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Antennae and the role of olfaction and contact stimulation in mate recognition by males of the pollinating fig wasp *Ceratosolen graveleyi* (Hymenoptera: Agaonidae)

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## KEYWORDS

Mating behaviour; olfactory cues; contact chemicals; cuticular hydrocarbon; chemoreceptor

## Abstract

Finding a mate is a crucial step in sexual reproduction. The mutualism between figs and their pollinators is exploited by many figwasps that develop within their galls and attain sexual maturity simultaneously. Male pollinators carry the specific burden of detecting and opening mating holes in galls containing conspecific females before mating with them. The mating behaviour and antennal sensilla of male pollinators associated with *Ficus semicordata* was investigated to understand mate recognition. Male *Ceratosolen graveleyi* located female-containing galls from a distance, but only attempted to chew a mating hole after antennal contact with the gall surface was made. They showed similar responses to females and body extracts but failed to respond to washed female bodies. This behaviour indicates that unidentified chemicals present on the body surfaces of females are sufficient to elicit olfactory attraction and tactile confirmation. Multiporous plate sensilla are candidates for olfactory reception from a distance while basiconic sensilla may be involved in contact chemoreceptor. All of the sensilla are highly localised, on the distal part of the terminal flagellomere, suggesting a response to selection for strong directionality in the complex odour environment of the fig in which messages produced by hundreds of females may confuse precise mate localisation

## Introduction

A set of signals (e.g. pheromones, coloration, displays) is attractive to the opposite sex, and is thus involved in mating pair formation. Several studies suggest that sympatric taxa show stronger mating discrimination than do allopatric taxa of the same genetic distance (Butlin 1987; Noor 1999; Ortiz-Barrientos et al. 2004; Lukhtanov et al. 2005). This enhanced species discrimination in sympatry appears to be the result of natural selection for pre-zygotic reproductive isolation, such that females preferentially mate with conspecific males in many species (Noor 1999; Lukhtanov et al. 2005; Langerhans and Makowicz 2013). This pattern is typically beneficial to both sexes. Females that mate preferentially with their own species can produce more offspring than those that mate at random. Males also benefit from mating preferentially with conspecific females rather than investing time and resources on heterospecifics. Hence, selection for non-random mating will favour the evolution and maintenance of species isolation among sympatric species (Noor 1999; Smadja and Butlin 2008; Langerhans and Makowicz 2013). This is particularly true for taxa that breed simultaneously on the same site. Fig wasps constitute such an example as sometimes thousands

of individuals from multiple species occur within a single breeding arena constituted by a fig (Weiblen 2002; Kjellberg et al. 2005b; Krishnan et al. 2014).

Fig wasps are hymenoptera that only develop inside the urn-shaped inflorescences of fig trees (called figs or syconia). Fig wasp species can be divided into two functional groups. One group consists of mutualistic pollinators that are specifically responsible for the pollination of fig trees: the fig trees completely depend on the pollinators for pollen dispersal between figs. The species-specific mutualism between the pollinators and fig trees is generally obligatory interaction, particularly in local region, although some exceptional cases, such as a fig tree hosting two (or more) pollinators and several pollinators sharing a fig, have been documented in recent years (Bain et al. 2016; Sutton et al. 2017). Pollinating fig wasps are all members of the chalcidoid family Agaonidae and have similar life cycles (Weiblen 2002; Kjellberg et al. 2005b). The other group includes parasitic non-pollinators that provide no apparent benefit to their associated fig species. They include gall inducing wasps, cleptoparasites and parasitoids. Some studies have shown that most non-pollinating fig wasp species are each associated with a single fig species, whereas a single fig species typically hosts multiple non-pollinator wasp species (Weiblen 2002; Cook and Rasplus 2003). Non-pollinating fig wasp species belong to several chalcidoid families and show a diversity of life cycles. Nevertheless, regardless of the species of parasite that oviposits inside the fig, the offspring of both pollinators and non-pollinators attain sexual maturity simultaneously in the cavity of the enclosed, urn-like fig. In pollinating wasps and some parasitic lineages, males emerge from their galls before females and mate with females still enclosed in their natal galls, while in other non-pollinator fig wasps, matings occur in the fig cavity or even outside figs. In such a close-knit nursery system, mate discrimination is a crucial step for the lifecycle of each species and finding females can be challenging. Efficient mechanisms facilitating sexual recognition within species are of particular importance in such systems. Indeed, in fig wasps, the wingless and shortlived (24–48 h) (Ghara and Borges 2010) males have to rapidly and accurately locate the conspecific female-containing galls. The signals and cues involved in mate discrimination in fig wasps are probably primarily chemical as locating receptive females probably involves an olfactory-guided approach associated with contact confirmation. Indeed, male fig wasps live in the restricted, dark cavity of the fig and have a pair of reduced eyes; therefore, unlike males of many other insect species, male fig wasps do not rely on visual cues to find potential mates in close proximity and they have to gnaw a hole into galls to reach the females (Greff et al. 2003; Kjellberg et al. 2005b; Krishnan et al. 2014; Ohyama et al. 2015). Nevertheless, using acoustic songs to locate receptive females from a distance is particularly challenging in a fig as several hundreds of conspecific receptive females may be present within the enclosed cavity. Hence the males have might evolved an odour reception system that avoids the risk of saturation of olfactory receptors and of olfactory confusion and still allows location of receptive females. Conversely the females have had to evolve a mode of signalling that can be adequately perceived in the fig cavity.

If male fig wasps rely on chemicals to recognise conspecific mates, then individual females must possess species-specific chemical signatures that allow males to discriminate among conspecific and heterospecific females that reach sexual maturity concurrently inside the fig. The cuticular hydrocarbons of the insect epicuticle compose a group of lipid compounds that contain various numbers of carbon atoms. These cuticular hydrocarbons are important in insect chemical communication and function in mate discrimination, kin discrimination and sexual

discrimination in numerous insect taxa, including fig wasps (Howard and Blomquist 2005; Nagamoto et al. 2005; Krishnan et al. 2014; Ranganathan et al. 2015). Cuticular hydrocarbons are relatively heavy and hence have limited volatility, a feature which is probably a favourable trait for efficient signalling within the closed fig cavity. Typically, antennae are the main receptors involved in insect perception (Schneider 1964). Male fig wasps might therefore depend on antennal sensation to detect the cuticular hydrocarbons on the surfaces of females or female-containing galls. For this hypothesis to be supported, behavioural and chemical evidence for the role of such chemicals must be found and a chemoreception system that allows the perception of such chemicals through chemoreceptors must be identified in the antennae of these species. However, no information regarding chemoreceptors on the antennae of male wasps is available. Ultrastructural analyses of such chemoreceptors, which could provide insight into behavioural characters and possible physiological functions, are lacking.

In this contribution we test the hypothesis that mate location and recognition by males is olfactory and involves a more focused sensing system than for instance the olfactory system of the female wasps that is used to locate figs from a distance. To demonstrate this, we conducted a series of mating observations to investigate the mating behaviour of the pollinating fig wasp *Ceratosolen gravelyi*, including the male's mating sequence, duration, and number of rounds. We test the behavioural reactions of males to stimuli from virgin and mated females as well as from extracts of female-containing galls and empty galls (from which females had been removed; see below). We then investigated the male's chemoreceptors at the ultrastructural level to gain insight into their functional morphology as they are likely involved in olfactory and tactile perception within the dark cavity of the fig.

We provide evidence that mate discrimination is mediated by the olfactory and contact perception of an as yet unidentified chemical message originating from the body surface of females. The ultrastructure of male antennal chemoreceptors is consistent with highly directional chemosensory function.

## **Materials and methods**

### **Species biology and source**

*Ceratosolen gravelyi* Grandi pollinates the dioecious fig *Ficus semicordata* and develops only within the male figs of this plant. As all fig pollinating wasps, the species is sexually dimorphic. Female pollinators have functional wings and large eyes and are responsible for colonising new figs. Male pollinators are wingless, are typically restricted to the cavity of their natal fig, and were observed to exhibit fighting behaviour with conspecific males (Supplementary materials). Although fighting behaviour was not quantified in the present study, this is the first report of fighting between males of the genus *Ceratosolen*. Males of fighting species move more rapidly than those of non-fighting species, which can facilitate behavioural monitoring (Greeff et al. 2003). The figs of *F. semicordata* are also parasitised by four species of non-pollinating fig wasps that belong to the subfamilies Sycophaginae (*Sycophaga cunia*) and Sycoryctinae (*Philotrypesis dunia*, *Sycoscapter trifemmensis* and *Apocrypta* sp.); these species show sexual dimorphism similar to that of the pollinator species. Males of all of these wasp species die inside their natal figs except for some male pollinator individuals that cut a hole through the fig bracts, thus allowing the mated female fig wasps to emerge from the fig.

To obtain galls containing only *Ceratosolen* wasps and no non-pollinating fig wasps, we manipulated figs to prevent non-pollinating fig wasps from ovipositing within them. This was done by enclosing developing figs of *F. semicordata* in mesh nylon bags. Five *Ceratosolen*

females were introduced per receptive fig, as an average of five foundresses were found within individual, naturally visited figs. We then collected these figs at the stage at which male wasps had exited their galls but female wasps were still within their galls. We cut the figs open to obtain male and female (virgin and mated) pollinating fig wasps as well as female-containing galls and empty galls.

### **Mating behaviour observations**

For the mating behaviour observations, one quarter of each of the collected predispersal figs was individually placed on the object stage of a dissecting microscope (SteREO Discovery V20, Zeiss, Heidelberg, Germany) under cold-light illumination and observed on a computer screen. Observations were focused on analysing the sequence of behaviours of male individuals that led to mating as well as the subsequent behavioural sequence. When a male reached a female-containing gall, we observed whether the male individual contacted the surface of the gall and how it made contact. We monitored the male's sequence of behaviour leading to mating. In fig-pollinating wasps, once a male has reached a female containing gall, the male probes the gall with its antennae. Then, the male chews a hole into the gall and, while maintaining its hold on the gall, extends its elongated genitalia under its body and inserts them through the hole in the gall and copulates with the female (Weiblen 2002; Kjellberg et al. 2005b). Mating duration was calculated as the time from the initiation of chewing a hole in the gall to the removal of the male's genitalia from the gall. Mating duration and number of mating round was recorded for each male. Each male was continually observed for at least 30 min. Data from a total of 38 males were recorded.

### **Bioassay**

To determine whether male *Ceratosolen* wasps recognise conspecific females using olfactory-guiding and tactile-confirming chemicals that are emitted by females, we employed three biological tests using a method similar to that of Krishnan et al. (2014). Each male was individually placed in the middle of a cell culture plate filled with 1% agarose gel and presented with a choice between two galls or extracts placed symmetrically on either side of the male at a distance of 3 mm. Each male was allowed 5 min to respond to odours. A choice of side was scored when the wasp approached one side. Meanwhile we recorded the male's behaviours, noting what part of the male's body first made contact and whether genital elongation occurred. A scoring system based on a dichotomous variable 'behaviour exhibited' or 'behaviour not exhibited' was used. If the behaviour was exhibited within 5 min, a score of 1 was recorded; if not, a score of 0 was recorded. All of the tests were performed using an activity recorder for studies of insect behaviour (SN2000, Camsonar, London, UK) under red light conditions (set value 100%) at  $25 \pm 1^\circ\text{C}$ , 70–80% RH. Observations were made using a self-adaptive camera for the pairwise choices and with a stereoscope for the male behaviours. Preliminary experiments with the control of solvent hexane showed males equally chose to either side. Choice tests included 40–58 replicates, and at least 35 individuals made a choice. Each male individual was used only once in the bioassay tests.

Experiment A: male response to different types of galls. Individual males were randomly assigned to one of the following four treatments: (1) a female-containing gall, (2) a male-containing gall, (3) an exited gall, or (4) a washed gall (control). The different types of galls

were determined on the basis of their external morphology. Washed galls were obtained by washing male/female-containing gall surfaces in 500  $\mu$ l of hexane in a 2 ml glass vial for 10 min.

Experiment B: male response to different types of female wasps. These treatments were similar to those of Experiment A. Individual males were randomly assigned to (1) a virgin female, (2) a mated female, or (3) a washed female (control). The virgin females were obtained by directly collecting females from intact female-containing galls. Mated females and males were separately collected in the fig cavity. The stimuli wasps were freeze-killed by exposing them to  $-80^{\circ}\text{C}$  for 10 min. Washed wasps were obtained by washing the body surface with hexane, a method similar to that described in Experiment A.

Experiment C: male response to insect extracts. Males were presented with a washed empty gall covered with female extracts, gall extracts, or an equivalent amount of solvent. Individual males were randomly assigned to (1) a washed gall with female-containing gall extracts, (2) a washed gall with female extracts, (3) a washed gall with empty gall extracts, or (4) a washed gall covered with hexane (control). Extracts were obtained by dipping 30 object individuals into 500  $\mu$ l of hexane for 10 min at room temperature, condensing the solution under nitrogen to a total volume of 50  $\mu$ l, and then maintaining the sample at  $-20^{\circ}\text{C}$  until needed. Extracts (5  $\mu$ l) were directly added to the gall surface using a micropipette, and the solvent was allowed to evaporate for 15–20 min.

### **Ultrastructural analysis**

Scanning electron microscopy (SEM) was used to determine the presence and location of antennal sensilla that may perceive chemicals for mate discrimination, and a transmission electron microscope (TEM) was used to examine the ultrastructure of these sensilla. For scanning electron microscopy, intact male individuals were washed with an ultrasonic cleaner and fixed in 2% glutaraldehyde. The samples were dehydrated in a graded ethanol series to 100%,  $\text{CO}_2$ -dried using a critical point dryer, sputter-coated with gold and examined at an accelerating voltage of 10 kV using a Zeiss EVO LS10 SEM. For transmission electron microscopy, the antennae were removed from living male individuals and immediately immersed in 2.5% glutaraldehyde containing 0.1 M phosphate buffer (pH 7.4) and 4% paraformaldehyde and maintained at  $4^{\circ}\text{C}$  for at least 4 h. The antennae were then briefly washed in 0.2 M PBS (overnight) and postfixed with 1% osmium tetroxide mixed with PBS at  $4^{\circ}\text{C}$  for 2 h. After dehydration in a graded series of ethanol, the antennae were embedded in Epon 618. Ultrathin sections were cut with a diamond knife using a Leica-U ultramicrotome and collected on copper grids. The samples were then contrasted with 4% uranyl acetate and 1% lead citrate and finally observed using a Hitachi JEM-1010 TEM. The sensillar terminology adopted follows Schneider (1964), Barlin and Vinson (1981), Ochieng et al. (2000), van Baaren et al. (2007) and Li et al. (2009).

### **Data analysis**

All data analyses were performed in R version 3.5.0 (R Core Development Team 2018, [www.R-project.org](http://www.R-project.org)). Graphs were produced using Sigmaplot 10.0 (Systat Software, San Jose, CA, USA). The null hypothesis was that the pairwise choices or behavioural displays occurred at

equal proportions between the two sides (odour sources). A Fisher's exact test was used to test whether males responded to the odours of conspecific females significantly more often than to the stimulus on the other side. If the male showed 'no choice', this result was excluded from the analyses but reported in the results. For the exhibited behaviours, the dichotomous (0, 1) variables were analysed with a Cochran's Q test. Post hoc pairwise comparisons were performed using McNemar's tests, and the p-values were adjusted with the Bonferroni procedure.

## **Results**

### **Mating behaviour**

The males were very active, with most of the observation time spent moving and interacting with females. All 38 males showed the following sequence of behaviour (Supplementary material). After emerging from its natal gall, the male held the natal gall with its fore legs and mid legs and extended its body, with its antennae oriented forward and its head up, slightly rocking and appearing to be continuously sensing environmental cues, a behaviour we called 'olfactory detection'. Once the male started to move, it moved directly towards a gall. Upon reaching the gall, the male would first touch the surface of the gall with its antennae, which was in most cases (96%) a female-containing gall, and then it moved forward and grasped the tip of the gall with its head down and touched the surface of the gall with its antennae. The contact could elicit 'contact confirmation' and then the male would start chewing a hole. Alternatively, generally for galls that did not contain a conspecific female, the abovedescribed subsequent behaviour did not occur following the antenna touching the surface of the gall; instead, the male moved away from the gall to search for a new nearby gall. Although most of the male individuals (67%) chewed holes prior to copulation, some individuals (33%), particularly those copulating multiple times with the same female, used holes that had been chewed for previous copulation events. Following contact confirmation and chewing of the mating hole, the male moved forward slightly, adjusted the position of its body, held the tip of the gall with its fore legs and mid legs, and attempted copulation by elongating its gaster and inserting it into the female-containing gall; we referred to this behaviour as 'genital touch'. Genital touch was followed by copulation. During the observation sequence, one male copulated with 1–11 different females, and the same individual copulated 1–7 times with the same female. However, over its successive matings with the same female, mating duration gradually decreased (Figure 1).

Fighting was typically observed when male individuals competed for the same female-containing gall, particularly for galls located closest to the fig cavity, where female-containing galls are more accessible (Yu and Compton 2012). When two males encountered each other, they pushed each other away, or one would directly attack another using its mandibles or bite the opponent's mid leg or hind leg from behind and pull them backward. We also observed one case in which two males competing for the same female showed no aggression towards one another. The duration of fighting was variable and ranged from 5 s to 258 s. No injuries resulting from fighting were recorded for any of the observed individuals, but the victors almost always copulated with the contested female.

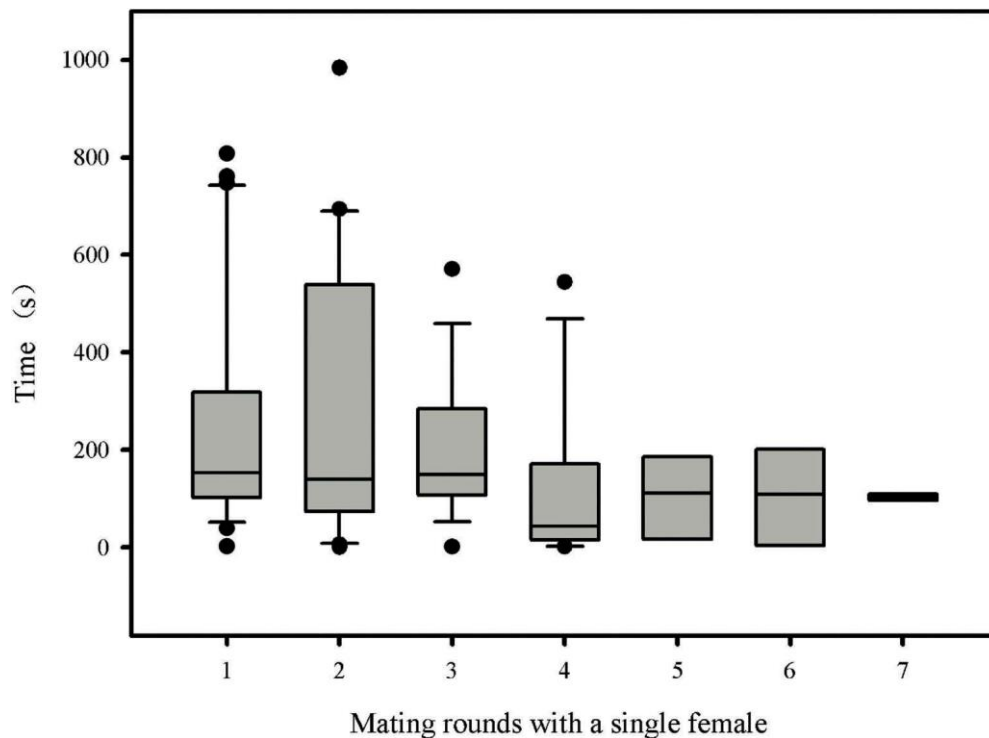


Figure 1. Duration of male *Ceratosolen gravelyi* copulation events with a single female.

### Bioassay

Experiment A: Male *C. gravelyi* individuals showed a significant preference for female-containing galls ( $p = 0.0032$ ) or male-containing galls ( $p = 0.0091$ ) over washed galls. Five of 42 individuals chose the empty galls, but none responded to the washed galls. When given a choice between female-containing and male-containing galls, males tended to prefer female-containing galls, but the difference was not significant ( $p = 0.469$ ) (Figure 2(a)). In the behavioural analysis, a significant difference in the frequency of contact confirmation was observed among the four different types of galls ( $Q = 78.172$ ,  $p < 0.0001$ ). This behaviour was exhibited toward female-containing galls and male-containing galls at rates of 82% and 69%, respectively. However post hoc pairwise comparison showed that the difference was not significant ( $\chi^2 = 3.368$ ,  $p = 0.199$ ). Similarly, a significant difference in the frequency of genital touch among the treatments following antennal contact was observed ( $Q = 93.571$ ,  $p < 0.0001$ ), with males showing no response to washed or empty galls. Post hoc pairwise comparison showed that the galls containing females were much more likely to elicit this behaviour (95% response rate) than were the male-containing galls (5% response rate) ( $\chi^2 = 12.410$ ,  $p = 0.0013$ ).



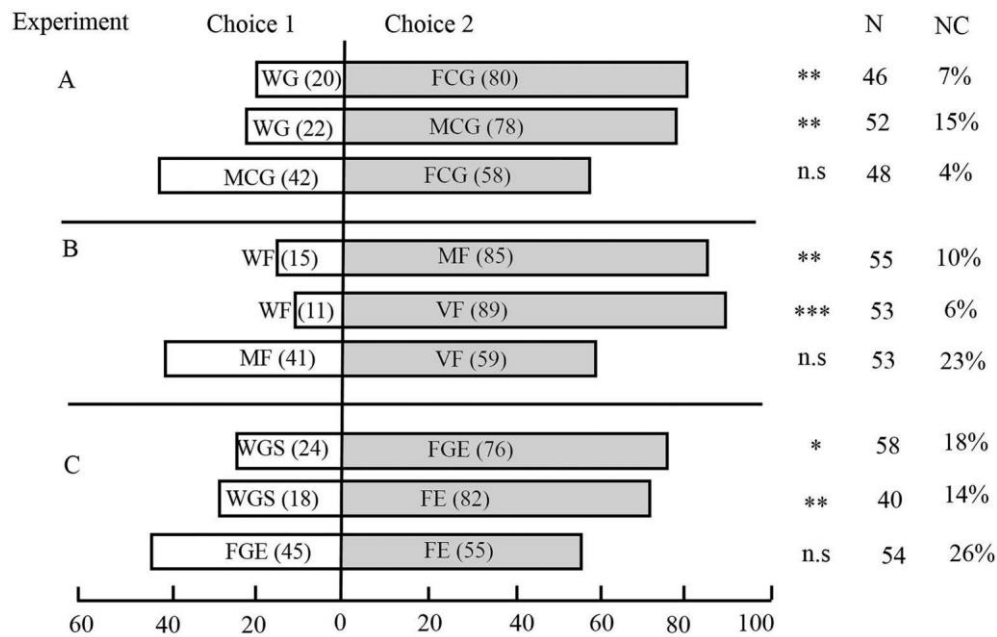


Figure 2. Choice experiment with male *Ceratosolen gravelyi* responding to cues from different types of galls (a) and wasps (b) as well as insect extracts (c). WG, washed galls; FCG, female-containing galls; MCG, male-containing galls; WF, washed females; MF, mated females; VF, virgin females; WGS, washed galls covered with solvent; FGE, female-containing gall extracts; FE, female extracts. Males that did not respond within 5 min were excluded from the chi-square test. The number following the abbreviation indicates the percentage of times that each side was chosen. NC = no choice, n.s = non-significant difference ( $p > 0.05$ ), \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , N indicates the total number of the tested wasps.

Experiment B: Most male individuals distinguished between mated or virgin females and washed females ( $p < 0.01$ ), but they did not distinguish virgin from mated females ( $p = 0.387$ ; Figure 2(b)). The Cochran's Q test revealed a significant difference in antennal contact ( $Q = 51.209$ ,  $p < 0.0001$ ) and genital touch ( $Q = 50.167$ ,  $p < 0.0001$ ) among the three types of stimulus females we presented. A post hoc pairwise comparison revealed that virgin and mated females were equally likely to elicit these behaviours (antennal confirmation:  $\chi^2 = 3.125$ ,  $p = 0.231$ ; genital touch:  $\chi^2 = 3.20$ ,  $p = 0.221$ ).

Experiment C: A significantly larger proportion of males chose the gall treated with female-containing gall extracts ( $p = 0.023$ ) or female extracts ( $p = 0.001$ ) over the control galls covered with solvent (Figure 2(c)). Nine of 52 male individuals chose the washed gall treated with the extract from empty galls, and no males responded to the control galls. Males did not distinguish between the galls treated with female-containing gall extracts and those treated with female extracts ( $p = 0.776$ ). A Cochran's Q test revealed a significant difference in the frequency of contact confirmation elicited among the three types of extracts ( $Q = 37.852$ ,  $p < 0.0001$ ). This behaviour was exhibited by 72% and 83% of male individuals in response to the galls treated with female-containing gall extracts and those treated with female extracts, respectively. A post hoc comparison showed that the female-containing gall extracts and the female extracts elicited similar frequencies of contact confirmation ( $\chi^2 = 4.0$ ,  $p = 0.137$ ). A significant difference was observed among the extract treatments in the frequency of genital touch following antennal contact ( $Q = 39.931$ ,  $p < 0.0001$ ), as the males did not display this behaviour in response to empty gall extracts or to the control solvents. A post hoc pairwise comparison showed that the

washed galls treated with female wasp extracts were more likely to elicit copulation attempts than were the control galls ( $\chi^2 = 12.0$ ,  $p = 0.0016$ ). A further result is that males attracted to female gall extracts and to female extracts against washed gall extracts in treatment C mostly continued the sequence of behaviour up to genital touch, i.e. exhibited the whole sequence of behaviour leading to mating in 48 instances out of 58.

### Ultrastructural analysis

The flagellum of the male antennae is composed of three flagellomeres (Figure 3(a,b)), but only the distal part of the terminal flagellomere (the third flagellomere, F3) presents antennal sensilla (Figure 3(c)). We identified three types of sensilla: multiporous plate sensilla (MPS) and basiconic sensilla types 1 (BS-1) and 2 (BS-2). These sensilla were considered to be chemosensory receptors on the basis of their external morphology (Figure 3(a–c)) and ultrastructure (Figure 3(d–i)).

**Multiporous plate sensilla:** MPS are botuliform and surrounded by a groove and a cuticular ridge (Figure 3(c)). These sensilla are slightly raised from the antennal surface and distributed between rows of BS-1 and BS-2. They are generally aligned in parallel with the longitudinal axis of the flagellomere segment. The dimensions of these plates vary little, with a length and width of approximately 10  $\mu\text{m}$  and 4  $\mu\text{m}$ , respectively. Each plate is innervated by numerous dendrites. These neurons are situated proximally in the hemolymph space of the antenna and project distally into the sensillum lymph along the sensillum axis. The dendrites expand quickly once they reach the distal end, which contains multiple cuticular pores (Figure 3(d,g)) that are suggestive of olfactory function.

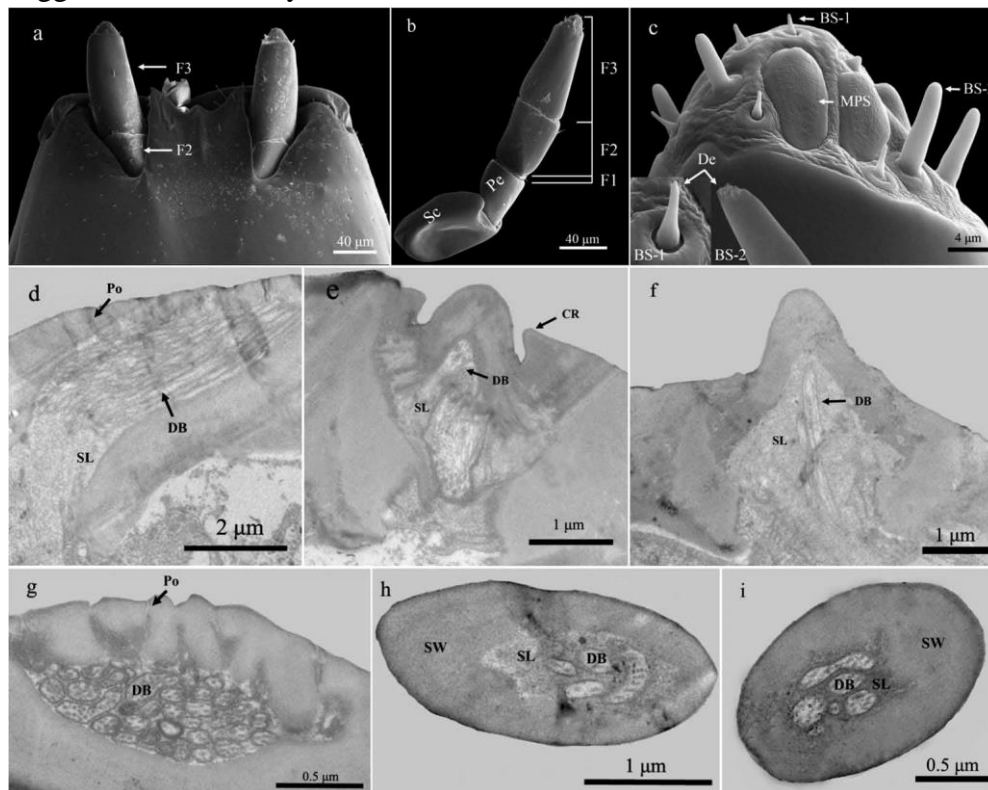


Figure 3. Micrographs of the antennae and chemosensory sensilla of male *Ceratosolen graveleyi*. (a) Scanning electron micrograph of the head and antennae, showing an exposed area of the 2nd–3rd flagellomeres (F2–F3). (b) Scanning electron micrographs of an excised antenna,

showing the scape (Sc), pedicel (Pe) and flagellum (F). (c) High magnification image of the terminal flagellomere (the 3rd flagellomere, F3) showing the three types of chemoreception sensilla: multiporous plate sensilla (MPS) and basiconic sensilla types 1 (BS-1) and 2 (BS-2). Note the terminal indentation (De) in the BS on the insets in the lower left corner. (d) Longitudinal section of a multiporous plate sensillum showing dendritic branches (DB) running parallel to the sensillar lymph (SL) and ending with cuticular pores (Po) at the sensillum surface. (e) Longitudinal section of the basal area of a basiconic sensilla type 1 inserted into a socket surrounded by a raised cuticular ring (CR). (f) Longitudinal section of the basal area of a basiconic sensilla type 2. (g–i) Cross-sections of a multiporous plate sensillum and basiconic sensilla types 1 and 2. The sensillar wall (SW) of BS-1 and BS-2 is non-porous at this level.

Basiconic sensilla type 1: BS-1 are short and peg-like in shape, with a blunt tip and a flexible socket (Figure 3(c)). Each sensillum has a thick, nonporous cuticular wall surrounding the inner lumen and is innervated by several dendritic branches (Figure 3 (e,h)). BS-1 length varies from 2.2  $\mu\text{m}$  to 3.3  $\mu\text{m}$ , and their basal diameter ranges from 0.9  $\mu\text{m}$  to 1.1  $\mu\text{m}$ . The tips of BS-1 presents multiple indentations (Figure 3(c), inset) that may allow some chemicals access to the sensillar lymph, and they are thus considered to have a contact chemoreception function.

Basiconic sensilla type 2: BS-2 are robust and project more obviously with respect to the antennal axis compared with BS-1 (Figure 3(c)). A thick, nonporous cuticular wall surrounds an inner lumen innervated by numerous dendritic branches (Figure 3(f,i)). The length of these sensilla varies from 7.2  $\mu\text{m}$  to 8.9  $\mu\text{m}$ , and the width ranges from 1.8  $\mu\text{m}$  to 2.3  $\mu\text{m}$ . Because of their orientation and size (Figure 3(c)), the tips of BS-2 extend substantially beyond the other sensilla, suggesting a contact chemoreception function.

## Discussion

Aggressive male behaviour is rare among pollinating fig wasps. Due to the female-biased offspring sex ratio, males face little competition in accessing potential mates. Greeff et al. (2003) reported that males of the Agaonidae genera *Alfonsiella*, *Nigeriella*, *Allotriozoon*, *Pegoscapus*, *Platyscapa* and *Courtella* can engage in aggressive behaviour and exhibit several fighting-related traits, including falcate mandibles, a large head, a long antennal scape, and modified legs and thorax. The present study is the first to report fighting between *Ceratosolen* males competing for an individual female; however, the morphology of *C. gravelyi* males is not consistent with the fighting traits of aggressive males (Greeff et al. 2003). Conversely, *C. gravelyi* males move more rapidly than males of the non-aggressive *C. fusciceps*, which move slowly when searching for females in the dark cavity of the fig (Krishnan et al. 2014). During our observations of male behaviour, we observed that fighting between males typically occurred in the centre of figs (Supplementary material), where access to females is easy, and females have larger body-size, suggesting higher reproductive value and more mating opportunities, while fighting was not observed for the fertilisation of females in galls of difficult access located close to the fig wall (Dunn et al. 2008; Yu and Compton 2012; Peng et al. 2014). Therefore, several males often compete for the same gall in the fig centre, leading to a localised male-biased operational sex ratio that favours male–male fighting. This fighting can lead to an arms race among relatives (Dawkins and Krebs 1979) if males gain more benefits from the particular ecological situations. Dispersal is an effective means for decreasing competition intensity (Greeff et al. 2003); however, in our experience, *C. gravelyi* males have never been observed

to disperse; e.g. males have not been observed to leave the natal fig and enter another fig on the same tree as was observed for males of some other Agaonidae (Greeff et al. 2003). Within *C. graveleyi*, therefore, the arms race must be restricted. The intensity of competition between brothers can be partially or fully eliminated through the precise adjustment of the offspring sex ratio by foundresses (Herre et al. 1997); however, several factors, such as multiple foundresses (Herre et al. 1997), male mortality (Hardy et al. 1998; Greeff et al. 2003) and parasitic wasps (Kathuria et al. 1999; Pereira and Prado 2005) can increase the male-to-female sex ratio. Because of a strategy to ensure sufficient numbers of males involving both laying more males and laying them in locations protected against parasites (Kjellberg et al. 2005a; Yu and Compton 2012), the fine adjustment of the sex ratio in fig wasp clutches may be regarded as a trade-off in the reproductive strategy (Scharf et al. 2013).

Another important finding of this study is that individual male pollinators may mate several times with the same female. This condition could result in a more female-biased sex ratios if sperm is limiting (Hamilton 1979; Ramírez-Benavides et al. 2009; Peng et al. 2014), since more gametes might be transferred over the successive matings. Occurrence of a male mating successively several times with the same female has also been documented to occur in the two pollinators of *Ficus ottoniifolia* in Gabon. In that situation, the species mating most rapidly was also the one that remated females more frequently, suggesting that quantity of sperm transferred can be limiting (Michaloud et al. 1985). Females being mated several times has also been recorded for a number of fig pollinating wasp species, but in those cases the males tried to find other females to mate before coming back and remating a female (Greeff et al. 2003). For example, males of *C. solmsi marchali*, which is associated specifically with the fig *Ficus hispida*, can repeatedly mate four (Peng et al. 2014) to ten times (Murray 1990). In *Kradibia tentacularis*, only offspring of a single male were found in single female broods, a result the authors attributed to lack of multiple matings but which could alternatively be due to sperm replacement (Zavodna et al. 2005). In this study, we found that *C. graveleyi* males participated in up to seven rounds of mating, and some individuals used previous mating holes chewed by themselves or other individuals to mate. Hence number of mating holes can underestimate the number of mating events (Greeff et al. 2003; Peng et al. 2014). Regarding mating duration, considerable time may be invested in moving nutrients or compounds other than sperm from the male to its mate. These substances may be beneficial for increasing clutch size or offspring viability, thereby increasing the female's reproductive success. For example, male pollinating fig wasps have higher nitrogen levels and lower phosphorus levels than do females (Zhang and Han 2010), and mating duration may be influenced by the transfer of these substances to their partners (Kay et al. 2005). However, over multiple mating events with the same male, the provision of such substances may decrease over time, leading to decreased mating duration. Foundresses have short lifespans, and their eggs are mature upon adult emergence (Ghara and Borges 2010). This may limit the role of a male resource donor; nonetheless, male provisioning may be vital in prolonging foundress lifespan, as adult females do not feed.

Consistent with the mating systems of other species in which the encounter of the sexes is mediated by cuticular chemicals (Howard and Blomquist 2005; Nagamoto et al. 2005) and in agreement with previous results of fig pollinating wasps (Krishnan et al. 2014), we found clear evidence for a role of cuticular surface chemicals originating from *C. graveleyi* females. In pollinator fig wasps, females develop and remain in their galls until males chew holes that allow them to escape. Females, in turn, are responsible for the pollination that maintains the stability

of the fig-wasp mutualism (Weiblen 2002; Kjellberg et al. 2005b). In such a system, selection favours females producing cuticular chemicals that facilitate their recognition by conspecific males and limiting fertilisation by heterospecific males, thus ensuring pre-zygotic isolation through assortative mating (Lukhtanov et al. 2005; Smadja and Butlin 2008; Langerhans and Makowicz 2013). In our experiments, the males were very active and moved more rapidly than do males of the non-aggressive, pollinating fig wasps (Krishnan et al. 2014). This allowed monitoring of numerous choices made by the males. Males were clearly attracted by cuticular chemicals emitted by the females, and their encounters with females involved olfactory-directional and tactile-confirmation cues. Our data also shows that female-emitted cuticular chemicals are sufficient to initiate the whole sequence of male behaviour leading to mating attempt. Hence, while we cannot exclude that males can use such cues as vibrations produced by females as previously suggested (Krishnan et al. 2014) we can state that such cues are not required to elicit the full sequence of male behaviour.

Males of *C. graveleyi* showed no differences in their attraction toward wasp-containing galls (either females or males) and types of females (virgin and mated), although some males did not respond to empty galls treated with extracts from these objects. Their responses thus suggest the existence of a chemical or a combination of chemicals common to both sexes, similarly to what has been observed in *Drosophila* spp. (Howard and Blomquist 2005), that does not allow the sex of conspecific individuals to be discriminated. In fig pollinating wasps, as females are much more numerous than males and as males rapidly emerge from their galls, males have limited opportunities to encounter galls containing males. There is therefore limited selection on them to rapidly detect that a gall contains a male. Nevertheless, the males did not chew mating holes into galls containing males, showing that in a second phase they detected the sex of the individual enclosed in the gall.

In most agaonid wasps, females do not avoid mating more than once (for exceptions see Greeff et al. 2003). This is a direct consequence of females waiting in their galls until the fig reaches the appropriate stage for their emergence into the fig cavity. Nevertheless while unmated females only produce sons (Weiblen 2002; Cook 2005; Kjellberg et al. 2005b), our data indicate that virgin and mated females are equally attractive to males.

Our results are consistent with the detection at a distance of cuticular compounds, allowing the males to reach a gall containing a female, followed by contact chemical stimulation. Detection at a distance and contact stimulation should be mediated by the multiporous plate sensilla and the basiconic sensilla (types 1 and 2), since no other chemoreceptors were observed on the antennae. Numerous pores of MPS occur along the botuliform plate, allowing the entry of the volatile molecules into the sensillar hemolymph space, which fulfil the morphological criteria of olfactory chemoreceptive mechanism underlying the olfactory function of the sensory neurons (Barlin and Vinson 1981; Ochieng et al. 2000; van Baaren et al. 2007). Much fewer sensillar sensory neurons were observed here in male *C. graveleyi* individuals than had been observed previously in females of another agaonid wasps species (Li et al. 2009) and more generally comparatively to other chalcidoid wasps (Barlin and Vinson 1981). However, within a fig, males could easily be confused by the chemical messages produced by the several hundreds of females. The simplified male antennae (Figure 3(a,b)), with olfactory distance and contact receptors located only at the tip of the distal flagellomere, may be an adaptation to easily manipulate the precise position of the chemoreceptors in a complex sensory environment. Male *Ceratosolen* can readily retract their antennae into their typical more or less closed antennal

grooves, and they steadily move around the tip of their antennae, probably probing their environment for the precise location of particular chemical cues at rather low distances. In such situations, a highly sensitive detection mechanism would probably be inefficient as it would systematically become saturated. We may suggest that MPS plays an important role for detection at a distance, while basiconic sensilla (see below) would be more involved in short distance or contact stimulation. It is probable that the MPS plays a similar role in the behaviour of the different fig wasp species that reproduce in *Ficus racemosa*, given that male non-pollinating fig wasps have been shown to exhibit olfactory discrimination between conspecifics and heterospecifics (Krishnan et al. 2014).

The two subtypes of BS that were present on the distal flagellomere concurrently are quite common on the antennae of hymenopteran parasitoids (Slifer 1969; Ochieng et al. 2000; van Baaren et al. 2007; Li et al. 2009). The BS-1 have a similar morphology to the thinwalled chemoreceptor in the pteromalid *Nasonia vitripennis* (Slifer 1969), and to the sensilla basiconica B in braconid *Microplitis croceipes* (Navasero and Elzen 1991), or multiporous BS in female agonids (Li et al. 2009). The shape of BS-2 was similar to the previously described sensilla in some parasitoids including sensilla basiconica A (Navasero and Elzen 1991), thickwall chemoreceptor (Slifer 1969), and uniporous BS (Li et al. 2009). Yet the two subtypes of BS are smooth and present a nonporous surface (Figure 3(e,f,h,i)). In spite of remarkable differences in size and cuticular attachment, both BS-1 and BS-2 had similar numbers of neurons and apical pores, suggesting a similar function of perceiving the contact signals, since they stood out above the antennal surface so that they must contact the gall surface when the male touches it with the tip of its antennae (Ochieng et al. 2000; van Baaren et al. 2007). More generally, the distribution pattern, size and orientation of both types of BS are consistent with a contact chemoreceptive role in several parasitic wasps (Navasero and Elzen 1991; Ochieng et al. 2000; van Baaren et al. 2007; Li et al. 2009).

In conclusion, males of *C. gravelyi* are aggressive, but they lack the suite of fighting traits exhibited by classical fighting male agaonid wasps. These results suggest that morphological adaptation to fighting in *C. gravelyi* may be limited by the necessity to access the females enclosed in galls located close to the fig wall, a localisation that also decreases the level of male–male competition. Males showed a fixed sequence of behaviour leading to mating, including olfactory direction, contact confirmation and genital touch. This species uses distinctive olfactory directional and tactile-confirmed cues to recognise conspecific females. An analysis of the major components of the cuticular chemicals, a test of single sensilla response to these chemicals and a direct assessment of their roles in mate recognition is now needed to confirm our interpretation of how male fig pollinating wasps locate females still enclosed in their galls.

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