Finding hidden females in a crowd: Mate recognition in fig wasps

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A B S T R A C T
Multi-species mating aggregations are crowded environments within which mate recognition must occur. Mating aggregations of fig wasps can consist of thousands of individuals of many species that attain sexual maturity simultaneously and mate in the same microenvironment, i.e., in syntopy, within the close confines of an enclosed globular inflorescence called a syconium—a system that has many signalling constraints such as darkness and crowding. All wasps develop within individual galled flowers. Since mating mostly occurs when females are still confined within their galls, male wasps have the additional burden of detecting conspecific females that are "hidden" behind barriers consisting of gall walls. In Ficus racemosa, we investigated signals used by pollinating fig wasp males to differentiate conspecific females from females of other syntopic fig wasp species. Male Ceratosolen fusiceps could detect conspecific females using cues from galls containing females, empty galls, as well as cues from gall volatiles and gall surface hydrocarbons.

In many figs, syconia are pollinated by single foundress wasps, leading to high levels of wasp inbreeding due to sibmating. In F. racemosa, as most syconia contain many foundresses, we expected male pollinators to prefer non-sib females to female siblings to reduce inbreeding. We used galls containing females from non-natal figs as a proxy for non-sibs and those from natal figs as a proxy for sibling females. We found that males preferred galls of female pollinators from natal figs. However, males were undecided when given a choice between galls containing non-pollinator females from natal syconia and pollinator females from non-natal syconia, suggesting olfactory imprinting by the natal syconial environment.

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1. Introduction

Mate recognition is often the first and most important step in reproduction. For those organisms that mate in large aggregations, mate recognition must occur in crowded conditions. Such aggregations can either consist of conspecifics as in garter snakes (Shine and Mason, 2001) and bark beetles (Byers and Wood, 1980) or of multiple species as in mycophagous Drosophila (Jaenike et al., 1992), fiddler crabs (Detto et al., 2006), Anopheles mosquitoes (Diabaté et al., 2006) and fig wasps (Janzen, 1979; Weiblen, 2002; Herre et al., 2008; Ghara and Borges, 2010). Mate recognition signals function not only as barriers to hybridisation in mixed-species aggregations, but are also important for reproductive isolation in sympatric species. The evolution of mate recognition signals can often be important in the radiation and maintenance of species isolation in species that are sympatric and syntopic sharing not only geographic ranges, but also habitats within these ranges (Symonds and Elgar, 2004; Mullen et al., 2007; Smadja and Butlin, 2009; Nanda and Singh, 2011). Species specificity of premating signals in sympatric species is higher than those between allopatric species (Butlin, 1987; Coyne and Orr, 1989; Noor, 1999). Such signal specificity is also high in sympatric species under syntopic conditions (Shine et al., 2002).

In crowded and dark spaces, chemical (Partan and Marler, 2005) and/or vibratory signals (Hebets and Papaj, 2005) could conceivably be more important than visual signals in mate
recognition. In crowded situations where several species are simultaneously sexually mature and are ready to mate within the same micro-environment, i.e. in syntopy, the ability of males to accurately find a conspecific female becomes crucial. Such systems afford valuable insights into mechanisms by which mate recognition could occur under signalling constraints. Since sex pheromones could function over longer or shorter distances depending on their volatility, it is expected that heavier relatively non-volatile cuticular hydrocarbons could serve as contact sex pheromones (Singer, 1998; Ginzel, 2010), while compounds with low volatility could serve as mate recognition signals over short distances without contact having to occur (Yoshida, 1978; Simser and Coppell, 1980). In species in which females are hidden by physical barriers, males have to find such hidden females using signals that can either penetrate or coat such barriers such as the retreat silk of ant-mimicking spiders (Borges et al., 2007) and the cocoons of hymenopteran parasitoids (Howard, 1993), or employ proxies for female signals as in male gall wasps which use altered host plant volatiles to find females hidden within plant stems (Tooker et al., 2002; Tooker and Hanks, 2004), or male Heliconius butterflies that use host plant volatiles and the presence of immature larvae to find females developing within pupal cases which they proceed to guard until female eclosion (Estrada and Gilbert, 2010; Estrada et al., 2010).

Mating recognition by male wasps in the nursery pollination mutualism between fig and fig wasps has to function under the constraints of crowding, darkness, syntopy and simultaneous sexual maturation of multiple fig wasp species as well as the possible requirement for proxies for the location of hidden females. In this mutualism, individual fig wasps develop in galled flowers within an enclosed, gloular, thick-walled inflorescence called the fig syconium, while many of the uniovulate females also develop seeds after being pollinated by mutualistic fig wasps. Wingless male fig wasps emerge first from their galls, and either release females from their galls before mating or insert genitalia into female-containing galls to mate with virgin females (Weiblen, 2002; Cook and Rasplus, 2003; Cook and Segar, 2010).

Depending on the fig species and syconium size, syconia may contain hundreds to thousands of flowers (Janzen, 1979; Verkerke, 1989; Kjellberg et al., 2001; Cook and Rasplus, 2003), with hundreds of developing wasps and seeds. Besides the mutualistic fig wasp species (belonging to Agaonidae), most species of figs are parasitised by other galler, inquiline or parasitoid fig wasp species (belonging to other subfamilies in the Chalcidoidea), all of which also develop within individual galls within the enclosed syconium. Between 1 and 30 species of sympatric fig wasps could occupy the syconia of a single fig species (Cook and Rasplus, 2003; Cook and Segar, 2010). In some fig species, two species of congeneric pollinating wasps may also develop within the same syconium (Michaloud et al., 1996). Therefore, if males are able to detect the pre-mating chemical signals of different fig wasp species, they can be expected to experience a chemical equivalent of the cocktail party problem encountered in acoustic communication in a noisy environment (Bee and Michely, 2008), i.e., the need to perceive specific mate recognition signals in a noisy chemical environment. Furthermore, since in some fig species such as Ficus racemosa, females cannot free themselves from their galls and rely upon males to do so (A. Krishnan, pers. observ.), males need to either use a species-specific chemical or vibratory signal that is emitted by females themselves and that can pass through the gall wall, or rely on proxy cues that coat the gall wall and indicate the presence of conspecific females within galls. Furthermore, since male wasps (especially the pollinator males) have a short lifespan of 24–48 h (Kjellberg et al., 1988; Ghara and Borges, 2010) within which they have to mate as well as cut an opening in the syconium wall to release pollinator females, male pollinator wasps should also be under intense selection pressure to identify galls containing conspecific females quickly and accurately.

Most often, fig wasp males mate with females found within their own syconium since males usually die within their natal syconium (Galil and Eisikowitch, 1968; Herre et al., 2008; Ghara and Borges, 2010). However, in some species, fig wasp males may leave their natal syconium and mate with females from other syconia (Greeff, 2002; Greeff et al., 2003, 2009). This may be to avoid inbreeding (Greeff et al., 2009), although it is believed that fig wasps with their haplodiploid sex determination system can tolerate high levels of inbreeding (McKey, 1989); often only a single or few foundress female wasps may lay eggs within individual syconia (Kathuria et al., 1999; Zavodna et al., 2007). Consequently, sib matings are likely to be quite frequent (Zavodna et al., 2007). However, whether male fig wasps prefer female signals from their natal syconia or from non-natal syconia is not clear, though studies on the pollinator Pegoscapus assuetus seem to indicate that males prefer females from natal syconia (Frank, 1985). The cues employed by P. assuetus males to differentiate between females from natal and non-natal syconia were not investigated.

With this background, we have used F. racemosa to understand how male pollinator fig wasps solve the problem of finding hidden females. We determined if male Ceratosolen fuscipes can distinguish between galls of conspecific females from those of non-pollinator females (which can co-occur with pollinators within the same syconium) on the basis of (1) whole galls (with the gall occupant within the gall); (2) empty galls (gall occupant removed to remove potential vibratory signals); (3) volatile signals from empty galls and (4) surface hydrocarbon signals of empty galls. We also conducted choice assays to test if males prefer galls containing females from non-natal fig syconia (which are definitely non-sibs) to females from natal fig syconia (which have higher probabilities of being sibs).

2. Materials and methods

2.1. Species biology and study site

The syconia of F. racemosa are pollinated by the mutualistic agaonid wasp Ceratosolen fusciceps Mayr and are also host to six other species of non-pollinating fig wasps in the subfamilies Sycophagineae and Sycoryctinae respectively (gallers — Apocryptophagus stratheni Joseph, Apocryptophagus testacea Mayr, Apocryptophagus fuscus Girault and the parasitoids — Apocryptophagus agranesis Joseph, Apocrypta westwoodi Grandi and Apocrypta sp. 2) (Ghara and Borges, 2010) that develop within them. Although wasp dispersal phase syconia containing all 7 species of wasps are rare, syconia containing 3–4 wasp species are quite common (Ghara, 2012). Pre-dispersal stage syconia are generally 13–35 mm in size and can contain 2000–7000 flowers. Each syconium can contain from 0 to 1200 developed wasps, of which 10–40 percent are males (M. Ghara, A. Krishnan and R.M. Borges, unpublished data). Males of all 7 species of wasps reproducing in F. racemosa are wingless, usually die within their natal syconia, and have never been observed to exhibit aggressive or fighting behavior. All species of wasps are sexually mature at the same time; females of all species leave the syconium concurrently.

We collected pre-dispersal stage syconia (in which male wasps had excited their galls, but female wasps were still within their galls) from in and around the Indian Institute of Science campus in Bangalore, India (12°58′N, 77°35′E). We cut open syconia to collect males of the pollinator wasp (C. fusciceps) and galls containing females of pollinator and the non-pollinating wasps.
A. testacea and Apocrypta sp. 2 (the gall contents were known only post-facto especially in those experiments involving intact galls). We chose these two non-pollinating wasps since they were the most common galler and parasitoid species in the sampled syconia.

2.2. Choice assays

We conducted choice assays in six-well cell culture plates (12.5 cm × 7.5 cm, each well = 3.6 cm diameter) with each well half-filled with 1% agarose in distilled water to provide a moist environment for the male wasps. Each well was conveniently used as an independent assay chamber for the experiments. In all experiments, we gave male pollinating wasps a choice between two galls (or two hydrocarbon extracts), with the galls/hydrocarbon extracts placed ~3 mm from a central point where the male pollinator was placed (Fig. 1). For all assays, the positions of the choices were interchanged between trials to counter directional biases. We conducted these assays in a room with dim lighting inside a darkened thermocol box with each assay lasting 5 min. Observations on males began as soon as they were released into the assay chamber through the transparent top of the chamber. We noted the first choice made by each male (generally made within 1 min of beginning the assay), along with its final choice at the end of 5 min. We conducted two sets of experiments (species recognition and natal syconium recognition experiments) involving four types of choice assays.

a) Whole gall choice assays — We used unopened galls with the occupant still within the gall (Fig. 1a). The identity of gall inhabitants were unknown during these assays and was noted when the galls were dissected at the end of the assays. b) Empty gall choice assays — We opened galls and removed their occupants just before using them for the choice assays (Fig. 1b).

c) Volatile choice assays — We made two slits in the agarose at a distance of about 2.5 mm on either side of the central point to prevent diffusion through the gel of non-volatile signals from the galls towards the male pollinators (Fig. 1c). We placed galls (which were opened and had their occupants removed just prior to the experiments) outside these slits, and conducted the assay as explained before. d) Hydrocarbon extract choice assays — We made hydrocarbon extracts of galls (which were opened and had their occupants removed just prior to the extractions) by adding ~1 ml of pentane to 20 galls in a glass vial. Pentane is a solvent proven to extract cuticular hydrocarbons from fig wasps efficiently (Ranganathan, 2012). We gently agitated the vials for 1 min and allowed them to remain in contact with the solvent for 10 min at room temperature. We then removed the galls from the solvent which was allowed to evaporate completely. We used the extracts either immediately, or stored them at ~20 °C till required. We reconstituted each cuticular hydrocarbon extract in ~50 μl of pentane, and used this solution for 5 assays. Into each well, we added 10 μl each of 2 different extracts such that the edge of the extracts was located ~3 mm from the central point where a male pollinator was placed for the assay (Fig. 1d). We allowed the pentane to evaporate, and traced out with a pin the spread of the extracts on the surface of the agarose as described in Krishnan et al. (2010) and carried out the assay as before. All trials for each assay type were performed together, generally over a period of one or several days depending on the number of males that were available for testing. All trials for whole gall choice assays were completed before beginning the trials for choices involving volatile cues. Trials for hydrocarbon choice assays were begun only after completing the trials for volatile cue assays.

1) Species recognition experiments — We performed these experiments to determine the nature of the signals used by male pollinating wasps to distinguish between conspecific and heterospecific female galls. We used two species of non-pollinators — one galler (A. testacea) and one parasite (Apocrypta sp. 2) — for all species recognition experiments. We utilised female pollinator galls, female non-pollinator galls and male pollinators for the assays from three different syconia to make sure that no natal syconium development preferences affected the choice of the male pollinators.

2) Natal syconium recognition experiments — We conducted these experiments to determine if male pollinators preferred galls containing female pollinators belonging to their natal syconia to those belonging to non-natal syconia, and to investigate the nature of the signals used by the males to make these choices. Assays involving whole galls, volatile cues and gall hydrocarbon extracts were conducted as described before for the species recognition experiments. Assays were carried out over a period of several days with males and galls from only two freshly opened syconia being used for all the trials (10–25) each day. As far as possible, we also tried to test equal numbers of males from the two syconia used in each day's trials. Due to a scarcity of syconia containing sufficient pollinators and non-pollinators within them to carry out such experiments, we were forced to pool together non-pollinators of several different species (A. testacea, A. fusca, A. agraensis and Apocrypta sp. 2) under the grouping of natal female non-pollinators. We were limited by the scarcity of such syconia to exploring the response of male pollinators to whole galls and volatile signals from galls of natal compared to non-natal syconia.
2.3. Statistical analysis

All sets of experimental pairs were analysed with $\chi^2$-tests using the software package R (version 2.14.1). All assays labeled “no choice”, i.e. when the male pollinator did not show a response, were excluded from the analyses, but are reported in the results.

3. Results

Most pollinator males made choices within one minute of starting the assay. In the whole gall choice assays, most males that chose female pollinator galls began chewing open the galls and attempted to mate with the females inside. Pollinator males did not attempt to open non-pollinator galls. In the choice assays with empty galls and volatiles, most males choosing female pollinator galls exhibited searching behaviour near the galls at the end of 5 min and a few tried to chew on the opened galls. In the surface hydrocarbon assays, most males choosing female pollinator gall extracts exhibited searching behaviour, and a few attempted to chew the extract-coated agarose. Signals from the natal syconium environment seemed to interfere with species recognition mechanisms when males were made to choose between non-pollinator galls from the natal syconia and pollinator galls from a non-natal syconium.

3.1. Species recognition experiments

A significantly larger number of male pollinators chose female pollinator galls (Figs. 2a, c) over $A.\ testacea$ galls using signals from whole galls (first choice, $\chi^2 = 15.87, p < 0.001$; 5 min, $\chi^2 = 4.45, p = 0.035$), empty galls (first choice, $\chi^2 = 7.07, p = 0.007$; 5 min, $\chi^2 = 4.57, p = 0.03$), volatiles (first choice, $\chi^2 = 19.88, p << 0.0001$; Fig. 2). Species recognition experiments to examine the response of male pollinators to cues from whole galls, empty galls, volatiles and gall surface hydrocarbons when given choices between these cues from female pollinators (FP) and female non-pollinators ($A.\ testacea$ and $A.\ sp. 2$). (a–b): First-choice responses. (c–d): Responses after 5 min. All female galls used in these experiments are from non-natal syconia. The choices (excluding the ‘no choice’ responses) were analysed using chi-square tests. n.s. = non-significant difference ($p > 0.05$); *$p < 0.05$; **$p < 0.01$; n includes male pollinators exhibiting no choice.
choosing natal over non-natal galls containing female pollinators remained, the result was not significant ($\chi^2 = 1.4, p = 0.24$), owing to an increase in the number of 'no-choice' males. A similar trend was observed when male pollinators were presented with hydrocarbon cues alone, with a significantly larger number of males choosing natal galls containing female pollinators over non-natal galls initially ($\chi^2 = 5.77, p = 0.016$), but with no significant difference in the number of males choosing natal over non-natal galls containing female pollinators after 5 min ($\chi^2 = 2.67, p = 0.1$). These results suggest that whole intact galls and empty galls from natal syconia provide cues that are more attractive than either volatile cues or cuticular hydrocarbon extracts from such syconia compared to non-native galls containing female pollinators. Furthermore, males receiving a smaller subset of cues in the form of either volatiles or hydrocarbons are more likely to be unresponsive. In the last set of experiments exploring the interaction of a natal gall effect and species recognition (Figs. 3b,d), male pollinators demonstrated a tendency, although non-significant, to choose whole galls containing natal female non-pollinator galls over non-natal female pollinators (Fig. 3) as evidenced by their initial choice ($\chi^2 = 3.0, p = 0.083$). After 5 min, male pollinators showed a tendency to choose non-natal galls of their own species over natal galls of other

### 3.2. Natal syconium recognition experiments

A significantly larger number of male pollinators chose female pollinator galls from natal figs over female pollinator galls from non-natal figs (Figs. 3a, c) on the basis of whole gall (first choice, $\chi^2 = 11.76, p < 0.001$; 5 min, $\chi^2 = 10.7, p = 0.001$) and empty gall signals (first choice, $\chi^2 = 10.7, p = 0.001$; 5 min, $\chi^2 = 9.94, p = 0.002$). However, when presented with only volatile signals, a significantly larger number of male pollinators chose natal galls containing female pollinators over non-natal galls only in their first choice ($\chi^2 = 6.0, p = 0.014$). After 5 min, although the trend of males

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**Fig. 3.** Natal syconium recognition experiments to examine the response of male pollinators to cues from whole galls, empty galls, volatiles and gall surface hydrocarbons when given choices between these cues from female pollinators (FP) and female non-pollinators (pooled between Apocryptophagus testacea, A. testacea, A. fusca, and Apocrypta sp. 2) from natal or non-natal syconia. (a–b): First-choice responses. (c–d): Responses after 5 min. The choices (excluding the 'no choice' responses) were analysed using chi-square tests. n.s. = non-significant difference ($p > 0.05$); *$p < 0.05$; **$p < 0.01$; n includes male pollinators exhibiting no choice.
species ($\chi^2 = 1.39, p = 0.24$). When male pollinators were given only volatile signals, they displayed a tendency to choose natal non-pollinator galls over non-natal pollinator galls (first choice, $\chi^2 = 2.9, p = 0.086$; 5 min, $\chi^2 = 2.19, p = 0.14$). This suggests that when male pollinators are presented female pollinator galls from a non-natal syconium together with non-pollinator galls from a natal syconium, the natal chemical signature seems to interfere with species recognition cues, possibly overriding mate recognition in this case. The statistical non-significance observed in some of these assays is unlikely to be due to low sample sizes as similar or lower sample sizes gave clearly significant results in other assays. Non-significance is more likely to be the results of the greater proportion of non-responding males with implications for mechanisms causing the lack of responsiveness.

4. Discussion

Mating aggregations can occur at oviposition sites (Jaenike et al., 1992), overwintering sites (Shine and Mason, 2001), feeding sites (Byers and Wood, 1980), leks (Diabaté et al., 2006) and adult emergence sites (Thorhill, 1976). Mating aggregations in fig wasps occur at adult emergence sites which are the enclosed interiors of fig syconia (Janzen, 1979; Weiblen, 2002; Herre et al., 2008). The interior of an F. racemosus syconium in wasp pre-dispersal phase is a crowded and dark arena which may contain an aggregate of up to a thousand wasps (A. Krishnan, unpublished data) composed of 3—4 different species on average (Ghara, 2012), all of which are sexually mature and mate at the same time. This system therefore provides us with a unique opportunity to study mate recognition in species that are not only sympatric, but also syntopic, under conditions that are crowded with the added complexity of females that are hidden behind physical barriers, i.e., within galls.

Our experiments on the pollinating fig wasp C. fusciceps indicate that the males of this species are capable of recognising female galls of their own species from female galls of other commonly co-occurring species with high specificity and accuracy on the basis of whole galls (occupant still within the gall), empty galls (occupant removed from the gall), and volatile and gall surface hydrocarbon cues from empty galls (Fig. 2). Since males can locate and recognise conspecific female galls even when they are empty, the gall surface could be considered as an extended phenotype (Dawkins, 1982) that can signal the species and sex of the hidden gall occupant. It is likely that the gall surface is impregnated with and perhaps permeable to species and sex specific chemicals which function as pheromones, a signalling system similar to that seen in the retreat silk of Myrmarche spiders (Borges et al., 2007) and the cocoons of the parasitoid Cephalonomia waterstoni (Howard, 1993).

Fig wasp males are likely to experience the chemical equivalent of a cocktail party effect, i.e., they are required to recognise mates of the appropriate species in the chemical babel of the syconium interior. In other systems where organisms need to contend against noisy background signals (Grafe et al., 2012), or diffuse chemical signals (Shine and Mason, 2001) to localise mates, multimodal cues are utilised in mate recognition (Shine and Mason, 2001; Heberts and Papaj, 2005; Partan and Marler, 2005; Grafe et al., 2012). Since anecdotal observations indicate that female fig wasps show movement within their galls (Murray, 1990; P. Yadav and R.M. Borges, pers. observ.), we suspect that mate recognition in fig wasps could be multimodal, involving vibratory and chemical signals from gall-bound females. Although we were unable to perform experiments to establish that male pollinators can identify conspecific female galls on the basis of vibratory signals alone, observations of male behaviour in the choice assays indicated that such signals could exist. In the whole-gall choice assays, many male pollinators often chewed open female pollinator galls and attempted to mate with the female inside; this behaviour was seldom exhibited by males in the empty-gall choice assays, which instead, demonstrated searching behaviour near the empty galls. In many assays, males often wandered away after making a first choice, thereby increasing the numbers of no-choices at the end of 5 min (Figs. 2 and 3). The increase in no-choice percentages at 5 min in the empty gall, volatile and surface hydrocarbon choice assays may be explained by the loss of some of the female mating signals when the female is removed from the gall. Furthermore, another possible explanation for this phenomenon could be due to a lack of further signals such as vibratory cues from female pollinators that may be necessary to initiate gall chewing and opening. Such cues may allow males to distinguish between empty galls or galls with dead or undeveloped females from those with viable females inside them. In the hydrocarbon choice assays, however, a few males were seen chewing the agarose coated with female pollinator gall extract. This suggests that surface hydrocarbons of the gall could also induce male chewing behaviour to open galls.

As male pollinators were able to use empty galls, volatiles and gall surface hydrocarbon cues individually to locate female galls, the multimodal mate recognition in this system could also be interpreted as exhibiting signal redundancy. This could be especially advantageous in improving the accuracy of mate recognition within physically and chemically noisy environments (Hebets and Papaj, 2005; Partan and Marler, 2005; Bro-Jørgensen, 2010) such as that of the syconium. Alternatively, these observations could also point to the use of multiple signals in a hierarchical manner—beginning with a volatile signal for species recognition and gall location, progressing to a tactile and/or gustatory signal to reinforce species recognition and perhaps to initiate gall opening and culminating in a vibratory signal that accelerates gall opening and mating. The pattern of increasing no-choice percentages at the end of 5 min was also observed in whole-gall choice assays. This is rather surprising, as in these assays the whole complement of cues are presented to the male pollinators, and such a response was not entirely expected. Since galls used in the assays were not examined to determine if they were previously perforated by male genitalia (indicating that the female pollinator within was already mated), and since female pollinators are generally assumed to mate only once (Zavodna et al., 2005; but see Murray, 1990), it is possible that the galls rejected by males after their first choice contained non-viable or mated females in which males had limited interest. It is also possible that vibratory signals from females could indicate their mated or virgin status, and that males and virgin females could engage in reciprocal vibratory communication as occurs in plant hoppers (Ichikawa, 1976).

Males of several hymenopteran species learn to recognise nestmates and prefer to mate with non-nestmates probably to avoid inbreeding (Ayasse et al., 2001). The ability to distinguish siblings from non-siblings could be mediated by the use of signal mixtures which are unique to individuals or nests unlike pheromones which are anonymous signals that mediate species recognition (Holldobler and Carlin, 1987; Wyatt, 2010). Although fig pollinating wasp populations have a high incidence of inbreeding (Zavodna et al., 2002; Molbo et al., 2004; Greeff et al., 2009; Kobmoo et al., 2009, 2010), fig wasps like most organisms with haplodiploid mating systems should be more resistant to inbreeding depression than diploid species (Antolin, 1999; Henter, 2003). Still, in some species of pollinators, males mate within their natal syconia and also disperse to find mates outside their syconia (Greeff, 2002; Greeff et al., 2003, 2009), thereby reducing inbreeding. In Ficus citrifolia, behavioural assays on males of the pollinating wasp indicated that they preferred a natal environment while searching for mates (Frank, 1985). In this study a majority of the marked male wasps confined between natal and non-natal syconium halves.
were found within the natal syconium halves at the end of 60 min. A major drawback in this experimental setup was that the movement of the males during their 60-minute confinement was unknown, and it is entirely possible that the males switched between the syconium halves several times. The experiments also did not distinguish between the males’ preference for sibling males or sibling females, nor did they explore the type of cues that the males may use to distinguish between natal and non-natal syconia. In our experiments, we show that males consistently prefer natal syconium galls containing female pollinators even in the absence of related males that could form ‘brother-mating groups’ (as hypothesised by Frank, 1985), and that males can distinguish between natal and non-natal galls using only volatile or gall-surface hydrocarbon cues (Fig. 3).

In F. racemosa, although syconia receive on average 2–10 foun-
dresses (M. Ghara and A. Krishnan, pers. observ.), microsatellite analysis of the C. fusciceps population from southeast Asia reveals high levels of inbreeding, probably due to unequal contribution to the brood by the first few foundresses (Kathuria et al., 1999; Zavadna et al., 2007; Kobmoo et al., 2009). Therefore, results of our study coupled with those of Frank (1985) are surprising and contrary to the expected result, which is that male pollinators either show no preference between the natal and non-natal galls or show a higher preference for non-natal galls. The preference of male pollinators for their natal environment is so strong that it seems to override even species-specific cues as seen in our experiments where males were offered choices between natal non-pollinator galls/volatiles and non-natal pollinator galls/volatiles. In these assays, males showed an initial preference for natal galls (though results for the first choices were not statistically significant) despite them belonging to female wasps of other species over non-natal galls of females of their own species, indicating that galls of individual fig syconia might have unique signature mixtures which attract male pollinators over and above species-specific signals. At a later time interval (5 min after initiating the assays), males show a tendency to choose galls of the right species although the result is not significant. These results might indicate olfactory memory of natal environments via conditioning during development as occurs in other insects (McCall and Eaton, 2001; Gupta and Stopfer, 2011; Geiselhardt et al., 2012) and that is manifest in the first choices made by male pollinators when they are faced with the “wrong” galls (non-pollinator galls) from the “right” syconia (natal syconia). This effect was not seen in the species recognition experiments, as the male pollinators had to choose between female pollinator galls and female non-pollinator galls originating only from non-natal syconia.

In conclusion, our studies have established that the males of the pollinating wasps of F. racemosa show high species specificity in choosing hidden females of their own species based on signals from whole intact galls, empty galls, volatiles and gall surface hydrocarbons, and that they can differentiate between galls from natal and non-natal syconia using similar cues. The strong preference for galls from natal environments is manifest in the indecisiveness shown by males when presented with non-pollinator galls from a natal environment together with galls containing pollinator females from a non-natal environment. These results suggest olfactory conditioning by natal environments which may override species recognition cues in certain contexts.

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