,67,76,78,83]. These behaviors all point at a strategy whereby the insects first locate a host plant where mating is most likely to takes place. To accomplish this they may engage in hierarchical plume switches by first fly upwind after detecting plant volatiles and abandon the such long-rang cues upon the detection and in favor of a plume carrying the sex pheromone (e.g. mate) [4,96]. For example, virgin males of *Cotesia* parasitoids are strongly attracted by HIPVs, but when the odors of virgin females and a host-damaged plant are offered at the same time, the males are predominantly attracted to the virgin females [47]. By integrating the two foraging behaviors together, insects possibly abandoned to synthesize and release some long-range pheromones which are extremely costly for them and possibly replaced by plant volatiles [47].

6. Concluding remarks and future perspectives

An important role of plant volatiles in mate location by insects is to be expected because of the close interactions between plants and many insect species, making plants ideal rendezvous sites for these insects. Volatiles released from different plant tissues, e.g. leaves, flowers or fruits, can affect and stimulate production and release of sex pheromone. In addition, the plant-provided airborne signals provide spatial information on the presence of plants in the habitat and where it is most likely to find mates. Because of their relatively large quantities and higher vapor pressures compared to typical pheromones, plant volatiles are expected to mainly serve as a long-range attractant, whereas we expect that pheromones be used at close-range to confirm the presence of a mate and to determine its precise location. Alternatively, volatiles of specific plants may also provide a consistent background odor, allowing for easier detection of pheromones than when the pheromones are released in unfamiliar habitats with more variable background odors. This review supports and expands on this background odor theory, which was originally used to explain host location of insects [4,96,99]. Locating mate and food (hosts) are two distinct but close-related key steps in insects, and combining them as part of the same behavioral process may have evolved as an efficient foraging strategy. Studying the different physical and chemical properties of odor blends released by plants and insects may further help us to understand their relative contribution to plume structures and how they provide spatial information to foraging insects.

Acknowledgement

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Suppl. table

Suppl. table 1. The normal boiling points of plant volatiles assisting mate location and pheromones of insect species.

Order*	species	plant volatiles (°C)	Mean (°C)	pheromones (°C)	Mean (°C)
Lep	*Helicoverpa zea*	ethylene; -104	106	(*Z*)-11-hexadecenal; 324	318
		linalool; 198		hexadecanal; 298	
		(*Z*)-3-hexenol; 157		(*Z*)-9-hexadecenal; 331	
		(*Z*)-3-hexenyl acetate; 174			
	Cydia pomonella	GLVs; 136[#]	136	(*E,E*)-8,10-dodecadien-1-ol; 271	271
	Heliothis virescens	(*Z*)-3-hexenyl acetate; 174	170	(*Z*)-9-tretradecenal; 297	310
		(*E*)-2-hexenyl acetate; 166		(*Z*)-11-hexadecenal; 324	
				(*Z*)-11-hexadecenol; 309	
	Spodoptera exigua	benzaldehyde; 179	187	(*Z*)-9-tetradecenyl acetate; 334	325
		linalool; 198		(*Z*)-9-tetradecen-1-ol; 307	
		phenylacetaldehyde; 198		(*Z,E*)-9,12-tetradecadienyl acetate; 335	
		(*Z*)-3-hexenyl acetate; 174			
	Eupoecilia ambiguella	(*Z*)-3-hexen-1-ol; 157	214	(*Z*)-9-dodecenyl acetate; 300	312
		(+)-terpinen-4-ol; 209		dodecenyl acetate; 291	
		(*E*)-β-caryophyllene; 268		octadecyl acetate; 344	
		methyl salicylate; 222			
	Plutella xylostella	(*Z*)-3-hexenyl acetate; 174	174	(*Z*)-11-hexadecenal; 324	336
				(*Z*)-11-hexadecenyl acetate; 348	
	Lobesia botrana	(*E*)-β-caryophyllene; 268	193	(*E,Z*)-7,9-dodecadienyl acetate; 310	297
		(*Z*)-3-hexenyl acetate; 174		(*E,Z*)-7,9-dodecadien-ol; 281	
		1-hexanol; 157		(*Z*)-9-dodecenyl acetate; 300	
		1-octen-3-ol; 174			
Coleo	*Melolontha hippocastani*	(*Z*)-3-hexen-1-ol; 157	157	1,4-benzoquinone; 180	180
	Melolontha melolontha	(*Z*)-3-hexan-1-ol; 158	158	toluquinone; 187	187
		(*E*)-2-hexen-1-ol; 159			
		1-hexanol; 157			
	Anthonomus grandis	(*E*)-2-hexen-1-ol; 159	159	Glandlure I; 211[◊]	211

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				Glandlure II; nd◊	
				Glandlure III; nd◊	
	Anthonomus rubi	1,4-dimethoxybenzene; 212	212	Grandlure I; 211◊	221
				Glandlure II; nd◊	
				(±)-lavandulol; 230	
	Leptinotarsa decemlineata	GLVs; 136#	136	(*S*)-3,7-dimethyl-2-oxo-6-octene-1,3-diol; 321	321
	Scolytus multistriatus	hexanal; 131	144	*α*-multistriatin; 207	217
		hexan-1-ol; 157		methyl heptanol; 178	
				α-cubebene; 266	
	Conotrachelus nenuphar	benzaldehyde; 179	179	grandisoic acid; 264	264
	Anaglyptus subfasciatus	methyl phenylacetate; 218	218	(*R*)-3-hydroxy-2-hexanone; 164	176
				(*R*)-3-hydroxy-2-octanone; 188	
	Rhynchophorus palmarum	ethyl acetate; 74	108	(*E*)-6-methyl-2-hepten-4-ol; 163	163
		isoamyl acetate; 142			
	Rhynchophorus cruentatus	ethyl acetate; 74	74	5-methyl-4-octanol; 189	189
	Gnathotrichus spp.	*α*-pinene; 155	155	(*S*)-(+)-sulcatol; 175	175
Di.	*Bactrocera oleae*	*α*-pinene; 155	155	1,7-dioxaspiro[5.5]undecane; 194	201
				nonanal; 191	
				methyl dodecanoate; 263	
				α-pinene; 155	
				(*Z*)-9-tricosene; 399	
Hemi	*Rhopalosiphum padi*	benzaldehyde; 179	201	(1*R*,4a*S*,7*S*,7a*R*)-nepetalactol; 271	271
		methyl salicylate; 222			
	Phorodon humuli	benzaldehyde; 179	201	(1*RS*,4a*R*,7*S*, 7a*S*)-nepetalactol; 271	271
		methyl salicylate; 222			
	Dysaphis plantaginea	hexyl butyrate; 207	221	(1*R*,4a*S*,7*S*,7a*R*)-nepetalactol; 271	271
		(*Z*)-3-hexenyl isovalerate; 232			
		hexyl 2-methylbutyrate; 214		(4a*S*, 7*S*,7a*R*)-nepetalactone; 271	
		(*E*)-2-hexenyl butyrate; 217			

The normal boiling points are predicted by Chemspider database (ACD/labs) (http://www.chemspider.com/). "*" different orders of insects: "Lep", Lepidoptera; "Coleo", Coleoptera; "Di", Diptera; "Hemi", Hemiptera. "#" GLVs, green leaf volatiles; the normal

boiling point of GLVs (136 °C) is the average of the normal boiling points of the components: n-hexanal (131 °C), n-hexanol (157 °C), (*E*)-2-hexenal (38 °C), (*E*)-2-hexenol (159 °C), (*E*)-3-hexenal (126 °C), (*E*)-3-hexenol (157 °C), (*Z*)-3-hexenal (127 °C), (*Z*)-3-hexenol (157 °C), (*Z*)-3-hexenyl acetate (172 °C). "◊" Glandlure I: racemic grandisol (210 °C), (*Z*)-2-isopropenyl-1-methylcyclo-butaneethanol (nd); II, (*Z*)-3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexaneethanol (nd) and III, 3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde (nd). nd: no data.

CONCLUSIONS and OUTLOOKS

The pheromone use of braconid parasitoids varies from species to species

Each species showed attraction to pheromones, but in somewhat different ways. Males of the two *Cotesia* species were attracted to virgin females (Chapter 2), whereas females of *M. rufiventris* were attracted to virgin males (Chapter 5). Males and females of *M. mediator* exhibited attraction to both sexes (Chapter 5). The cuticular aldehyde heptanal is an anti-aphrodisiac for *C. glomerata*, but not for *C. marginiventris* (Chapter 3 and 4). All sections (head, thorax and abdomen) of *C. marginiventris* females are attractive to conspecific males, suggesting the pheromone compounds are probably emitted by female cuticle (Chapter 4). However, in *C. glomerata*, only the extract of female abdomens, but heads or thoraxes, is attractive to males, indicating the sex pheromone is probably produced by a gland located on abdomen (Chapter 3). The strongly attractive components of sex pheromones in both *Cotesia* species have relatively big retention times in chromatographic analyses (GC-EAD and GC-PFC), supporting the pheromone components are possibly with high molecular sizes (Chapter 3 and 4).

The pheromones of two Cotesia *parasitoids*

The gregarious parasitoid *C. glomerata* has an exceptional pheromone system (Chapter 3): Males normally emerge a bit earlier than sibling females on natal patches. Virgin females, even at their emerging stages, are attractive to new-emerged males with female sex pheromones. A male probably uses the anti-aphrodisiac heptanal to repel other males when he is waiting an emerging female. Therefore, males are able to mate successfully on a natal patch with attraction of emerging females and repellence to other males. This pheromone controls possibly alter the sibling mating structure when different sex ratio of offspring (range from 25-67%, percentage of males) is produced. When the offspring is female-biased (25%), more sibling males are probably attracted to stay at natal patches and involve in inbreeding. In contrast, a male-biased offspring (e.g. 67%) probably drives more males to disperse from natal patches with a high concentration of heptanal released by other males and mate with ones from other colonies (Chapter 3).

Interestingly, heptanal synergistically increases the attractiveness of other components of sex pheromones in the solitary species *C. marginiventris*. For both *Cotesia* species, some nonspecific compounds synergistically enhance the attractiveness of the main constituents of

the sex pheromones of each species. The specificity of sex pheromones is probably retained by two specific compounds which evoke biggest electroantennographic (EAG) responses of male antennae of respective species. Therefore, we reveal that certain cuticular lipids have a strong flexibility to evolve into distinct pheromonal functions according to the specific habitat of each species.

Alternative cues, such as plant volatiles, direct mate-seeking insects.

When insects try to locate a mating partner, they frequently use both specific pheromones and other volatile cues (such as host-associated volatiles). For example, the solitary parasitoid *M. mediator* does not use a highly volatile pheromone, but both virgin females and males are robustly attracted by host-damaged plants which probably act as rendezvous sites for the mating-seeking individuals. The result remains similar with other tested parasitoid species: the gregarious parasitoid *C. glomerata*, and the solitary parasitoid species *C. marginiventris*, *M. rufiventris*, even though they indeed use volatile sex pheromones. When males of both *Cotesia* species detect host-damaged plants and conspecific females (pheromone source) at the same time, they are dominantly attracted by the sex pheromones. However, the females of *M. rufiventris* are attracted by the combination of males (pheromone source) and host-damaged plants more than by each element alone. All together, these tests indicate that braconid wasps use the pheromones and host-damaged plant volatiles in combination for mate location (Chapter 5).

Plant volatiles not only direct parasitoids, but also herbivorous insects and pollinators to find mate. Different roles of insect pheromones and relevant plant volatiles could be expected after studying on the chemical property of these compounds (such as volatility) and recent theoretical and experimental advances of odour plume. Plants generally emit a bouquet of volatiles which are with low molecular weights, lipophilic character and high vapour pressures, and easily meander a few meters away from the source in nature environment. In contrast, insect sex pheromones are normally consisted with relatively higher molecular weights and are only released under a certain occasion when the emitters are ready to mate. Therefore, plant volatiles more likely play as background odour in which locating mate is much easier than only with pheromones for many insect species, whereas the pheromone is a high specific indication of mating partners in a relatively short range (Chapter 6).

Future works

Identification of sex pheromones of two *Cotesia* species needs more effort. In the GC-PFC and GC-EAD analyses, we found that the both *Cotesia* species had a specific component of sex pheromone. But when we tried to identify the specific pheromone compound of each species, its quantity seemed to be small and they were failed to be identified. For the *C. glomerata*, the specific sex pheromone component was difficult to separate from other compounds in our chromatographic analyses. Therefore, a higher concentrated sample needs to be collected, and other techniques of chromatographic analysis, such as selecting specific irons, should be applied.

The gregarious parasitoid *C. glomerata* has evolved divergently many times from the solitary ancestor in the genus *Cotesia*, and I hypothesize that the cuticular aldehyde heptanal is possibly an important factor. When the foundresses tried to produce many progenies with one host caterpillar, the deadly mate competition among offspring will not happen due to the anti-aphrodisiac compound. Therefore, whether an anti-aphrodisiac compound is commonly used by other gregarious *Cotesia* species and whether the compound possibly supports the gregarious parasitoids in *Cotesia* genus to evolve is an interesting topic to study.

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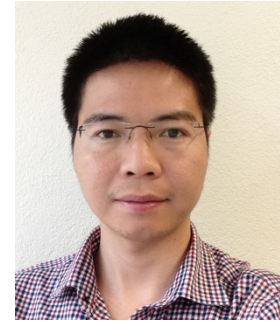# CURRICULUM VITAE

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Publications:

1. **Xu, H.**, Desurmont, G., Degen, T., Zhou, G., Laplanche, D., Henryk, L., & Turlings, T. C. (2016). Combined use of herbivore-induced plant volatiles and sex pheromones for mate location in braconid parasitoids. *Plant, Cell & Environment* (cover story).

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3. Erb, M., Veyrat, N., Robert, C. A., **Xu, H.**, Frey, M., Ton, J., & Turlings, T. C. (2015). Indole is an essential herbivore-induced volatile priming signal in maize. *Nature communications*, 6.

4. Hu, G. L., Yan, G., **Xu, H.**, & Hua, B. Z. (2015). Molecular phylogeny of Panorpidae (Insecta: Mecoptera) based on mitochondrial and nuclear genes. *Molecular phylogenetics and evolution*, 85, 22-31.

5. **Xu, H.**; Veyrat, N.; Degen, T.; & Turlings, T.C.J. (2014). Exceptional use of sex pheromones by parasitoids of the genus *Cotesia*: males are strongly attracted to virgin females, but are no longer attracted to or even repelled by mated females. *Insects*, 5, 499-512.