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Private channel: a single unusual compound assures specific pollinator attraction in *Ficus semicordata*

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Summary

1. Floral scents have been suggested to play a key role in the obligate pollination mutualism between figs and fig wasps. However, few studies have determined whether pollinator-attractive compounds could alone assure species-specificity ('private channel'), or whether specificity is mediated by more complex 'floral filters', of which scent is only one component.

2. We examined changes in the floral volatile compounds of *Ficus semicordata*, a dioecious fig species, during and after pollination using headspace collection and compound identification by Gas Chromatography/Mass Spectrometry (GC/MS). One benzenoid compound, 4-methylanisole, was strongly predominant (94–98%) among the volatile compounds emitted by both male and female receptive figs of *F. semicordata*, whereas it was totally absent in the volatiles emitted by figs 4 days after pollination, as well as in receptive-stage volatiles emitted by two other sympatric fig species, *Ficus racemosa* and *Ficus hispida*.

3. Bioassays using the specific pollinator of *F. semicordata*, *Ceratosolen gravelyi*, in a Y-tube olfactometer showed that 4-methylanisole was attractive to *C. gravelyi* in a wide range of concentrations (from $1{\cdot}22 \times 10^{-2}$ ng/100 μL to $1{\cdot}22 \times 10^{6}$ng/100μL). Moreover, chemical blends lacking 4-methylanisole were unattractive to *C. gravelyi*. These non-active odour sources included volatile compounds emitted by receptive figs of the two other sympatric fig species and volatiles of *F. semicordata* post-pollination figs.

4. All these results suggest that 4-methylanisole is the main signal compound in the floral scent of *F. semicordata* that attracts its obligate pollinator to the host figs at the precise stage required for pollination and oviposition. Furthermore, the high proportion of 4-methylanisole in the odours of receptive figs of both sexes was consistent with the hypothesis of chemical mimicry in dioecious figs.

5. A simple signal comprised of one compound that is unusual among *Ficus* and that is an infrequent, usually minor, component of other floral odours, may thus function as a private channel in this specialized obligate mutualism.

Key-words: 4-methylanisole, behavioural tests, *Ceratosolen gravelyi,* chemical mediation, dioecy, nursery pollination mutualism, olfactory signal, post-pollination changes

Introduction

Floral scents are complex blends of volatile organic compounds which have been suggested to be an ancient medium of chemical communication in plant–pollinator interactions (Pellmyr & Thien 1986; Visser 1986; Dobson 1994; Raguso 2001, 2008b; Knudsen *et al.* 2006). These olfactory cues are often essential for pollinators to locate their host plant and find floral rewards (Grison-Pigé, Bessière & Hossaert-McKey 2002a; Dufaÿ, Hossaert-McKey & Anstett 2003; Raguso 2008a). In turn, scent-driven pollinator preference and constancy have been invoked as isolating mechanisms for many diverse angiosperm taxa (Dodson *et al.* 1969; Knudsen 2002; Raguso 2008b). Pollinators can also detect floral scent changes and use them in orienting to available flowers and avoiding reward-depleted flowers (Schiestl *et al.*

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2003; Theis & Raguso 2005). The isolation between plant species and the stage-dependent attraction of pollinators, both of which are driven by floral scent variation, increase the effectiveness of pollination (Pellmyr 1986; Weiss 1991; Theis & Raguso 2005; Muhlemann *et al.* 2006; Terry *et al.* 2007; Proffit *et al.* 2008). In some obligate interactions, such as nursery pollination systems, odours have been shown to facilitate partner encounters (Grison-Pigé, Bessière & Hossaert-McKey 2002a; Dufaÿ, Hossaert-McKey & Anstett 2003; Svensson, Pellmyr & Raguso 2006).

Among the very specialized plant–pollinator interactions is the fig/fig wasp pollination mutualism. The genus *Ficus* (Moraceae) consists of about 750 species of mainly tropical plants which are involved in an obligate nursery pollination mutualism with fig wasps (Agaonidae, Chalcidoidea) (Wiebes 1979; Berg 1989; Cook & Rasplus 2003). Each fig species is generally pollinated by females of a single species-specific agaonid wasp and provides, in return, oviposition sites for the pollinating wasp and food for its larvae (Janzen 1979; Wiebes 1979; Anstett, Hossaert-McKey & Kjellberg 1997; Cook & Rasplus 2003). *Ficus* produce urn-shaped inflorescences called figs, the inside of which is lined by tiny unisexual flowers. When the female flowers are receptive, specific mutualist pollinating wasps are attracted to the figs. Loaded with pollen, they enter the fig, pollinate and oviposit in some of the ovules. Their larvae feed exclusively on the developing ovule tissues of the host. Some weeks later the wasps emerge into the fig cavity at a time when the fig's male flowers are mature and have released pollen. The winged female wasps become loaded with pollen (or actively load pollen in specialized pollen pockets), mate with wingless male wasps, and leave the fig in search of a new receptive fig of the same species in which to oviposit. At the adult stage, female pollinating wasps do not feed and live for only a few hours (Kjellberg, Doumesche & Bronstein 1988). Because figs serve as the only host for their pollinating wasp, neither partner can successfully reproduce in the absence of the other.

Specialized chemical attraction of the wasp promotes species-specificity in fig/pollinator interactions (Grison-Pigé, Bessière & Hossaert-McKey 2002a). When figs are receptive, the female pollinating fig wasps, attracted by specific volatile signals emitted by the host fig, crawl into the fig cavity through the sole entrance of the fig, the bract-lined ostiole (Ware *et al.* 1993; Hossaert-McKey, Gibernau & Frey 1994; Gibernau *et al.* 1998; Song *et al.* 2001; Grison-Pigé, Bessière & Hossaert-McKey 2002a; Chen & Song 2008; Proffit *et al.* 2009). Post-pollination changes in the fig floral scent may provide an effective olfactory signal to fig pollinating wasps enabling them to avoid figs that have already been pollinated (Proffit *et al.* 2008).

Whether floral scent alone can assure species-specificity, acting as a 'private channel' that attracts only the appropriate pollinator, is not known. In addition to odour, physical characteristics of the fig, such as ostiolar morphology (Verkerke 1989), could be part of a 'floral filter' assuring specificity (Gibernau *et al.* 1998).

Floral scent, however, may be particularly critical in the reproduction of fig species that are dioecious. About half of fig species are anatomically gynodioecious but functionally dioecious, with trees producing either male or female figs. In 'male' figs, female flowers are present but have very short styles, allowing pollinators to oviposit in all of them. Male figs thus produce only pollen and pollen vectors, and no seeds. In female figs, no functional male flowers are present and because the length of the style is much longer than the pollinator's ovipositor, the wasps cannot oviposit in the ovule of the flowers. Female figs produce only seeds (for more details, see Kjellberg *et al.* 1987; Cook & Rasplus 2003). Thus, in dioecious fig species, an extreme conflict occurs between the fig and its pollinating wasp: wasps entering female figs cannot oviposit in these figs and act only as pollinators, receiving no reward and dying in the fig without reproducing (Kjellberg *et al.* 1987; Patel *et al.* 1995; Anstett, Hossaert-McKey & Kjellberg 1997). Female figs are pollinated by deceit. In dioecious species, differences in floral scent emitted by receptive figs of the two sexes could be used by the pollinator to avoid the non-rewarding sex (female figs). However, on the fig side, selection for chemical mimicry between sexes is expected to maintain the reproductive success of both sexes (Grafen & Godfray 1991; Patel *et al.* 1995; Borges, Bessière & Hossaert-McKey 2008; Proffit *et al.* 2008).

To examine the role of plant volatiles in the specificity of pollinator attraction, to explore post-pollination changes in volatiles emitted, and to test the hypothesis of chemical mimicry between sexes, we collected and analyzed the volatile chemicals emitted by both male and female figs of the functionally dioecious species *F. semicordata* J.E. Smith (Fig. 1). We also tested the behavioural responses of *Ceratosolen gravelyi* Grandi (Agaonidae), the specific pollinator of this fig species (Wang, Yang & Peng 2003; Zhen *et al.* 2005), to volatile signal(s) emitted by receptive figs of its specific host or to scents of sympatric non-host figs. We aimed to answer the following questions: (i) What floral compounds are emitted by male and female figs of *F. semicordata*, and which of these compounds are responsible for pollinator attraction at the receptive stage? (ii) What are the post-pollination changes in floral scent of *F. semicordata*, and how do pollinators respond to these changes? (iii) Are odours of the two sexes in this dioecious species more similar at the receptive stage than at other stages, as predicted by the chemical-mimicry hypothesis? (iv) Could floral scent alone assure the specificity of the attraction of *C. gravelyi*?

Materials and methods

STUDY SITE AND NATURAL HISTORY OF THE SPECIES

This study was carried out in Xishuangbanna Tropical Botanical Garden (21°41′N, 101°25′E), Chinese Academy of Science, Yunnan Province, southwestern of China.

Ficus semicordata J.E. Smith (subgenus *Sycomorus*, section *Hemicardia*), a functionally dioecious fig species, is only pollinated by *C. gravelyi* Grandi. The trees studied occur naturally in the garden.

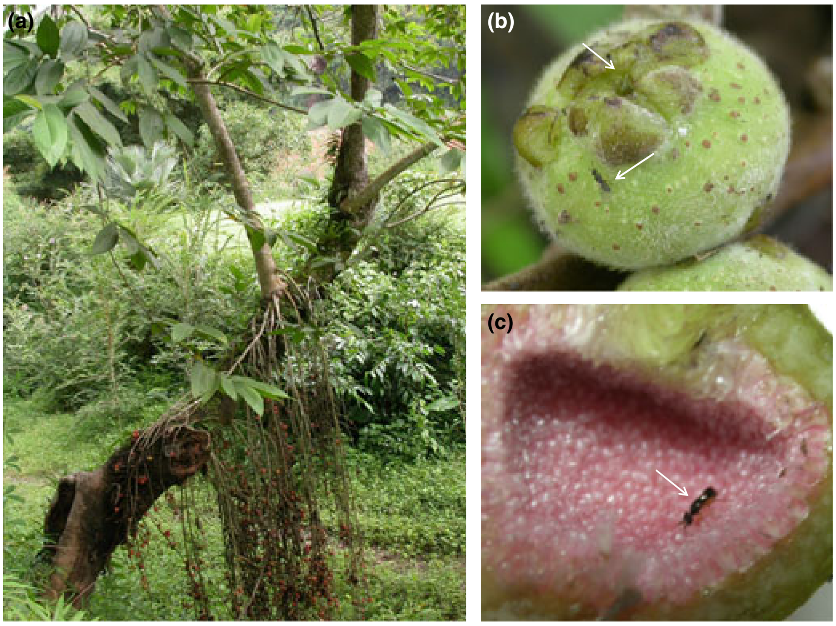

Fig. 1. (a) *Ficus semicordata* (male tree) in Xishuangbanna Tropical Botanical Garden. (b) fig at receptive stage; (c) pollinating fig wasp, *Ceratosolen gravelyi* within the fig cavity. White arrows indicate the pollinating fig wasp.

All the volatile extractions were performed from Oct. 2006 to July 2007 and the behavioural tests were performed from April 2007 to July 2007.

In the behavioural tests of host specificity of chemical attraction of *C. gravelyi*, three species of figs were tested: the host plant *Ficus semicordata* J.E. Smith (subgenus *Sycomorus*, section *Hemicardia*), a second functionally dioecious fig species, *F. hispida* L. (subgenus *Sycomorus*, section *Sycocarpus*) and a monoecious fig species *F. racemosa* L. (subgenus *Sycomorus*, section *Sycomorus*). All three of these *Ficus* species are sympatric in Yunnan province and flower and fruit year-round in our study site.

COLLECTION OF VOLATILE COMPOUNDS

Pre-receptive figs of *F. semicordata* of very similar diameter from male and female trees were covered with mesh bags to exclude pollinators. Once most of these experimental figs loosened their ostiolar bracts and began to emit a strong smell which could be detected by human noses, a change accompanied by the arrival of large numbers of pollinating wasps that perch on the bags enclosing the figs, we began headspace collection of volatiles of the receptive-stage figs. After this first collection, the figs were uncovered from the bags and marked, and a second collection was conducted 4 days later (post-pollination stage). Because this species, like most figs, exhibits strong within-tree flowering synchrony, 4 days was long enough to ensure that all of the experimental figs were pollinated (Khadari *et al.* 1995). We ran volatile collections on five female trees and five male trees. Among them, two female fig trees that flowered during the rainy season failed to be pollinated and their figs were aborted. From these two trees odours were thus collected only at receptive stage.

Volatile compounds released by figs were collected by adsorption-desorption (dynamic headspace technique) (Grison-Pigé, Bessière & Hossaert-McKey 2002a; Proffit *et al.* 2008). Branches with 30–120 figs were enclosed in polyethylene terephtalate (Nalophan®) bags (Kalle Nalo GmbH, Wursthüllen, Germany) for collection. Airflow was maintained through the bags by pumps connected to flowmeters. A charcoal filter (Supelco, Bellefonte, PA, USA) was connected to the entrance of the bag at a flow rate of 300 mL min^{-1}, and a glass cartridge (7 mm Internal Diameter) filled with 100 mg of Super Q absorbent (ARS Inc., Gainesville, FL, USA) was connected to the exit of the bag at a flow rate of 280 mL min^{-1}. Empty bags were used as ambient controls to check for possible contaminations during our volatile collections. Odour collection was performed for 3 h during the same period of the day (about 11.00–14.00 h). After volatile collection, the filters were twice eluted with 150 μl dichloromethane. Two internal standards, octane (Fluka Chemie GmbH, Buchs, Switzerland) and decyl acetate (Alfa Aesar, Heysham, Lancashire, UK), were added to each sample for gas chromatography (octane, 117·00 ng μL^{-1}; decyl acetate, 144·17 ng μL^{-1}). Purity of standards was as follows: octane (≥99%), decyl acetate (≥98%). The extracts were then concentrated to 100 μl by solvent evaporation in a graduated vial and stored at −18°C in glass vials until analysis.

TENTATIVE IDENTIFICATION OF COMPOUNDS

To identify the compounds in samples of volatiles, the extracts were injected into a coupled Gas Chromatography-Mass Spectrometry (GC/MS) system (Thermo Finnigan Trace DSQ mass spectrometry, Thermo Electron Corporation, Watham, MA, USA), equipped with a 30 m DB-5 column (methyl siloxane, 0·25 μm film thickness, 250 μm ID, J&W Scientific, Folsom, CA, USA) with helium as carrier gas. Ionization was by electron impact (70eV, source temperature 200°C). For each sample, 0·2 μl was injected (split with a 20 : 1 ratio). The injector temperature was 200°C. The column temperature was kept at 40°C for 4 min with a following program of 4°C min^{-1} up to 180°C.

Tentative compound identification was based on computer matching of the mass spectra with NIST'02 libraries for Xcalibur 1·3 and on retention indices reported in the literature (Adams 2001) and when possible by injection of reference compounds. The quantities of volatile compounds produced by figs were estimated by using the average peak area of the two internal standards as a scale. The relative proportions of all compounds emitted were also calculated for each sample.

STATISTICAL ANALYSES OF THE VOLATILE BLENDS

All statistical analyses were performed using R version 2.7.0 (R Development Core Team, 2008).

In order to compare scent composition among samples, we used the function metaMDS (Vegan package, Oksanen *et al.* 2008). Data were first square-root transformed and then standardized using a Wisconsin double standardization to normalize the data. Then a data matrix of pairwise comparisons among samples was calculated using the Bray-Curtis distance index (Bray & Curtis 1957), ranging between 0 and 1. A non-metric multidimensional scaling (NMDS) was used to find the best low-dimensional representation of the distance matrix. NMDS is a non-parametric dimension reduction technique that works without the assumption of linear or unimodal response and escapes many of the distortions of eigenvector techniques (Legendre & Legendre 1998). To evaluate how well (or poorly) the particular configuration produces the observed distance matrix, the stress value is given. The smaller the stress value, the better the fit of the reproduced distance matrix to the observed distance matrix (Clarke 1993). In this analysis, several iterative processes were run until we obtained two similar configurations with minimum stress values. Finally, the solution was scaled by rotating the solution so that the largest variance of samples was on the first axis. The null hypothesis of no difference in patterns of scent composition (between sexes at the receptive stage and post-pollination stage, and between stages within each sex) was tested with a multiple response permutation procedure (MRPP) on the matrix of dissimilarity with 10 000 permutations. MRPP is a non-parametric multivariate test appropriate for community data sets which fail to meet assumptions of normality or homogeneity of variances (McCune & Grace 2002). MRPP calculates a within-group agreement A that is based on the fraction of permuted pairwise dissimilarities that are less than the observed pairwise dissimilarities between the sampling plots (McCune & Grace 2002). $A_{\max} = 1$ when all items are identical within groups, $A = 0$ when heterogeneity within groups equals expectation by chance, and $A < 0$ when there is more heterogeneity within groups than expected by chance.

In order to test if there were significant differences among the total quantities of volatiles emitted by figs of the two sexes at receptive and post-pollination floral stages, we used one-way analysis of variance (ANOVA) followed by Tukey's 'Honest Significant Difference' (Tukey HSD) method for pairwise multi-comparison tests. A log transformation of the explained variable was performed to assure the normality of the residuals (Shapiro-Wilk test, $P = 0{\cdot}14$).

Relative amounts of the dominant compound, 4-methylanisole, could not be transformed to fit the assumptions of ANOVA. Therefore non-parametric statistical comparisons were performed. Kruskal–Wallis tests and Mann–Whitney *U*-tests for paired comparisons were used to test for differences in the relative amounts of this compound between sex and stage of development of the figs with a false discovery rate (Benjamini & Hochberg 1995) control to adjust *P* values.

BEHAVIOURAL TESTS

To test the responses of pollinating fig wasps to odours of fresh figs or synthetic compound(s), a Y-tube olfactometer (stem 8 cm; arms 9 cm; at 55° angle; i.d. 1·5 cm) was used (Tooker, Crumrin & Hanks 2005; Chen & Song 2008). Each arm was connected to a glass container or a Nalophan® bag containing an odour source. Air was drawn through Teflon tubing by an air pump and passed through a charcoal filter and distilled water. The cleaned and humidified air stream, regulated to a flow rate of 200 mL min^{-1} with two flowmeters, was split via a 'Y' hose junction to create two equal air streams. To limit visual distractions for the insects, the olfactometer was placed in the centre of a flat white table illuminated with three 40-W cool white fluorescent tubes placed above the arms of the Y-tube. The room was kept dark by covering the window with black curtains. The air temperature was monitored and maintained at ~26°C.

Each wasp was tested independently and was given 5 min to move in the olfactometer. A choice of the left or right arm of the olfactometer was noted when the insect went 1 cm (decision line) past the Y junction and stayed there more than 1 min. Wasps which did not reach the decision line after 5 min were removed and recorded as having exhibited 'no choice'. The treatments were switched between the two arms of the Y-tube every four bioassays to avoid any influence of unforeseen asymmetries in the setup. The olfactometer was rinsed with pure ethanol and then dried after each bioassay. Two different experiments were conducted.

Experiment A: responses of C. gravelyi *to odour sources of fresh figs*

To distinguish between the response of *C. gravelyi* to odours of receptive vs. post-pollination figs of the host, as well as between odours of host vs. non-host figs, we conducted two-choice experiments, in which the wasps were offered a choice of fig odour vs. clean air (control). Different types of fig odour were successively tested: scent emitted by: male receptive figs of *F. semicordata*; female receptive figs of *F. semicordata*; male pollinated figs of *F. semicordata*; female pollinated figs of *F. semicordata*; male receptive figs of *F. hispida*; and receptive figs of *F. racemosa*. In each case, three fresh figs were placed into a Nalophan® bag and used as the odour source. A bag of identical size was used as control for supplying clean air. The figs were replaced with new ones every 1–2 h. Each test (= one type of odour tested) was replicated until a sufficient number of wasps (n = 24~32) chose one of the two odour sources in the Y-tube. An individual wasp was used for only one test and was never used again.

Experiment B: responses of C. gravelyi *to different concentrations of synthetic compound(s)*

After identifying the floral scent compounds of *F. semicordata*, we tested the attraction of *C. gravelyi* to the main compound emitted by figs of this species at receptivity. Different dilutions in dichloromethane of pure synthetic 4-methylanisole (Fluka Chemie GmbH, Buchs, Switzerland) (purity: ≥98%) were prepared (from 1×10^{-11} μL/100 μL to LμL/100 μL) and tested separately. The different solutions were separately dropped (100 μL) on a piece of cellulose sponge (1 × 1 × 0·2 cm). In the same way, a piece of cellulose sponge with pure solvent (100 μL of dichloromethane) was prepared as a control. After 15 min of solvent evaporation, each piece of sponge was stored in a pipette tube. The tube was put into a 30 mL glass container, and the container was connected with one arm of the Y-tube. The compound put into the pipette tube was used as a sample of odour source and tested individually on one wasp. Each pipette tube was made from a 7 cm long, 1 mL polypropylene pipette, from which 3 cm of the base and 1·5 cm of the tip were removed. Trials were repeated until at least 40 wasps responded to the odour sources or to the control in the Y-tube.

First, using only pure air in each arm, we tested if there was any bias in our olfactometer, with wasps entering one of the arms (left or right) more often than the other. The behavioural choice made by each individually tested wasp was recorded by distinguishing three modalities: choice for fig odour, choice for pure air, or no choice. We considered that wasps made no choice when they stayed motionless for 5 min in the departure section and/or the central arm (i.e. before bifurcation) of the olfactometer. Then, to determine if wasps were attracted by fig odours, for each type of odour tested, data were arranged in a 2 × 2 contingency table. We compared the proportion of wasps that chose the right olfactometer arm or the left, when the tested fig odour was on the left vs. on the right (excluding the 'no choice' response). We then examined if there was a difference of attraction between the different types of odours by comparing the proportions of wasps that chose each odour. For all comparisons, the response of wasps was statistically analysed using $\chi 2$-tests with Yates' correction (when observed values in at least one cell of the contingency table were < 5).

Table 1. Occurrence and relative amounts of volatile compounds emitted by male and female figs of *Ficus semicordata* at receptive and post-pollination stages

		Female receptive (n = 5 trees)		Female post-pollination (n = 3 trees)		Male receptive (n = 5 trees)		Male post-pollination (n = 5 trees)	
Compounds	RI	%	O	%	O	%	O	%	O
Fatty acid derivatives									
Dodecane	1200	0·06 ± 0·03	4	0·32 ± 0·07	3	0·08 ± 0·05	3	—	
Tetradecane	1400	0·05 ± 0·03	2	0·16 ± 0·00	3	0·01 ± 0·01	2	0·15 ± 0·10	2
Pentadecane	1500	0·05 ± 0·02	3	0·26 ± 0·02	3	0·03 ± 0·02	3	0·57 ± 0·39	3
Mean of total percent		*0·15*		*0·74*		*0·12*		*0·72*	
Terpenes									
Monoterpenoids									
α-Thujene	922	—		1·34 ± 0·44	3	—		4·27 ± 4·03	3
α-Pinene	930	0·10 ± 0·05	5	7·27 ± 3·24	3	0·02 ± 0·01	2	7·61 ± 3·71	4
Sabinene	968	0·28 ± 0·18	5	22·00 ± 4·34	3	0·02 ± 0·01	2	8·47 ± 2·40	4
β-Pinene	972	0·02 ± 0·01	2	3·23 ± 2·73	3	0·01 ± 0·00	2	1·81 ± 1·49	3
β-Myrcene	985	—		0·61 ± 0·16	3	0·00 ± 0·00	1	0·22 ± 0·13	2
Limonene	1024	—		0·84 ± 0·18	3	—		1·63 ± 1·32	3
1,8-Cineole	1025	0·23 ± 0·23	1	14·66 ± 6·51	3	—		4·83 ± 2·32	3
(*Z*)-β-ocimene	1030	0·00 ± 0·00		1·55 ± 0·20	3	—		0·68 ± 0·44	2
(*E*)-β-ocimene	1043	1·14 ± 0·58	4	34·21 ± 3·85	3	0·09 ± 0·04	3	33·80 ± 14·00	3
γ-Terpinene	1052	0·00 ± 0·00		0·59 ± 0·08	3	0·00 ± 0·00		0·11 ± 0·11	1
Terpinolene	1087	—		0·19 ± 0·19	1	0·01 ± 0·01	1	—	
Linalool	1098	0·07 ± 0·05	2	0·44 ± 0·44	1	—		—	
Perillene*	1103	0·40 ± 0·18	4	—		0·18 ± 0·09	5	—	
Mean of total percent		*2·25*		*86·92*		*0·32*		*63·40*	
Sesquiterpenoids									
α-Ylangene	1371	0·03 ± 0·01	3	0·00 ± 0·00		0·00 ± 0·00		0·64 ± 0·57	2
α-Copaene	1375	0·53 ± 0·22	5	0·72 ± 0·35	3	0·03 ± 0·02	3	2·93 ± 1·22	4
β-Panasinsene	1378	—		0·15 ± 0·09	2	—		—	
β-Cubebene	1384	0·01 ± 0·01	2	—		—		—	
β-Elemene	1386	0·00 ± 0·00	1	0·13 ± 0·07	2	—		—	
α-Gurjunene	1407	0·02 ± 0·02	1	—		0·00 ± 0·00	1	0·05 ± 0·05	1
β-Caryophyllene	1419	1·35 ± 0·14	5	9·52 ± 9·52	1	0·34 ± 0·16	3	20·30 ± 14·36	3
α-Humulene	1450	0·32 ± 0·08	5	0·91 ± 0·66	3	0·03 ± 0·02	3	5·07 ± 2·43	4
Alloaromadendrene	1455	0·03 ± 0·01	3	—		—		5·22 ± 3·23	2
γ-Muurolene	1477	0·01 ± 0·00	2	0·07 ± 0·07	1	—		—	
Germacrene D	1480	0·03 ± 0·01	3	0·10 ± 0·10	1	0·00 ± 0·00	1	0·09 ± 0·09	1
β-Selinene	1485	0·05 ± 0·03	3	0·21 ± 0·10	2	0·00 ± 0·00	1	—	
α-Selinene	1495	0·00 ± 0·00	1	0·15 ± 0·15	1	—		—	
α-Muurolene	1499	0·02 ± 0·01	3	—		—		—	
(*E*,*E*)-α-farnesene	1506	—		0·26 ± 0·26	1	—		0·97 ± 0·60	3
δ-Cadinene	1520	0·04 ± 0·02	3	0·12 ± 0·07	2	0·00 ± 0·00	1	0·05 ± 0·05	1
Mean of total percent		*2·43*		*12·34*		*0·42*		*35·33*	
Shikimic compounds									
4-Methylanisole	1018	93·57 ± 0·78	5	—		98·36 ± 0·85	5	—	
1,4-Dimethoxybenzene	1184	0·02 ± 0·02	1	—		0·04 ± 0·03	2	—	
Indole	1296	1·58 ± 0·80	4	—		0·74 ± 0·71	2	0·55 ± 0·51	2
Mean of total percent		*95·17*		*0*		*99·14*		*0·55*	

The compounds detected for each type of floral scent emitted by figs are divided into classes based on general biosynthetic origin (Knudsen *et al.* 2006). Within each class they are listed in order of the retention index (RI). Relative amounts = mean ± SE of the proportion of each compound in the total bouquet (%). For each compound, we reported the number of trees in which the compound was detected (O ['occurrence']).

*Perillene or (E)-4,8-dimethyl-1,3,7-non-atriene.

Results

COMPOUND IDENTIFICATION

The floral scent composition emitted by syconia of *F. semicordata* at their receptive stage and 4 days after pollination are summarized in Table 1. In total, 35 compounds were found, including fatty acid derivatives, mono- and sesquiterpenoids, and shikimic compounds. As in the known fig volatiles identified from other species, mono- and sesquiterpenoids were the most numerous compounds in the odour components of *F. semicordata*. More surprisingly, one benzenoid compound, 4-methylanisole, represented more than 90% of the total quantity of compounds emitted by figs of both sexes at receptivity (Fig. 2). There was a significant effect of both sex and stage of the figs in the relative proportion of 4-methylanisole in the floral scent emitted (Kruskal–Wallis test, $\chi^2 = 15{\cdot}86$, $P = 0{\cdot}001$). At receptivity, this proportion was significantly higher for male figs than female figs, and for each sex it was significantly higher at the receptive than at the post-pollination stage (for all comparisons, Mann–Whitney *U*-test, $P = 0{\cdot}035$). However, when expressed as total quantity emitted by figs, the quantity of 4-methylanisole produced at receptivity by male figs ($1{\cdot}59 \pm 0{\cdot}31$ ng fig^{-1} h^{-1}) and female figs ($0{\cdot}83 \pm 0{\cdot}16$ ng fig^{-1} h^{-1}) was not significantly different (Wilcoxon rank test, $P = 0{\cdot}09$).

Ordination (NMDS) of the different odour samples based on Bray-Curtis distance is presented in Fig. 3. The volatile chemical profiles of figs of *F. semicordata* varied significantly within each sex between receptive and post-pollination stages (MRPP, $A = 0{\cdot}23$, $P < 0{\cdot}001$). However, the intersexual variation of these profiles was not significant either at receptivity or after pollination (respectively, MRPP, $A = 0{\cdot}06$, $P = 0{\cdot}09$; A $= 0{\cdot}035$, $P = 0{\cdot}25$). There was, however, significant temporal variation in the volatile chemicals produced by male and female figs after pollination (respectively, $A = 0{\cdot}19, P = 0{\cdot}008; A = 0{\cdot}25, P = 0{\cdot}033$).

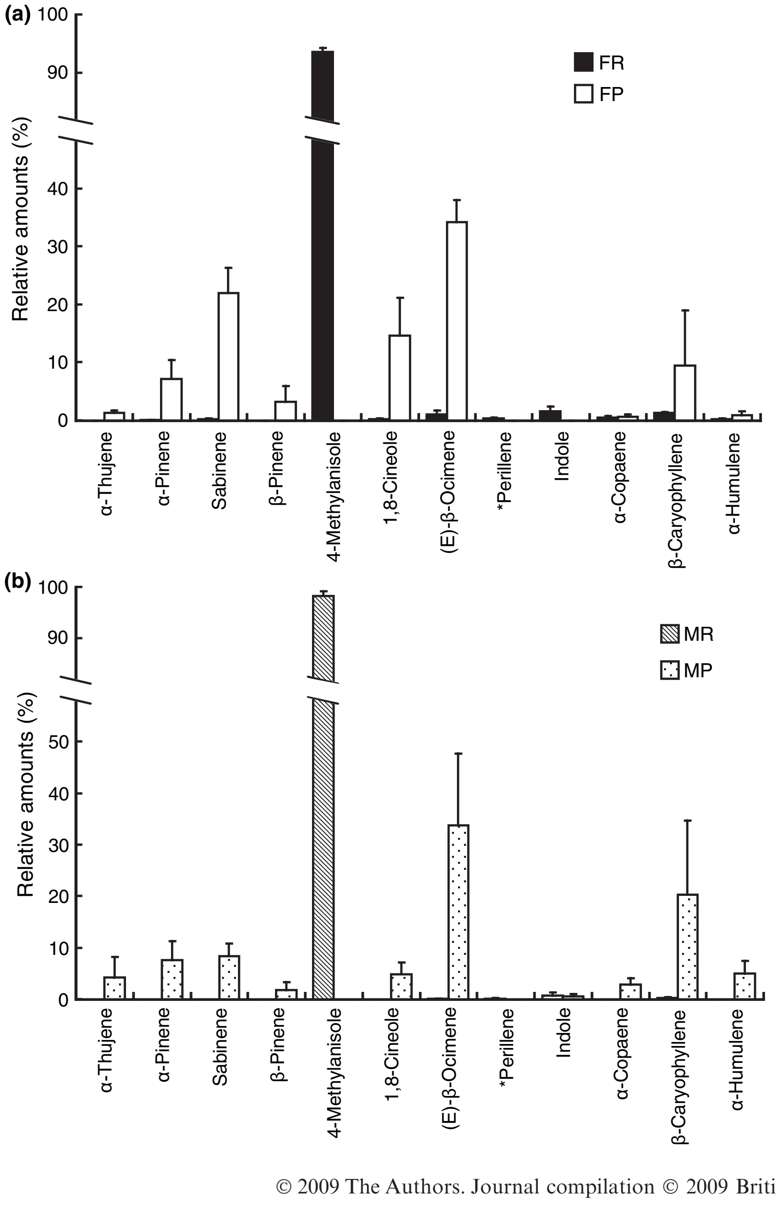

Fig. 2. Relative amounts of the main volatiles emitted from male and female figs of *Ficus semicordata* at receptive and post-pollination stages [(a) FR: female receptive, FP: female post-pollination, (b) MR: male receptive, MP: male post-pollination]. Relative amounts = mean ± SE of the proportion of each compounds in the total bouquet. * In our compound identification, perillene cannot be differentiated from (E)-4,8-dimethyl-1,3,7-non-atriene.

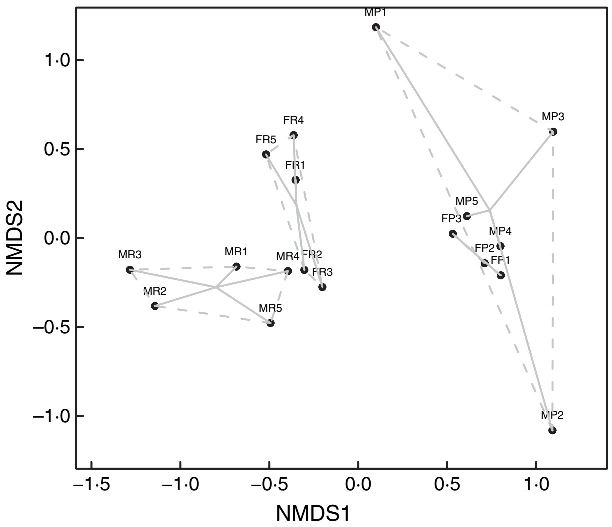

Fig. 3. Non-metric multidimensional scaling (NMDS) ordination of chemical composition of figs of both sexes of *Ficus semicordata* at receptive and post-pollination stages based on Bray-Curtis distance, rotated by principal component. The figs from different sexes and floral stages of *F. semicordata* were labelled as follows: MR = male receptive, MP = male post-pollination, FR = female receptive, FP = female post-pollination. Stress = 0·12.

The total amount of scent produced varied significantly between sex and stage of the figs (ANOVA $F_{3,14} = 23{\cdot}63$, $P < 0{\cdot}001$). Tukey's tests on pairwise comparisons showed no significant intersexual variation (both $P > 0{\cdot}28$) in the total amount of scent produced by figs at receptivity (female: 0·888 ± 0·388 ng μL^{-1}; male: 1·619 ± 0·706 ng μL^{-1}) and post-pollination (female: 0·039 ± 0·005 ng μL^{-1}; male: 0·024 ± 0·032 ng μL^{-1}; means ± SD). However, the quantity of scent produced decreased significantly after pollination for both male and female figs (both $P < 0{\cdot}01$).

Furthermore, different compounds showed different patterns of temporal change before and after pollination. Some compounds were mainly present in higher proportion in the odours of receptive figs, including 4-methylanisole, perillene and indole (Fig. 2, Table 1). In contrast, some volatile compounds were only present in the odours of pollinated figs of both sexes, including α-thujene, limonene, (*Z*)-β-ocimene, γ-terpinene and (*E*,*E*)-α-farnesene. Finally, several terpenoid compounds emitted by pollinated figs (with proportions up to 5% and detected at least twice in our sampled figs) were also identified in the compounds emitted by receptive figs, including α-pinene, sabinene, 1,8-cineole, (*E*)-β-ocimene and β-caryophyllene.

BEHAVIOURAL BIOASSAYS OF WASP RESPONSE TO FIG ODOUR

Experiment A: responses of C. gravelyi *to odour sources of fresh figs*

Both male and female figs of *F. semicordata* at their receptive stage were attractive to the pollinating wasp *C. gravelyi*, while figs at post-pollination stage were unattractive (Fig. 4a.). In our olfactometer experiments, receptive-stage figs of two non-host species, *F. racemosa* and *F. hispida*, repelled *C. gravelyi* compared with clean air (Fig. 4a).

Experiment B: responses of C. gravelyi *to different concentrations of 4-methylanisole*

Our compound identifications indicated that 4-methylanisole was the main volatile compound emitted by receptive figs of both sexes of *F. semicordata*. Considering also the absence of 4-methylanisole from the floral scent of figs of *F. semicordata* after pollination, we concluded that this compound is probably the main attractant for *C. gravelyi*.

In the bioassays with synthetic pure 4-methylanisole (Fig. 4b.), female pollinators were attracted even when the concentration of 4-methylanisole was as low as $1{\cdot}22 \times 10^{-2}$ ng/100 μL (in our result one fig produced about 1·5 ng h^{-1} of 4-methylanisole). Among the tested concentrations of $1{\cdot}22 \times 10^{-4}$ ng ~ $1{\cdot}22 \times 10^{7}$ ng, only the two lowest concentrations ($1{\cdot}22 \times 10^{-4}$ ng, $1{\cdot}22 \times 10^{-3}$ ng) and the highest tested concentration ($1{\cdot}22 \times 10^{7}$ ng) were significantly not attractive to the pollinators. For all the other concentrations of 4-methylanisole, the wasps were significantly attracted to the branch of the olfactometer with the tested compound (Fig. 4b).

Discussion

Our results provide evidence that olfactory cues play a role in facilitating the location by the pollinating wasp *C. gravelyi* of receptive-stage figs of its host *F. semicordata*. Moreover, they provide strong support for three key hypotheses about the functioning of fig-fig wasp interactions: (i) species-specificity mediated by scent alone, (ii) stage-dependent pollinator attraction and (iii) intersexual chemical mimicry between male and female figs. Another striking result of our study is the simplicity of the signal used to facilitate this interaction. A single compound, 4-methylanisole, accounted for more than 90% of the volatile compounds emitted by receptive figs of both sexes, and we show evidence that the sole presence of this compound can be used as a specific signal by the pollinator of *F. semicordata*. This compound thus appears to act as a private channel (see Raguso 2008b) assuring specificity in this highly specialized plant–pollinator interaction.

In the approximately 40 fig species so far studied for their floral scents, at least two to five major compounds account for the majority of the total volatiles emitted by receptive figs (Ware *et al.* 1993; Grison, Edwards & Hossaert-McKey 1999; Song *et al.* 2001; Grison-Pigé *et al.* 2002b; Proffit *et al.* 2009). These major volatiles emitted by receptive figs are generally not rare compounds in floral fragrances and the species-specificity of wasp attraction is thus usually not likely to result from the presence of one single specific compound (Grison, Edwards & Hossaert-McKey 1999; Grison-Pigé *et al.* 2002b). Though 4-methylanisole is not rare in floral fragrances, having been detected in floral scents of plants from

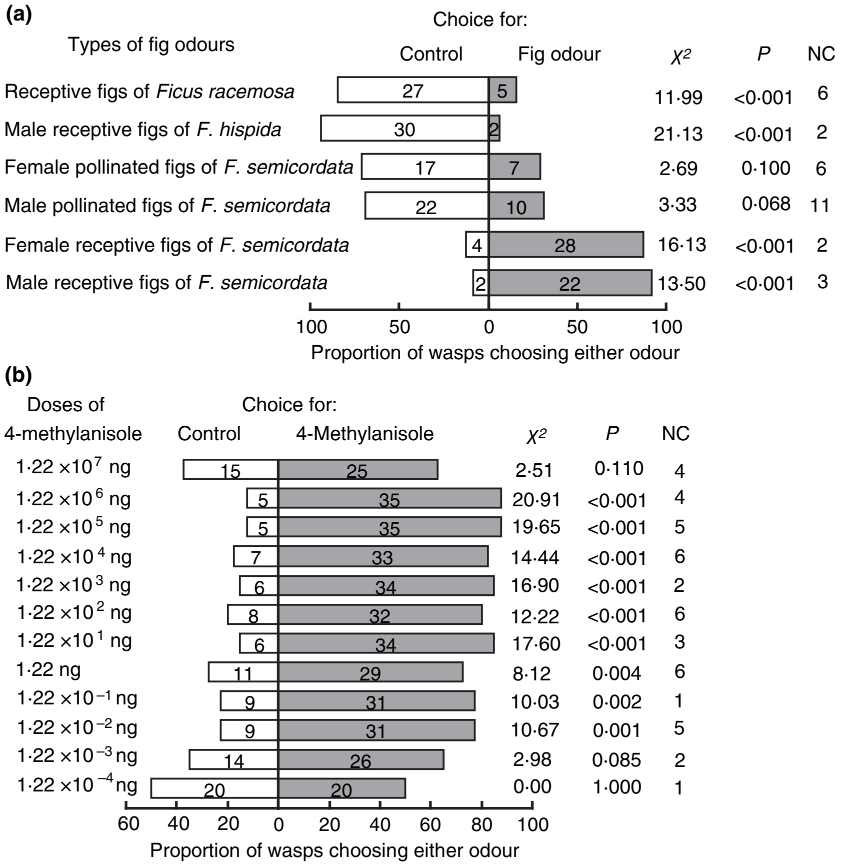

Fig. 4. Number of females of *Ceratosolen gravelyi* responding to natural or synthetic fig volatiles in a Y-tube olfactometer. (a) Choices made by *C. gravelyi* between volatiles of host/non-host figs of different developmental stages and clean air. The tested volatiles included those from male receptive figs of *Ficus semicordata*, female receptive figs of *F. semicordata*, male post-pollination figs of *F. semicordata*, female post-pollination figs of *F. semicordata*, male receptive figs of *F. hispida* and receptive figs of the monoecious *F. racemosa*. (b) Choices made by *C. gravelyi* between 4-methylanisole diluted in dichloromethane ($1{\cdot}22 \times 10^{-4}$ ng ~ $1{\cdot}22 \times 10^{7}$ ng/100 μL) and pure dichloromethane (100 μL). Each of the tested compounds and the pure dichloromethane was allowed to evaporate 15 min at room temperature. Numbers in the bars indicate the number of wasps that chose either arm. Wasps that did not respond within 5 min [no choice (NC)] were excluded from the statistical analysis.

17 families [of the 90 families included in the review by Knudsen *et al.* (2006)], this is the first time that this benzenoid compound has been reported in the floral scent of a *Ficus* species (Grison, Edwards & Hossaert-McKey 1999; Grison-Pigé *et al.* 2001; Song *et al.* 2001; Proffit *et al.* 2009). To our knowledge, no previous study has shown that 4-methylanisole is attractive to pollinators of any plant, or that this compound could by itself mediate the specificity of any mutualistic interaction.

In this study, the species-specificity of wasp attraction to *F. semicordata* seems to be due mainly, if not entirely, to this single major compound, 4-methylanisole, which also is known not to be produced by the two other sympatric *Ficus* species studied here (Proffit *et al.* 2008, 2009). Moreover, in our behavioural tests, the responses of *C. gravelyi* to 4-methylanisole in concentrations ranging from very low to relatively high (from $1{\cdot}22 \times 10^{-2}$ ng/100 μL to $1{\cdot}22 \times 10^{6}$ ng/100 μL) support the conclusion that 4-methylanisole is the main olfactory cue for pollinator attraction in *F. semicordata*. Our results provide a unique example of simplification of the chemical message facilitating partner encounter in this highly specific set of mutualistic interactions.

Our study also highlights the precise relationship between chemical mediation of pollinator attraction and floral phenology. Although figs emit volatiles during the entire period over which syconia develop, only the odour at receptivity includes the chemical signals responsible for pollinator attraction (Grison-Pigé *et al.* 2001; Proffit *et al.* 2008). In fig pollination mutualisms, a rapid post-pollination decrease in pollinator attraction limits pollinator visits, and thereby minimizes the overexploitation of the ovaries by these mutualists, which are also seed predators (Khadari *et al.* 1995). In plants, odour changes after pollination are often due to a decrease in the total amount of odour production and to changes in the relative amounts of certain compounds (Schiestl & Ayasse 2001; Negre *et al.* 2003; Theis & Raguso 2005). Studies of temporal changes in odour production and composition of *F. carica* and *F. hispida* showed that they conformed to these patterns (Grison-Pigé *et al.* 2001; Proffit *et al.* 2008). Similarly, the quantity of odour emitted in *F. semicordata* was significantly higher in receptive figs than in post-pollination figs. However, this change in the emission of the main compounds after pollination in *F. semicordata* was divergent from patterns observed in *F. hispida* and *F. carica*. In the odours of both the latter species, the main compounds, such as linalool, usually were present in maximal concentration at receptive stage but also occurred in lower amounts after pollination. As shown in this study, the main signal compound in floral scent of *F. semicordata* disappeared totally after pollination.

The last point in this study concerns chemical mediation in dioecious figs. Chemical mimicry between male and female figs, which prevents pollinators from discriminating between sexes, often explains the maintenance of 'deceptive' female figs (Grafen & Godfray 1991; Patel *et al.* 1995; Anstett, Hossaert-McKey & Kjellberg 1997; Moore *et al.* 2003; Borges, Bessière & Hossaert-McKey 2008; Proffit *et al.*

2008). In this study, our behavioural experiments showed that pollinating fig wasps were strongly attracted to volatile compounds emitted by both male and female receptive figs. Furthermore, odours produced by figs of the two sexes at the receptive stage were qualitatively and quantitatively very similar, with 4-methylanisole being the strongly predominant compound. Although the intersexual variation of the relative proportion of this compound at receptivity was significant, its concentration was similar in odours of male and female figs. These results are consistent with the hypothesis that in dioecious figs, pollinators are unable to distinguish between male and female figs because of intersexual chemical mimicry (Grafen & Godfray 1991; Patel *et al*. 1995; Anstett, Hossaert-McKey & Kjellberg 1997; Moore *et al*. 2003; Borges, Bessière & Hossaert-McKey 2008; Proffit *et al*. 2008).

Our results on the emission of volatile compounds by figs of *F. semicordata* and our behavioural tests on its pollinator provide evidence that olfactory cues play a central role in allowing pollinating wasps to locate their specific host plant at the appropriate stage of fig development. The system studied here provides an example of co-evolution of the signal emitted by one partner in a specific and obligatory pollination mutualism and of the other partner's behavioural response to it, assuring their encounter in a species-rich, complex olfactory landscape.

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