



Utilisation of chemical signals by inquiline wasps in entering their host figs



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ARTICLE INFO

Article history:

Received 12 May 2013

Received in revised form 13 August 2013

Accepted 13 August 2013

Available online 22 August 2013

Keywords:

6-Methyl-5-hepten-2-one

Body odour

Chemical signal

Diaziella yangi

Inquiline wasp

Lipothymus sp.

ABSTRACT

The fig tree, *Ficus curtipes*, hosts an obligate pollinating wasp, an undescribed *Eupristina* sp., but can also be pollinated by two inquiline (living in the burrow, nest, gall, or other habitation of another animal) wasps, *Diaziella yangi* and an undescribed *Lipothymus* sp. The two inquilines are unable to independently induce galls and depend on the galls induced by the obligate pollinator for reproduction and, therefore, normally enter receptive *F. curtipes* figs colonised by the obligate pollinators. However, sometimes the inquilines also enter figs that are not colonised by the pollinators, despite consequent reproductive failure. It is still unknown which signal(s) the inquilines use in entering the colonised and non-colonised figs. We conducted behavioural experiments to investigate several possible signals utilised by the inquilines in entering their host receptive figs. Our investigation showed that both inquiline species enter the receptive *F. curtipes* figs in response to the body odours of the obligate wasps and one of the main compounds emitted by the figs, 6-methyl-5-hepten-2-one. The compound was not found in the pollinator body odours, suggesting that the two inquiline wasps can utilise two signals to enter their host figs, which is significant for the evolution of the fig-fig wasp system. These inquilines could evolve to become mutualists of the figs if they evolve the ability to independently gall fig flowers; there is, however, another possibility that a monoecious *Ficus* species hosting such inquilines may evolve into a dioecious one if these inquilines cannot evolve the above-mentioned ability. Additionally, this finding provides evidence for the evolution of chemical communication between plants and insects.

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1. Introduction

Many insects are capable of inducing galls on plants (estimates range from 21,000 to 211,000 with an average of 132,930 species, Espírito-Santo and Fernandes, 2007), and galls provide the gall-inducing insects with nutrition, a favourable microclimate and protection from natural enemies and environmental stresses (Stone et al., 2002; Raman et al., 2005). Nevertheless, insect galls are usually exploited by other inquiline insects that are unable to induce galls independently and may decrease the gall makers' fitness by competing for gall tissue (Ronquist, 1994; Ronquist and Liljeblab, 2001; Stone et al., 2002; van Noort et al., 2007). In general, gall inquiline insects are highly specialised (Miller, 2004), and this means these insects have the ability to locate their host galls accurately. However, heretofore there are few studies on utilisation of signals by inquiline insects in their process of locating their host galls (Raman et al., 2005; Hossaert-McKey et al., 2010).

Fig wasps (Agaonidae, Chalcidoidea) gall and pollinate the flowers of fig trees (*Ficus* spp., Moraceae). In this obligate nursery pol-

ination mutualism, most of the nearly 750 *Ficus* species rely on a single specific species of agaonid wasp for pollination, and the wasp also depends strictly on its host *Ficus* for reproduction by galling some female flowerlets in the fig (Wiebes, 1979; Anstett et al., 1997; Herre et al., 2008). Each fig is an enclosed inflorescence (syconium) lined with male and uniovulate female flowers on its inside surface. The fig often has a globular shape with a small opening (the ostiole, constituted of a series of bracts) at the outward end that allows access to agaonid wasps. Fig development is synchronous on the same trees and can be divided into 'pre-receptive', 'receptive', 'interfloral', 'male', and 'postfloral' stages (Galil and Eisikowitch, 1968). The volatiles of figs at each stage differ from each other in quantity and quality and play a key role in mediating the attraction and dispersal of specific pollinators (Proffit et al., 2008; Gu et al., 2012). When receptive (the figs can be entered and their female flowers pollinated), figs emit specific volatile compounds for attracting the associated pollen-carrying female agaonid wasps (Grison-Pigé et al., 2002a; Chen et al., 2009; Hossaert-McKey et al., 2010; Gu et al., 2012). The wasps enter the figs through the ostiole. Once inside, the wasps pollinate the flowers and lay their eggs in some of the flowers. Fig pollination by the wasps is active or passive, depending on the species of figs and

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wasps. Actively pollinated figs have few male flowers and anthers do not shed their pollen (Kjellberg et al., 2001), and the wasps show distinctive behaviours for collecting and depositing pollen (Galil and Eisikowitch, 1969; Frank, 1984). In contrast, passively pollinated figs have relatively more male flowers and their mature anthers dehisce naturally for releasing pollen; the wasps passively carry pollen on various parts of their body surfaces and show no active pollination behaviour (Kjellberg et al., 2001).

In *Ficus*, there are two breeding systems, monoecy and dioecy. The figs of monoecious species contain both male and female flowers and produce pollen, seeds, and wasps. In dioecious fig trees, female and male figs occur on separate plants. Female figs contain only female flowers and produce only seeds. Male figs contain both female flowers (which either become galls or are aborted) and male flowers. Female flowers produce only wasps and pollen is transported by the wasps from the male figs to the female figs.

Fig. also support a diverse community of non-agaonid fig wasps belonging to other chalcidoid families, and these wasps are usually divided into three ecological groups (West et al., 1996; Herre et al., 2008; Cook and Segar, 2010; Chen et al., 2013): (1) gallers that are of similar size to or larger than pollinators and induce galls inside the figs from the exterior or by entering figs, (2) inquilines that are also of similar size to or larger than pollinators and dependent on the galls of pollinators for reproduction due to their inability of inducing galls, and (3) parasitoids that are of similar size to pollinators and prey on the larva of pollinators or other wasps. Some of these non-agaonid wasps oviposit internally and in passively pollinated *Ficus* species can pollinate figs (Jousselein et al., 2001; Zhang et al., 2008). Once inside, the gallers oviposit their eggs individually into flowers as do the pollinators, and the inquilines and parasitoids may lay their eggs only into flowers that already contain an egg or eggs of the pollinators or of other wasps.

Ficus curtipes is a passively pollinated monoecious fig tree. It hosts three internally ovipositing wasps: one obligate pollinating wasp, an undescribed *Eupristina* sp., and two inquiline wasps: *Diaziella yangi* and an undescribed *Lipothymus* sp. The two inquiline wasps are larger than the *Eupristina* pollinator, and can enter and efficiently pollinate the *F. curtipes* figs at receptivity (Zhang et al., 2008). Once inside, the inquilines deposit their eggs only in the flower ovules containing the eggs oviposited by the obligate pollinators (Chen et al., 2013). Moreover, the two inquilines usually enter the figs already colonised by the obligate pollinators because they cannot induce galls and will fail to reproduce without the galls of the obligate pollinators (Zhang et al., 2008). Therefore, we hypothesise that the inquilines use the body odour of the obligate pollinators as a cue to enter receptive *F. curtipes* figs colonised by *Eupristina* sp.

However, approximately 13% of one or both inquilines have been observed to enter and pollinate receptive (and more mature, Gu, personal observation) figs without *Eupristina* pollinators (Zhang et al., 2008), although doing so will result in their own reproductive failure. The signals the two inquilines use in entering the receptive figs in which *Eupristina* pollinators are absent is unknown. Previous studies have shown that non-agaonid fig wasps can use fig volatiles to locate their host figs at appropriate stages (Proffitt et al., 2007, 2009). Whether the two inquilines are also capable of utilising the volatiles of the receptive *F. curtipes* figs to enter these figs, and, the compound(s) in the volatiles they might respond to, are unknown. Two volatile compounds (6-methyl-5-hepten-2-ol and 6-methyl-5-hepten-2-one) are associated with the receptive figs of *F. curtipes* (Gu et al., 2012), and their relative amounts increase markedly with the artificial and/or natural prolongation of the receptive phase of figs (Gu, 2012; Gu et al., 2012). Moreover, only small traces of the two compounds have been identified in the volatiles of a few other fig species (Grison et al., 1999; Song et al., 2001; Grison-Pigé et al., 2002a,b; Proffitt

et al., 2008, 2009; Chen et al., 2009; Proffitt and Johnson, 2009). Thus, we propose that the two inquilines utilise one or both of the two compounds as a signal for entering the un-colonised receptive figs.

To test our hypotheses we painted early receptive *F. curtipes* figs with 6-methyl-5-hepten-2-ol, 6-methyl-5-hepten-2-one, and the body odour of the female *Eupristina* sp., and observed whether *D. yangi* and *Lipothymus* sp. would enter these figs artificially loaded with odours. Because colonisation of receptive *F. curtipes* figs by one *D. yangi* and one *Lipothymus* sp. or two individuals of each have been observed under natural conditions (Zhang et al., 2008), we also investigated whether *D. yangi* or/and *Lipothymus* sp. would enter early receptive figs artificially loaded with the body odours of the females of *D. yangi* and/or *Lipothymus* sp.

2. Materials and methods

The study was conducted at the Xishuangbanna Tropical Botanical Garden (XTBG), Yunnan Province, China (21°55'N, 101°25'E). Five *F. curtipes* trees bearing pre-receptive figs (immature figs that cannot be entered and pollinated by their pollinating wasps) were selected. The twigs bearing the pre-receptive figs were covered with mesh bags to prevent wasps or other insects from entering or damaging these figs. Mesh bags were removed and experiments carried out once the figs developed into early (2- or 3-day-old) receptive figs (i.e., figs can be entered and pollinated). Female *Eupristina* sp., *D. yangi* and *Lipothymus* sp. were collected from male-stage figs and stored separately in nylon bags. Individuals used as body odour stimuli were killed by placing them in a freezer at -20 °C for 30 min. 6-methyl-5-hepten-2-ol and 6-methyl-5-hepten-2-one were purchased from Sigma-Aldrich (St. Louis, MO, USA).

The receptive figs were randomly selected. Each fig and each wasp were used in a single one-odour treatment. Each odour was tested using a minimum of 20 figs and the same numbers of *D. yangi* or *Lipothymus* sp. To assess whether an odour could stimulate fig-entry behaviour in *D. yangi* and *Lipothymus* sp., we painted a fig with the odour and placed a wasp on the fig to observe whether the wasp entered the fig within 10 min (based on personal observations of maximum time to entry of 378 s under natural conditions). Five micro litre of pure 6-methyl-5-hepten-2-ol or 6-methyl-5-hepten-2-one was dropped on a piece of filter paper with a micropipette, and the paper used for spreading the chemical on the bracts of the fig ostiole. Because wasps often expel body fluid when squeezing themselves through the ostiole, to simulate body odour, a freeze-killed female wasp was squashed onto a piece of filter paper, and the paper loaded with the body odour was used for spreading the body odour on the bracts of the ostiole of a fig. Receptive figs wiped with filter paper alone were used as controls. Each filter paper was used for a single test.

Chi-square tests were used for comparing the numbers of wasps that entered figs.

3. Results and discussion

Both 6-methyl-5-hepten-2-one and the body odours of the *Eupristina* sp. females stimulated both *D. yangi* and *Lipothymus* sp. to enter the receptive figs (Fig. 1). This entry behaviour was not stimulated by 6-methyl-5-hepten-2-ol or the body odours of the two inquiline species (Fig. 1).

Results of this study support our hypothesis that both *D. yangi* and *Lipothymus* sp. are capable of utilising the body odours of the *Eupristina* sp. females and 6-methyl-5-hepten-2-one as signals to enter their shared host receptive figs. More importantly, 6-methyl-5-hepten-2-one is one of the main compounds emitted

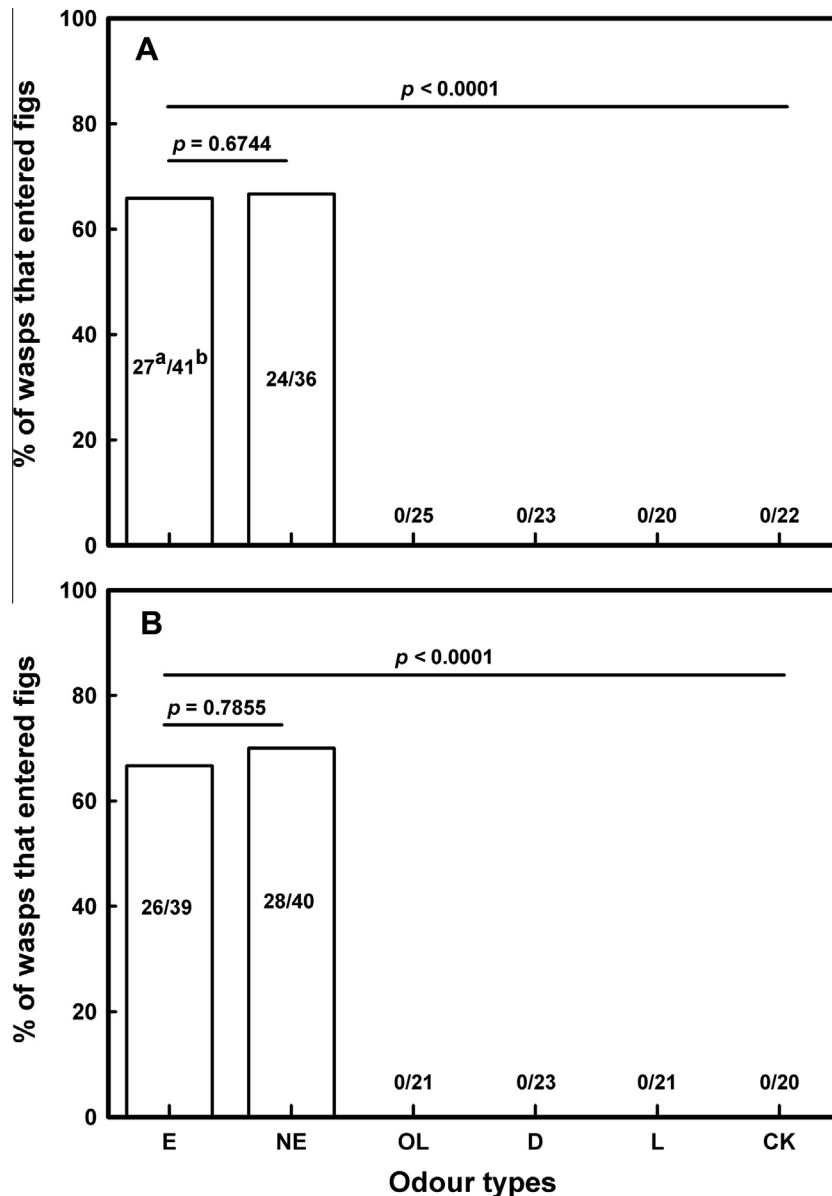


Fig. 1. Six odours tested for their role as cues used by two inquilines, *D. yangi* (A) and *Lipothymus sp.* (B), to enter the receptive figs of their shared host *F. curtipes*. E, body odour of the pollinator *Eupristina sp.*; NE, 6-methyl-5-hepten-2-one; OL, 6-methyl-5-hepten-2-ol; D, body odour of *D. yangi*; L, body odour of *Lipothymus sp.*; CK, blank control, i.e., filter paper. Numbers in each bar represent the number of wasps that entered figs (a) and the total number of wasps tested (b) in each odour treatment. *P*-values were obtained from Chi-square test for comparing the numbers of wasps that entered figs.

by the figs (Gu, 2012; Gu et al., 2012), and this chemical is not found in the body odours of the *Eupristina sp.* females (Gu, 2012). This means that the two inquilines can utilise two different signals to enter their host figs.

Under natural conditions, the observed proportion of the receptive figs occupied by only one or both of the two inquiline wasps was about 13% from February to May (dry season) at XTBG (Zhang et al. 2008). This proportion in our investigation was about 30% ($n = 107$ figs in four trees) when we sampled figs in the rainy seasons and in a dense forest (Gu and Yang, unpublished data), suggesting that this phenomenon may be more common in the rainy season and in dense forests. This may be because there is a lack of *Eupristina sp.* under these conditions, which prolongs the receptive phase of figs and thus induces the figs to emit more 6-methyl-5-hepten-2-one than figs at early receptive stage (Gu, 2012; Gu et al., 2012). The relative amount of 6-methyl-5-hepten-2-one in the volatiles emitted by the older (9–12-day-old) receptive

F. curtipes figs is over twice that emitted by the early (2–3-day-old) receptive ones (Gu, 2012). However, further investigation is needed to address why inquilines have adopted the seemingly maladaptive behaviour of entering figs that do not contain *Eupristina* pollinators.

Although the two inquiline species are able to pollinate *F. curtipes* figs (Zhang et al., 2008) the early receptive *F. curtipes* figs do not emit enough 6-methyl-5-hepten-2-one to provoke the two inquilines to enter and pollinate them. Perhaps, owing to reproductive failure of the two inquilines, the figs pollinated by an inquiline cannot implement their male function (i.e. their pollen cannot be transported) and this is disadvantageous to the long-term coexistence of the fig trees and the inquilines. The body odours of the obligate pollinators should be the major signal channel for the two inquilines entering the receptive figs, and 6-methyl-5-hepten-2-one may be an alternative signal to the body odours of the obligate pollinators for both the figs and the two inquilines.

Most importantly, however, this finding has great significance for understanding the evolution of the fig tree-fig wasp system. Inquiline wasps associated with some fig trees are effective pollinators (Jousselin et al., 2001; Zhang et al., 2008), and can independently enter their receptive host figs by utilising the volatiles emitted by the figs. If inquiline wasps evolve the capability to independently make galls for reproduction, they will, like the obligate agaonid wasps, establish a mutualistic relationship with their host figs. There is, however, another possibility. If the inquiline wasps capable of independently entering and pollinating figs do not make galls for their own reproduction, as supposed by Frank (1989), a monoecious *Ficus* species hosting such inquilines may evolve into a dioecious one to avoid inquiline wasps adversely affecting its male reproductive success.

Our findings also have significance in terms of the evolution of chemical communication between plants and insects. Inquiline wasps use 6-methyl-5-hepten-2-one as an alternative cue to the body odours of the obligate pollinators to enter receptive figs. This is an example of the existence of the starting point for the evolution of chemical communication between plants and insects (Steiger et al., 2011). In the fig-fig wasp system, the existence of the starting point could further increase the possibility that the inquiline wasps establish a direct mutualistic or parasitic relationship with their host fig trees. Furthermore, our finding that the inquilines use two different signals in entering their host figs under different conditions supports the hypothesis that the chemical(s) used for communication among species could change with environmental pressures (Steiger et al., 2011). Therefore, this case may be a good model for studying the evolution of chemical communication between plants and insects.

In summary, our results suggest that the two inquiline species, *D. yangi* and *Lipothymus* sp., can utilise the body odour of the galls' legitimate inhabitants (*Eupristina* sp.) and the odour of their 'houses' (the receptive figs but not the galls *per se*) to enter their 'houses', and that insects have the ability to use alternative signals. This way of inquiline insects utilising signals may be beneficial to the maintenance of a stable relationship among plants, gall insects and gall inquiline insects and to the evolution of inquiline insects. This study, to our knowledge, is the first to look at chemical signal utilisation by inquiline insects.

Acknowledgements

We thank two anonymous reviewers for their thoughtful and constructive comments, and Dr. Pelin Kayaalp for her valuable comments and help with the language. This work was supported by the Chinese Natural Science Foundation (30970403 and 31200292) and the CAS 135 Program (XTBG-F01).

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