

# ‘Push’ and ‘pull’ responses by fig wasps to volatiles released by their host figs

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**Abstract** In the specific mutualism between fig trees (*Ficus*) and their obligate pollinating fig wasps (Agaonidae), it is crucial that fig wasps can recognize the developmental stages of their host figs. However, the responses of fig wasps to volatiles released from figs during their developmental phases are less clearly understood and are the focus of this study. We extracted and identified the volatiles released from the figs of *Ficus curtipes* throughout their development. Using Y-tube choice experiments, we also compared the behavioural responses of the tree’s pollinator (*Eupristina* sp.) to figs at different developmental stages, and compared these results to those obtained by trapping fig wasps as they arrived at a tree with a developing fig crop. The chemical composition of the fig volatiles changed during fig development with the blends exhibiting clear segregation among figs at different developmental phases. Male phase figs had the most distinct blend. Fig wasp females were preferentially attracted to receptive figs, but figs at most other developmental phases were also attractive. Conversely, male phase figs had a repellent effect. These results were supported by the behaviour of the wasps under natural conditions, with small

numbers of fig wasps arriving at the tree before and after receptive figs were present. These results indicate a more complex relationship between fig volatiles and fig wasp behaviour than previously realized, with volatiles mediating both the initial meeting of the mutualists to achieve pollination and egg laying and the subsequent departure of the next generation of fig wasps. This offers an explanation for the specialization and long-term coexistence of figs and fig wasps.

**Keywords** Agaonidae · Behavioural response · Chemical communication · *Ficus* · Mutualism · Obligate pollinator

## Introduction

Mutualisms are interspecific interactions in which all the associated species receive net benefits from their partners (Boucher et al. 1982; Bronstein 1994, 2009) and are ubiquitous in all ecosystems (Herre et al. 1999; Bronstein 2001). The most thoroughly studied examples are plant–insect mutualisms (Bronstein 1994). Among plant–insect mutualisms, the nursery pollination mutualisms, in which the plant provides oviposition sites and shelter or food for the offspring of the pollinator (Biere and Honders 2006), are the most specialized relationships because both partners in these systems depend directly on each other for their reproduction. Therefore, it is crucial that the partners get into contact at correct time in the nursery pollination mutualisms.

Figs (*Ficus*, Moraceae) and their obligate pollinating wasps (Agaonidae, Chalcidoidea) constitute, perhaps the most tightly specialized nursery pollination mutualism (Machado et al. 2005). Each of the nearly 750 *Ficus*

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species generally relies on a single specific species of pollinating agaonid wasps for pollination, and the wasp also depends strictly on its host *Ficus* for reproduction (Wiebes 1979; Berg 1989; Cook and Rasplus 2003).

In tropical forests, many *Ficus* species co-occur (Harrison 2005) and a single fig species is patchily distributed, i.e. the density of a single fig species is often low (Anstett et al. 1997). Furthermore, fig development is synchronous on the same trees and can be divided into discrete phases, i.e. ‘pre-receptive’ (when the figs are immature), ‘receptive’ (when the figs can be entered and their female flowers pollinated), ‘interfloral’ (when the figs contain developing wasp larvae and seeds) and ‘male’ (when the next generation of fig wasps emerge from their galls, collect pollen and then disperse to other trees). In addition, a post-pollinated phase has been inserted recently between receptive and interfloral phases (Proffit et al. 2008; Chen et al. 2009). We also distinguished ‘post-pollinated’ figs, which had been entered by pollinators more than 24 h but <48 h. For details of the fig development phases, see Galil and Eiskowitch (1968) and Harrison (2005). Moreover, the female wasps can enter their host figs only at the receptive phase, and the life span of female wasps are short (a few hours to 2 or 3 days) (Kjellberg et al. 1988; Harrison 2005; Dunn et al. 2008). Therefore, the signals responsible for partner encounter are crucial, and they must be species-specific and developmental stage-specific to guide the fig wasps to their host figs at receptivity, i.e. fig wasps could recognize receptive figs of their specific host *Ficus* species from among figs at other developmental stages and of other species through the species-specific signals and the developmental stage-specific signals. It has been shown that these signals are volatile chemicals emitted by host figs (Grison-Pigé et al. 2002a; Hossaert-McKey et al. 2010), and the species specificity of chemical signals in fig–fig wasp interactions is confirmed (Ware et al. 1993; Grison-Pigé et al. 2002a, b; Chen et al. 2009). However, there are few data on developmental phase-related variation in fig volatile chemicals and the effect of the variation on fig wasps’ behavioural responses. As far as we know, only one paper reported the changes in floral scents of figs among their pre-receptive, receptive, post-pollinated and interfloral phases (Proffit et al. 2008), and few investigations concerned volatiles of figs at male phase and behavioural responses of fig wasps to figs at different developmental phases (Borges et al. 2008).

Therefore, there is little evidence for the hypothesis that fig wasps could recognize receptive figs of their specific host *Ficus* species from figs at other developmental stages through developmental stage-related volatiles. To obtain the evidence, in this study, we firstly investigated whether there were developmental stage-related changes in fig volatiles of *Ficus curtipes* (Corner), and (if so) then how its

pollinating fig wasp *Eupristina* sp. responded to these changes. For these purposes, we collected and analysed the volatile chemicals emitted by *F. curtipes* figs at different developmental stages, and we tested the behavioural responses of *Eupristina* wasps to these figs through two-choice bioassays in the laboratory and observed the behaviour of the wasps visiting their host fig tree through sticky traps in the field. We aimed to answer the following questions: (1) what differences are there among floral scents emitted by figs at different developmental phases? (2) Besides attracted by the receptive figs, whether female *Eupristina* wasps are attracted by other non-receptive *F. curtipes* figs in the absence of receptive figs? If so, (3) can the wasps distinguish the receptive *F. curtipes* figs from these attracting non-receptive figs? (4) Do the wasps avoid the male phase figs from which they escape? (5) Under natural conditions, are there any wasps arriving at the tree before and after receptive figs are present?

## Materials and methods

### Study site and organisms

*Ficus curtipes* is widely distributed in seasonal tropical forests of Southeast Asia and is also cultivated in cities and villages as an ornamental tree. It produces monoecious figs that contain both male and female flowers and produce both seeds and the offspring of pollinator fig wasps. The figs are produced in the leaf axils and reach about 12 mm in diameter when mature (Zhang et al. 2009). They develop in well-synchronized crops, with different trees having figs at different developmental stages throughout the year. At the Xishuangbanna Tropical Botanical Garden, Yunnan Province, China (21°55′N, 101°25′E), *F. curtipes* is pollinated by an undescribed species of *Eupristina* (Hymenoptera, Agaonidae) (Zhang et al. 2008, 2009). Pollinator females that enter a fig rarely re-emerge (Gu D, personal observation). Foundress numbers per fig range between 1 and 2 (mean foundress numbers =  $1.02 \pm 0.22$  SD,  $n = 81$ ) (Gu D and Yang DR, unpublished data). *Eupristina* sp. adult females are short-lived, rarely surviving more than 1 day under natural condition (Gu D, personal observation).

### Floral scent collections

Volatiles were extracted from *F. curtipes* figs collected from five trees at different developmental phases between September 2009 and March 2010. Prior to collection, pre-receptive figs were covered with mesh bags to exclude fig wasps. Volatiles of pre-receptive figs were collected 2–4 days after they first became visible. When there were

many pollinating wasps walking on the surface of the mesh bags, we regarded the figs receptive. The figs were then removed for volatile collection or left on the trees to be pollinated. We collected volatiles of these pollinated figs within 48 h of the bags being removed (post-pollination phase). Volatiles of interfloral phase figs were collected 7–15 days after pollination, and those of male phase figs were collected when the male flowers contained pollen grains and their wasps were about to emerge from their galls.

Volatiles emanating from the figs were collected by dynamic headspace sampling (Grison-Pigé et al. 2002a; Chen et al. 2009; Proffit and Johnson 2009) in a closed system. An air stream produced by a pump was passed sequentially through a flow meter for controlling flow rates, two activated carbon filters for cleaning, a glass sampling bottle (1.25 L) containing figs, a trap for collecting volatiles and another flow meter, and then the air went back to the pump. Sufficient figs were added to fill the glass sample bottle, so the number present was dependent on fig size (ranging from 189 large male phase figs to 436 small pre-receptive figs). Air in the sample system was also collected as a control to identify any background contaminants.

The traps for collecting volatiles were glass tubes (internal diameter 3 mm, length 7 cm) packed with 100 mg of Super-Q absorbent (ARS Inc., Gainesville, FL, USA). Both entrance and exit flow rates (controlled by flow meters) were 300 ml/min. Trapping of volatiles started at 0900 hours and continued for 3–4 h. The air in the sample system was cleaned for 1–2 h with two activated carbon filters before each collection. The collected volatiles in Super-Q traps were eluted with 160 µl *n*-hexane. The extracts were stored at –18 °C in brown glass vials until chemical analysis and were concentrated to 40 µl before analysis.

#### Chemical analyses of floral scents

Extracts were analysed by gas chromatography–mass spectrometry (GC–MS, GC, HP-6890; MS, HP-5973; Agilent Technologies, USA). The GC was equipped with an HP-5MS silica capillary column (30 m × 0.25 mm × 0.25 µm film thickness), a flame ionization detector and linked to the MS. Two microlitres of each sample was injected in splitless mode. Helium was used as the carrier gas at the velocity of 1 ml/min, and the injector temperature was 250 °C. The column temperature was programmed at 60 °C for 1 min and then increased by 5 °C/min to 260 °C. Mass spectra were recorded at 70 eV in the electron impact ionization mode, and the ionic source temperature was 230 °C.

The volatile compounds were identified by comparing their mass spectra with those reported in the Wiley7n.l and NIST08.L library. Their relative amounts were calculated with the peak area normalization method.

#### Choice experiments

Behavioural assays with *Eupristina* pollinators were performed between July and December 2010. A glass Y-tube (binary choice) olfactometer was used to test the response of the *Eupristina* pollinators to volatiles released from freshly collected *F. curtipes* figs. Each arm of the Y-tube olfactometer (internal diameter 1.5 cm, length of each arm 9 cm, stem 8 cm) was connected to a glass container (500 ml) inside which an odour source was located. Flow rates through each arm were maintained at 100 ml/min using a flow meter. The airflow through each arm was drawn through Teflon tubes by an air pump and passed through a charcoal filter and distilled water. Further details of the olfactometer setup are given in Chen and Song (2008) and Chen et al. (2009). All experiments were performed between 0800 and 1200 hours in a darkened room with three 40-W cool white fluorescent tubes placed above the arms of the Y-tube.

Female fig wasps were individually introduced at the basal stem of the olfactometer and were given 5 min to move into either of the two arms of the olfactometer. When a wasp went into one of the two arms and reached 1 cm or more past the Y junction, it was considered that the wasp had made a choice. Fig wasps that did not enter either arm within the given time period were recorded as expressing no choices and were excluded from statistical analysis. Fig wasps had emerged from male phase figs and each wasp was tested only once on the same day. The two arms were alternated regularly (every five wasps tested) to avoid position effects, and the odours were switched between the two arms of the Y-tube every 15 wasps tested to avoid any influence of unforeseen asymmetries in the setup. The olfactometer was rinsed with pure ethanol and then dried after each bioassay.

A total of 19 pairs of volatile sources were examined, using six different sources (Table 1). Because figs release larger quantities of volatiles during their receptive phase (Grison-pigé et al. 2001; Proffit et al. 2008), we applied 1:4 ratios for the number of receptive figs versus figs from other developmental phases. All other ratios were 1:1. Conditions and procedures involving fresh figs in the behavioural experiments were the same as those described in “Floral scent collections”. Clean air, purified by passing through a charcoal filter and distilled water, was used as the control odour source. The figs were replaced hourly. In all experiments, the responses of at least 30 or more wasps were tested.

#### Timing of fig wasp arrivals at a developing fig crop

Sticky traps (15 cm × 20 cm) were placed in a tree (from November 5 to December 29, 2009) where a new crop of

**Table 1** Behavioural tests conducted

Experiment	Type and (number of figs) in the arms of the Y-tube	
	Odour 1	Odour 2
1	Pre-receptive figs (20)	Clean air
2	Receptive figs (20)	Clean air
3	Post-pollinated figs (20)	Clean air
4	Interfloral figs (20)	Clean air
5	Male phase figs (20)	Clean air
6	Receptive figs (20)	Pre-receptive figs (20)
7	Receptive figs (10)	Pre-receptive figs (40)
8	Receptive figs (20)	Post-pollinated figs (20)
9	Receptive figs (10)	Post-pollinated figs (40)
10	Receptive figs (20)	Interfloral figs (20)
11	Receptive figs (10)	Interfloral figs (40)
12	Receptive figs (20)	Male phase figs (20)
13	Receptive figs (10)	Male phase figs (40)
14	Pre-receptive figs (20)	Post-pollinated figs (20)
15	Pre-receptive figs (20)	Interfloral figs (20)
16	Pre-receptive figs (20)	Male phase figs (20)
17	Post-pollinated figs (20)	Interfloral figs (20)
18	Post-pollinated figs (20)	Male phase figs (20)
19	Interfloral figs (20)	Male phase figs (20)

The sets of behavioural tests and the number of figs used for each set

figs was developing. Four sticky traps were hung around the tree in four directions and were located 2 m above the ground and at least 1 m from the nearest parts of the tree. The traps were changed daily and the numbers of trapped *Eupristina* sp. were recorded. Under natural conditions, there is a gradual transition between predominantly receptive phase and interfloral phase figs on a tree until all the figs are pollinated. In order to generate an abrupt transition between these phases, we placed bags around ~600 pre-receptive phase figs to prevent pollinator entry. Once all the other figs on the tree had been pollinated, we removed the bags and the figs were immediately pollinated. The sticky traps were located at least 1 m from the nearest bagged figs.

### Statistical analyses

Correspondence analysis (CA) (Härdle and Simar 2007) was used to explore the relationship between developmental phases of *F. curtipes* figs and the composition of their volatile compound blends using SPSS 13.0 for windows (SPSS Inc.). Preferences in binary choice experiments were analysed with Chi-square ( $\chi^2$ ) tests in R 2.12. In the analyses on trapped wasp numbers, we totalled the number of wasps on the four traps per day and calculated the means and standard errors (SE) every 5 days. All figures were plotted with Sigmaplot 10.0 (Systat Software, Inc).

## Results

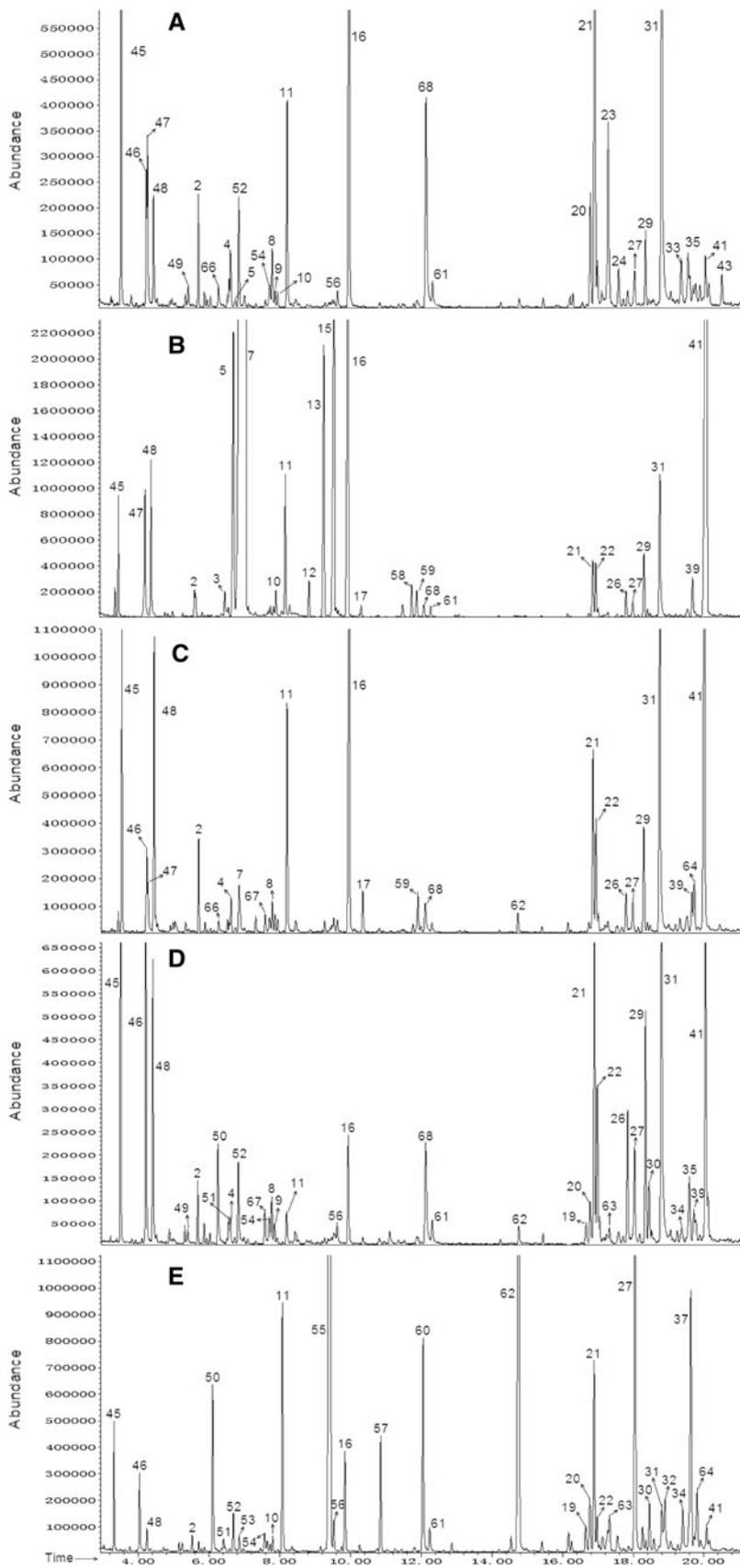
### Floral scent analyses

The composition of the bouquet of the figs changed with their developmental phases (Fig. 1). In total, 68 compounds were identified (pre-receptive phase, 47 compounds; receptive phase, 52 compounds; post-pollination phase, 54 compounds; interfloral phase, 40 compounds; and male phase, 41 compounds) (Table 2). The 68 compounds included 43 terpenoids, 42 aliphatic compounds and 3 aromatic compounds. The two former groups accounted for over 95 % of the volatile compounds from each phase and they were dominant both in terms of the number of compounds and the quantities produced (Fig. 2; Table 2). Twenty-three compounds were recorded from figs of all five phases, and most compounds were shared by two or more phases, but the composition of the blends (the number of compounds and their relative amounts) was unique to each phase. Eleven compounds were unique to a single phase (Table 2).

The relative amounts of the three classes of chemicals, especially the terpenoids and aliphatic compounds, changed substantially between developmental phases (Fig. 2). During the pre-receptive, receptive and post-pollination phases, terpenoids accounted for 77.15, 84.71 and 88.51 % of the totals, respectively, with aliphatic compounds accounting for <20 % (Fig. 2). The proportion of aliphatic compounds then rose during the interfloral and male phases to 40.40 and 60.68 %, respectively. Alkanes were the main components (51.86 %) during the male phase (Table 2). Among the 68 compounds, the main compounds (>5 % mean relative amounts in one or more developmental phases) comprised just 13 compounds (Table 2). The composition of the 13 main compounds also varied with developmental phase (Fig. 3).

Thirty-one compounds, each with >2 % mean relative amounts in one or more developmental phases, were included in the correspondence analysis (see Table 2). The first two axes of the CA together explained 80.2 % [dimension 1, 53.3 %; dimension 2, 26.9 %;  $\chi^2 = 517.90$ , degrees of freedom ( $df$ ) = 120,  $P < 0.001$ ] of the variance in volatile compounds (total inertia = 1.214) across the five developmental stages. The first axis of the CA distinguished the male phase blends from the rest, while axis 2 emphasized the major difference between receptive figs and the other earlier developmental phases (Fig. 4). Some compounds (located close to the origin in the CA) occurred during two or more phases, but at each phase one or more compounds were prominent (Fig. 4; Table 2). For example, the chemistry profile of receptive figs was mainly dominated by 6-methyl-5-hepten-2-ol, 6-methyl-5-hepten-2-one and linalool (compounds 5, 7 and 15),

**Fig. 1** The total ion chromatograms (TICs) of the volatiles emitted by *Ficus curtipes* figs at their pre-receptive (a), receptive (b), post-pollinated (c), interfloral (d) and male (e) phases. The numbers marked on peaks represent compounds with the same numbers listed in Table 2



**Table 2** Occurrence and mean ( $\pm$ SE) relative amounts (%) of volatile compounds emitted by *Ficus curtipes* figs at their pre-receptive (PR), receptive (R), post-pollinated (PP), interfloral (IF) and male (M) phases

Compounds	RT <sup>a</sup>	Phases					
		PR ( <i>n</i> = 2)	R ( <i>n</i> = 5)	PP ( <i>n</i> = 3)	IF ( <i>n</i> = 2)	M ( <i>n</i> = 2)	
Terpenoids							
1	$\alpha$ -Thujene	5.36	–	–	0.03 $\pm$ 0.03	–	–
2	$\alpha$ -Pinene <sup>b</sup>	5.52	2.28 $\pm$ 0.51	1.27 $\pm$ 0.31	3.21 $\pm$ 0.87	1.22 $\pm$ 0.11	0.26 $\pm$ 0.06
3	Sabinene	6.36	0.47 $\pm$ 0.37	0.23 $\pm$ 0.08	0.58 $\pm$ 0.16	–	–
4	$\beta$ -Pinene <sup>b</sup>	6.46	3.75 $\pm$ 2.35	0.59 $\pm$ 0.17	1.67 $\pm$ 0.66	0.69 $\pm$ 0.10	–
5	6-Methyl-5-hepten-2-one <sup>b</sup>	6.59	0.28 $\pm$ 0.23	2.0 $\pm$ 0.38	0.98 $\pm$ 0.54	–	–
6	$\beta$ -Myrcene	6.70	–	0.05 $\pm$ 0.03	0.07 $\pm$ 0.07	–	–
7	6-Methyl-5-hepten-2-ol <sup>b</sup>	6.75	–	32.46 $\pm$ 7.44	2.03 $\pm$ 0.65	0.83 $\pm$ 0.13	0.43 $\pm$ 0.12
8	$\alpha$ -Limonene <sup>b</sup>	7.65	2.98 $\pm$ 1.83	0.67 $\pm$ 0.20	1.50 $\pm$ 0.39	1.09 $\pm$ 0.11	0.25 $\pm$ 0.07
9	1,8-Cineole	7.74	0.96 $\pm$ 0.65	0.35 $\pm$ 0.12	0.71 $\pm$ 0.16	0.50 $\pm$ 0.11	0.08 $\pm$ 0.03
10	( <i>Z</i> )- $\beta$ -Ocimene	7.81	0.50 $\pm$ 0.18	0.31 $\pm$ 0.05	0.66 $\pm$ 0.17	–	0.33 $\pm$ 0.08
11	( <i>E</i> )- $\beta$ -Ocimene <sup>b</sup>	8.07	5.82 $\pm$ 1.98	2.27 $\pm$ 0.58	18.01 $\pm$ 6.45	3.62 $\pm$ 2.77	4.81 $\pm$ 1.06
12	( <i>Z</i> )-Linalool oxide (furanoid)	8.76	–	0.17 $\pm$ 0.06	0.02 $\pm$ 0.02	–	–
13	( <i>E</i> )-Linalool oxide (furanoid)	9.16	–	1.42 $\pm$ 0.38	0.38 $\pm$ 0.10	–	–
14	$\alpha$ -Terpinolene	9.18	–	0.50 $\pm$ 0.37	0.28 $\pm$ 0.17	–	–
15	Linalool <sup>b</sup>	9.44	0.10 $\pm$ 0.10	6.58 $\pm$ 2.12	1.69 $\pm$ 0.73	0.63 $\pm$ 0.23	–
16	( <i>E</i> )-4,8-Dimethyl-1,3,7-nonatriene <sup>b</sup>	9.84	8.71 $\pm$ 3.13	16.1 $\pm$ 4.93	19.77 $\pm$ 2.18	11.00 $\pm$ 8.48	2.47 $\pm$ 0.94
17	1,3,8- <i>p</i> -Menthatriene <sup>b</sup>	10.27	0.82 $\pm$ 0.82	0.36 $\pm$ 0.16	2.53 $\pm$ 0.91	–	0.19 $\pm$ 0.09
18	Camphor	10.76	0.47 $\pm$ 0.47	0.07 $\pm$ 0.05	0.07 $\pm$ 0.07	0.49 $\pm$ 0.49	–
19	$\alpha$ -Cubebene	16.67	1.14 $\pm$ 0.64	0.20 $\pm$ 0.12	0.52 $\pm$ 0.14	0.24 $\pm$ 0.24	0.33 $\pm$ 0.01
20	$\alpha$ -Ylangene	16.78	1.26 $\pm$ 1.26	0.05 $\pm$ 0.04	0.09 $\pm$ 0.09	0.62 $\pm$ 0.62	0.89 $\pm$ 0.10
21	$\alpha$ -Copaene <sup>b</sup>	16.90	11.94 $\pm$ 3.13	1.92 $\pm$ 0.47	4.68 $\pm$ 0.30	4.25 $\pm$ 3.58	3.88 $\pm$ 0.46
22	Calarene <sup>b</sup>	16.97	1.58 $\pm$ 0.55	1.38 $\pm$ 0.57	1.43 $\pm$ 0.81	2.40 $\pm$ 1.61	0.84 $\pm$ 0.09
23	$\beta$ -Elemene <sup>b</sup>	17.32	3.22 $\pm$ 1.81	0.19 $\pm$ 0.12	0.74 $\pm$ 0.38	–	–
24	$\alpha$ -Gurjunene	17.58	0.48 $\pm$ 0.48	–	–	–	–
25	Cyperene <sup>b</sup>	17.61	0.77 $\pm$ 0.77	0.49 $\pm$ 0.30	2.06 $\pm$ 1.03	0.40 $\pm$ 0.40	0.46 $\pm$ 0.08
26	( <i>Z</i> )- $\alpha$ -Bergamotene	17.84	0.38 $\pm$ 0.38	0.45 $\pm$ 0.23	0.37 $\pm$ 0.37	1.89 $\pm$ 1.49	–
27	$\beta$ -Caryophyllene <sup>b</sup>	18.03	0.50 $\pm$ 0.50	0.26 $\pm$ 0.13	0.35 $\pm$ 0.35	1.40 $\pm$ 1.40	8.60 $\pm$ 1.16
28	$\beta$ -Gurjunene	18.28	1.05 $\pm$ 1.05	0.18 $\pm$ 0.13	0.57 $\pm$ 0.29	–	0.62 $\pm$ 0.16
29	( <i>E</i> )- $\alpha$ -Bergamotene <sup>b</sup>	18.35	0.82 $\pm$ 0.82	1.12 $\pm$ 0.57	0.92 $\pm$ 0.92	3.71 $\pm$ 2.08	0.37 $\pm$ 0.00
30	$\alpha$ -Guaiene	18.47	–	0.02 $\pm$ 0.02	–	0.79 $\pm$ 0.79	1.05 $\pm$ 0.17
31	( <i>E</i> )- $\beta$ -Farnesene <sup>b</sup>	18.82	11.21 $\pm$ 4.77	3.72 $\pm$ 1.50	5.39 $\pm$ 2.78	7.88 $\pm$ 5.87	1.58 $\pm$ 0.49
32	$\alpha$ -Caryophyllene	18.90	–	–	–	–	1.67 $\pm$ 0.35
33	$\gamma$ -Elemene	19.38	0.73 $\pm$ 0.73	–	–	–	–
34	$\gamma$ -Selinene <sup>b</sup>	19.41	0.53 $\pm$ 0.53	0.42 $\pm$ 0.26	2.16 $\pm$ 0.85	0.29 $\pm$ 0.29	0.93 $\pm$ 0.02
35	Germacrene-d <sup>b</sup>	19.59	2.15 $\pm$ 0.02	0.33 $\pm$ 0.12	1.08 $\pm$ 0.22	1.20 $\pm$ 1.20	–
36	Bicyclogermacrene	19.61	–	0.10 $\pm$ 0.06	0.40 $\pm$ 0.20	–	0.90 $\pm$ 0.08
37	$\beta$ -Selinene <sup>b</sup>	19.63	2.01 $\pm$ 2.01	0.49 $\pm$ 0.30	1.84 $\pm$ 1.01	–	7.25 $\pm$ 1.90
38	Viridiflorene	19.72	0.27 $\pm$ 0.27	–	–	–	–
39	$\beta$ -Bergamotene	19.74	–	0.31 $\pm$ 0.13	0.35 $\pm$ 0.35	0.90 $\pm$ 0.21	–
40	$\alpha$ -Selinene	19.96	0.29 $\pm$ 0.29	0.10 $\pm$ 0.06	0.40 $\pm$ 0.20	–	–
41	$\alpha$ -Farnesene <sup>b</sup>	20.13	1.97 $\pm$ 0.56	6.56 $\pm$ 2.74	6.7 $\pm$ 6.05	5.77 $\pm$ 5.21	0.49 $\pm$ 0.06
42	$\alpha$ -Bulnesene	20.17	0.36 $\pm$ 0.36	–	–	–	–
43	$\delta$ -Cadinene	20.55	0.38 $\pm$ 0.38	–	–	–	–

**Table 2** continued

Compounds	RT <sup>a</sup>	Phases					
		PR ( <i>n</i> = 2)	R ( <i>n</i> = 5)	PP ( <i>n</i> = 3)	IF ( <i>n</i> = 2)	M ( <i>n</i> = 2)	
Aliphatics							
44 Ethyl butyrate	3.29	–	0.40 ± 0.33	0.21 ± 0.11	–	–	
45 Hexanal <sup>b</sup>	3.31	5.48 ± 1.96	3.06 ± 1.43	2.59 ± 1.03	10.82 ± 1.45	1.23 ± 0.04	
46 (E)-2-Hexenal <sup>b</sup>	4.02	1.42 ± 0.05	2.16 ± 1.21	0.46 ± 0.46	3.77 ± 0.10	0.80 ± 0.19	
47 (Z)-3-Hexen-1-ol <sup>b</sup>	4.06	2.16 ± 0.80	2.14 ± 1.21	0.46 ± 0.46	3.77 ± 0.11	–	
48 Hexanol <sup>b</sup>	4.24	2.27 ± 0.51	4.33 ± 2.88	1.97 ± 1.97	12.47 ± 7.64	0.33 ± 0.04	
49 Methyl <i>n</i> -hexanoate	5.24	0.20 ± 0.20	–	–	0.49 ± 0.11	0.13 ± 0.03	
50 Methyl (E)-2-hexenoate <sup>b</sup>	6.10	–	–	–	1.37 ± 0.94	2.88 ± 0.40	
51 Butyl 2-methacrylate <sup>c</sup>	6.41	1.06 ± 0.56	0.28 ± 0.07	0.58 ± 0.20	0.69 ± 0.09	0.38 ± 0.06	
52 2,2,4,4,6,6-Pentamethyl heptane <sup>b,c</sup>	6.68	2.26 ± 0.25	–	–	0.48 ± 0.48	0.43 ± 0.11	
53 Decane	6.86	–	–	–	–	0.25 ± 0.01	
54 2-Ethyl-1-hexanol <sup>c</sup>	7.57	0.29 ± 0.29	0.16 ± 0.09	0.15 ± 0.15	0.51 ± 0.18	0.63 ± 0.28	
55 Undecane <sup>b</sup>	9.40	–	–	–	–	26.74 ± 0.54	
56 Nonanal	9.52	0.20 ± 0.20	0.13 ± 0.07	0.11 ± 0.11	0.74 ± 0.12	0.73 ± 0.21	
57 Isododecane <sup>c</sup>	10.86	–	–	–	–	1.28 ± 0.54	
58 (Z)-3-Hexenyl isobutyrate <sup>c</sup>	11.70	–	0.67 ± 0.27	0.07 ± 0.07	–	–	
59 Hexyl butyrate	11.84	–	0.53 ± 0.23	0.37 ± 0.37	–	–	
60 Dodecane <sup>b</sup>	12.06	–	–	–	–	3.27 ± 0.38	
61 Decanal	12.24	0.46 ± 0.46	0.16 ± 0.08	0.12 ± 0.10	0.68 ± 0.02	0.78 ± 0.30	
62 Tridecane <sup>b</sup>	14.77	–	–	0.50 ± 0.27	0.26 ± 0.26	16.44 ± 2.18	
63 Tetradecane	17.35	–	0.17 ± 0.09	0.14 ± 0.14	0.26 ± 0.26	1.35 ± 0.17	
64 Pentadecane <sup>b</sup>	19.84	0.38 ± 0.38	0.15 ± 0.10	0.99 ± 0.49	0.25 ± 0.25	2.11 ± 0.37	
65 Hexadecane	22.20	0.83 ± 0.83	0.18 ± 0.11	0.77 ± 0.40	0.61 ± 0.61	–	
Aromatics							
66 Benzaldehyde	6.14	0.27 ± 0.27	–	0.13 ± 0.13	–	–	
67 4-Methylanisole	7.46	–	0.06 ± 0.06	0.16 ± 0.16	0.42 ± 0.42	0.08 ± 0.03	
68 Methyl salicylate <sup>b</sup>	12.11	3.51 ± 2.15	0.52 ± 0.10	1.18 ± 0.23	2.65 ± 0.70	–	

<sup>a</sup> Retention time<sup>b</sup> Compounds were used for correspondence analysis<sup>c</sup> Compounds were also found in the air controls

while that of male phase figs contained predominantly undecane, dodecane and tridecane (compounds 55, 60 and 62) (Fig. 4).

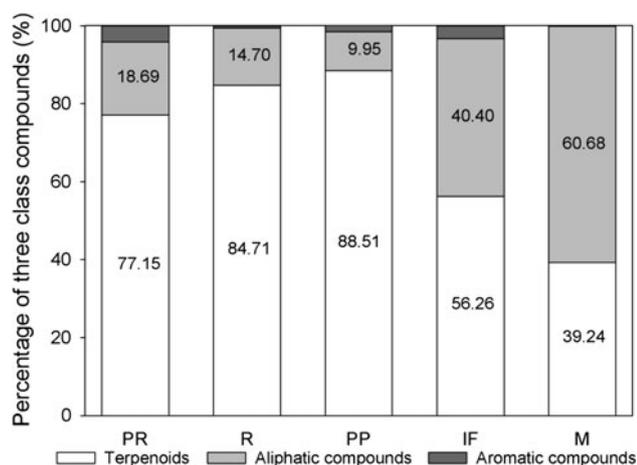
#### Choice experiments

When *Eupristina* sp. females were presented with a choice between *F. curtipes* figs and clean air, they showed a significant preference for figs at their pre-receptive, receptive, post-pollination and interfloral phases, but were repelled by figs that had reached the male phase (Fig. 5). When presented with a choice between receptive figs and figs at other developmental phases, the pollinators always showed a strong preference for receptive figs (Fig. 5). The fig wasps did not discriminate between pre-receptive and post-

pollination and interfloral phase figs, but showed a strong preference for post-pollination figs over interfloral figs (Fig. 5). Male phase figs were significantly less attractive than pre-receptive, post-pollination and interfloral phase (Fig. 5).

#### Timing of fig wasp arrivals at a developing fig crop

Female pollinators were captured throughout the trapping period, not just when receptive phase figs were present on the tree (Fig. 6). The numbers of pollinators attracted to the tree were nonetheless far higher during the period when receptive figs were present, and there was a rapid decline in captures once the bags were removed from the receptive figs (and were entered by the wasps).

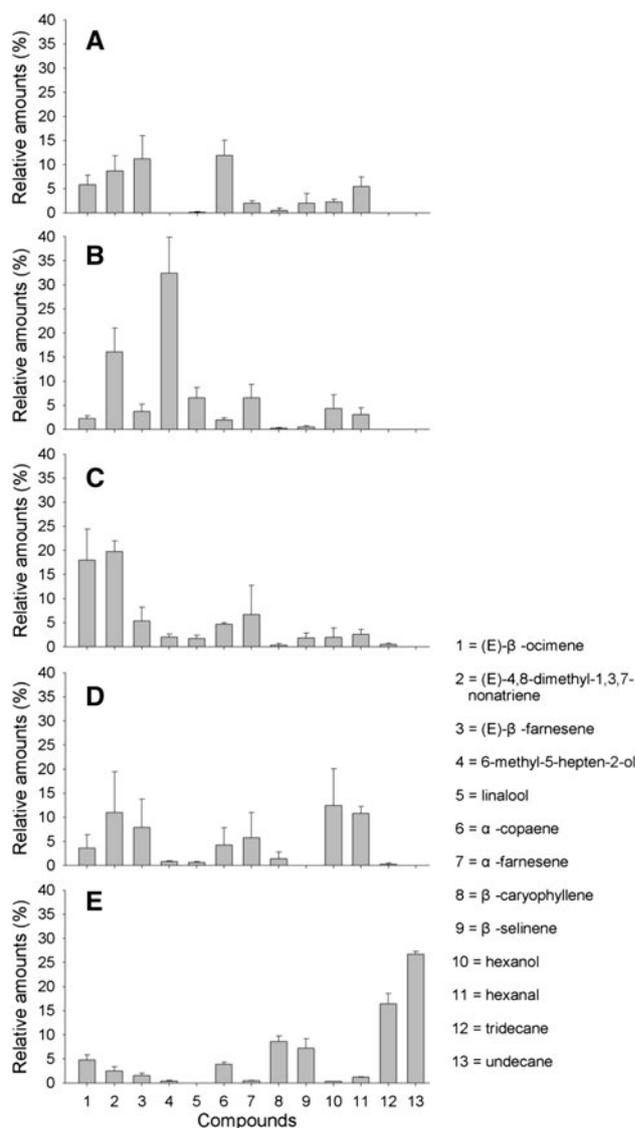


**Fig. 2** Percentage of terpenoids, aliphatics and aromatics changes with developmental phases. *PR*, *R*, *PP*, *IF* and *M* indicate figs at pre-receptive, receptive, post-pollinated, interfloral and male phase, respectively

## Discussion

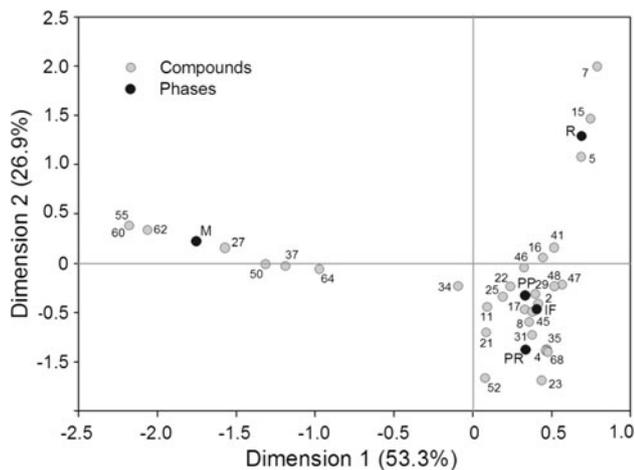
Our results demonstrate that (1) variation in the composition of volatile compounds emanating from *F. curtipes* figs is related to the phases of their development, (2) the *Eupristina* sp. pollinators of the figs show a preference for receptive figs over figs in other developmental phases, (3) the fig wasps were nonetheless attracted to pre-receptive, post-pollination and interfloral phase figs, (4) they actively avoided male phase figs, and (5) under natural conditions, some wasps arrive at the tree before and after receptive figs are present. In short, the floral scents from *F. curtipes* figs change with fig development, and the tree's pollinators respond differently to figs at different phases, with attraction during most phases, strong attraction to receptive figs and avoidance of male phase figs.

The volatile blends emitted by *F. curtipes* figs changed both in quantity (the relative amount of compounds) and quality (the number of compounds) during their development. The chemical profiles of figs in their pre-receptive, post-pollination and interfloral phase were relatively similar, whereas the volatile profiles during the male and receptive phases were quite different from each other and from those of the other three phases. When compared with the chemical profiles of figs of other *Ficus* species (Grison et al. 1999; Song et al. 2001; Grison-Pigé et al. 2002a, b; Borges et al. 2008; Proffit et al. 2008, 2009; Chen et al. 2009; Proffit and Johnson 2009), *F. curtipes* figs share many compounds with figs of different species and figs of different developmental phases, but some of the compounds have not been reported previously from other species or occur in very different proportions. For example, the proportion of 4-methylanisole in *F. curtipes* volatiles

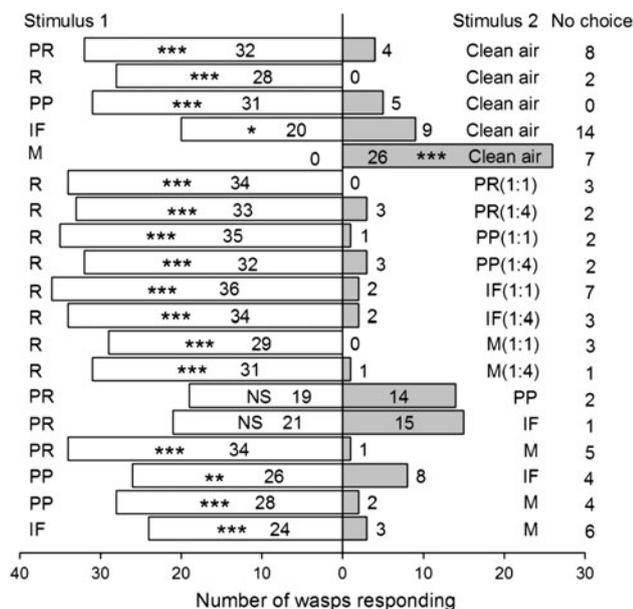


**Fig. 3** Changes (mean  $\pm$  SE) of 13 main compounds (>5 % mean relative amounts in one or more developmental phases) during the developmental phases (a pre-receptive phase, b receptive phase, c post-pollinated phase, d interfloral phase and e male phase) of figs of *Ficus curtipes*

was <1 %, yet it comprises >90 % of the volatile emissions of receptive stage *F. semicordata* figs (Chen et al. 2009). Previously unrecorded components of fig volatile blends included (E)-4,8-dimethyl-1,3,7-nonatriene, a common component of herbivore-induced vegetative emissions (Dudareva et al. 2006; Wiemer et al. 2009), and 6-methyl-5-hepten-2-ol (Song et al. 2001; Grison-Pigé et al. 2002a, b; Borges et al. 2008; Proffit et al. 2008, 2009; Chen et al. 2009; Proffit and Johnson 2009). Other notable components among the volatiles from *F. curtipes* figs were (E)-4,8-dimethyl-1,3,7-nonatriene, which occurred during all phases of development, and 6-methyl-5-hepten-2-ol, which

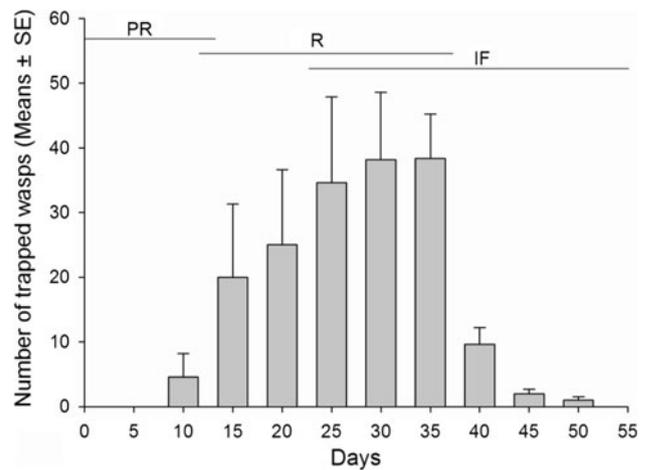


**Fig. 4** Plot of CA of the relationships between *Ficus curtipes* figs at various developmental phases and their volatile compounds. Distances between dots are Chi-square distance. Thirty-one compounds (>2 % means of relative amounts in one or more developmental phases) were selected for this analysis. This plot represents 80.2 % of the total information embedded in the original data matrix. PR, R, PP, IF and M indicate figs at their pre-receptive, receptive, post-pollinated, interfloral and male phases, respectively. The numbered compounds correspond to compounds with the same numbers listed in Table 2



**Fig. 5** Behavioural responses of female *Eupristina* wasps to *Ficus curtipes* figs at different phases in Y-tube olfactometer tests. The ratios of number of receptive figs versus figs from other phases were set as 1:1 and 1:4 and that of figs in other pairs 1:1.  $\chi^2$  test: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , NS:  $P > 0.05$ . PR pre-receptive figs, R receptive figs, PP post-pollinated figs, IF interfloral figs, M male phase figs)

was most abundant during the receptive phase. Additionally, the relative amounts of the three major contributors (i.e. 6-methyl-5-hepten-2-ol, 6-methyl-5-hepten-2-one and



**Fig. 6** The numbers of female *Eupristina* wasps trapped around a *Ficus curtipes* tree with a developing fig crop. PR, R and IF indicate the tree bearing figs at their re-receptive, receptive and interfloral phases, respectively

linalool) of the chemistry profile of the receptive *F. curtipes* figs were gradually or abruptly increased with the prolongation of the figs' receptive phase (Gu D and Yang DR, unpublished data), suggesting that the three compounds or one or two of them may be the attractant of pollinators.

Few studies have compared the behavioural responses of fig wasps to floral scents throughout the development of the figs (Chen and Song 2008; Chen et al. 2009). In particular, and probably because the wasps are rarely observed on non-receptive phase figs (Van Noort et al. 1989; Ware and Compton 1994a), comparisons of the behavioural responses of pollinating wasps to figs at receptive and non-receptive phases have seldom been made. We found that the pollinator of *F. curtipes* figs prefers receptive figs over figs at other phases of development, but that the other earlier developmental phases were nonetheless also attractive to the fig wasps, although to a reduced extent. *Ceratosolen solmsi marchali* similarly prefers receptive figs over interfloral figs of its *F. hispida* host, and yet is also attracted to interfloral figs (Chen and Song 2008). These results question the generally held assumption that pollinator fig wasps are only attracted to figs at their receptive stage, and ignore figs that are at other phases of development.

Although the overall volatile profiles of *F. curtipes* figs at different developmental stages are readily distinguished, most of the volatile chemicals released by its receptive figs also occur in pre-receptive, post-pollinated and interfloral figs. This overlap may be responsible for the attraction displayed by non-receptive figs in the Y-tube olfactometer. The amounts of volatiles released by receptive figs are nonetheless much higher than during other periods (Grisonpigé et al. 2001; Proffit et al. 2008), which will reinforce

their relative attraction over figs at other phases under natural conditions. The relative preference shown for pre-receptive and recently pollinated ('post-pollinated') figs over interfloral figs that had been pollinated several days later also points to a gradual, rather than abrupt, transition in the extent of attraction before and after it peaks during the receptive period. This presumably reflects relatively gradual changes in odour composition (and perhaps quantity) before and after receptivity, with the latter being possibly stimulated by the entry of fig wasps into the figs.

Our conclusions based on the laboratory experiments are consistent with the results from the field. The numbers of pollinators attracted to the tree peaked at the time when many of the figs were receptive, and the loss of the last receptive phase figs when we opened the bags led to an abrupt decline in the numbers of fig wasps recorded. Clearly, receptive figs were much more attractive than figs at other stages. Small numbers of pollinators were nonetheless present before we could find any receptive phase figs on the tree, and they continued to be present beyond the period when we had experimentally extended the presence of receptive figs by bagging some of them. Additionally, the sticky traps were placed around a non-fig tree in the neighbourhood of the sampled fig tree, and no wasps were observed (Gu D and Yang DR, unpublished data). Therefore, we could be confident that non-receptive phase figs remained to be attractive to the wasps.

The repellent effects of male phase figs of *F. curtipes* represent a previously unrecognized component of the chemical communication and co-evolution between fig trees and their pollinators. Most monoecious fig trees produce relatively synchronized crops. This forces pollinators to disperse from their natal trees in search of figs at a suitable stage for oviposition, and thereby ensures outbreeding among the trees (Janzen 1979; Windsor et al. 1989). Synchrony is not absolute, however, particularly when fig crops are large, and some interfloral phase figs will generally still be present on the natal trees when pollinators are emerging from their figs. The repellent effects of the male phase figs from which the newly adult fig wasps are emerging should help encourage them to fly away from the figs, where they can then be dispersed by the wind (Ware and Compton 1994b; Ahmed et al. 2009). The ecological function of floral scents from these male phase figs is therefore to "push" the fig wasps, in contrast to the "pull" exhibited during earlier stages of development. "Push and pull" is a feature of several other pollination systems (Schiestl et al. 1999; Schiestl and Ayasse 2001; Kessler and Baldwin 2007; Terry et al. 2007). The "pull and push" effect in the fig–fig wasp system probably maximizes the reproductive chance of the partners in this system and then promotes the coexistence between figs and their pollinating wasps.

The changing floral scents of figs play a key role in mediating the meeting and parting of the partners in the fig tree–fig wasp nursery mutualism. They also continue to be significant after the fig wasps have departed, because new volatile compounds are produced that serve to attract birds, bats and other vertebrates that will eat the figs and disperse the seeds (Borges et al. 2008).

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