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Maintenance of specificity in sympatric host-specific fig/wasp pollination mutualisms

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Background: Fig/wasp pollination mutualisms are extreme examples of species-specific plant-insect symbioses, but incomplete specificity occurs, with potentially important evolutionary consequences. Why pollinators enter non- typical hosts, and the fates of pollinators and the figs they enter, are unknown.

Methods: We studied two closely related sympatric pollinating fig wasps, *Ceratosolen emarginatus* and *Ceratosolen* sp., whose typical hosts are *Ficus auriculata* and *F. hainanensis*, respectively, recording frequencies of each wasp in figs of the non- typical host. We measured ovipositor lengths of each wasp species, and style lengths in male figs, in both typical and non- typical hosts. Volatile organic compounds (VOCs) emitted by receptive figs of each species were identified using GC-MS. We tested attraction of wasps to floral scents in choice experiments, and detected electrophysiologically active compounds by GC-EAD. We introduced *C. emarginatus* foundresses into figs of both species to reveal the consequences of entry into a non- typical host.

Results: Pollinators entered a low proportion of figs of the non-typical host, and produced offspring in a small proportion of them. Despite differences in the VOC profiles of the two fig species, they included shared semiochemicals. Although *C. emarginatus* females prefer receptive figs of *F. auriculata*, they are also attracted to those of *F. hainanensis*. *Ceratosolen emarginatus* that entered male figs of *F. hainanensis* produced offspring, as their ovipositors were long enough to traverse the style, but broods were larger and offspring smaller than in the typical host. Female figs of *F. hainanensis* failed to produce seeds when visited by *C. emarginatus*. These results provide new insights into the processes maintaining specificity in mutualist interactions and highlight both facilitating and vetoing introgression mechanisms whose potential malleability may intermittently permit hybridization-induced diversity fluxes.

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Abstract

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Introduction

Obligate species-specific pollination mutualisms are important and unique components of ecosystems that facilitate efficient reproductive isolation between plant species (Schiestl & Schlüter 2009). However, species-specificity is not absolute and numerous examples of non-typical host-use by pollinators have been recorded (Kawakita 2010; Rasplus 1996; Starr et al. 2013; Zhang et al. 2012). The ongoing occurrence of such events without vetoing mechanisms that regulate species-specificity have the potential to undermine extant biodiversity patterns by either creating hybrid swarms among closely related species or by instigating hybrid-induced speciation events (Coyne & Orr 2004). The proximate mechanisms that facilitate non-typical host-use and those that help maintain species-specificity, alongside the potential evolutionary consequences, are largely unknown.

Partners in species-specific mutualisms have evolved private codes including production of, and response to, particular mixes of volatile organic compounds (VOCs) that mediate host/pollinator encounter, and matching morphological traits that enforce specificity of the interaction (Chen et al. 2009; Ibanez et al. 2010; Okamoto et al. 2007). Such relationships have evolved between several plant and insect lineages, including *Ficus* (Moraceae) and their fig wasp pollinators (Cook & Rasplus 2003), Yucca (Asparagaceae) and yucca moth pollinators (Pellmyr et al. 1996), and Phyllantheae and leafflower moth pollinators (Kawakita 2010). In these systems, pollinators rear offspring exclusively within the reproductive structures of their host plants and they are the plants' sole pollinators (Dufaÿ & Anstett 2003).

Strict specificity is predicted to lead to co-diversification over evolutionary timescales that should lead to one-to-one interaction patterns. However, numerous exceptions to this have been reported. They may involve multiple pollinators breeding on a single host, or, less frequently, a pollinator species locally interacting with different hosts (Kawakita 2010; Rasplus 1996; Starr et al. 2013; Zhang et al. 2012). Among fig wasps, cases where a pollinator uses two hosts may result in interspecific hybridization among both hosts and wasps. While interspecific introgression may be a genetic dead-end if selection counters hybridization, it can also promote speciation (Abbott et al. 2013; Wang et al. 2016). Cases of incomplete specificity in species-specific mutualisms have received considerable attention (de Vienne et al. 2013; Whittall & Hodges 2007), but several important questions have not been addressed. For example, why would an exclusive pollinator associating with its own obligate host species interact with a non-typical host plant that presumably provides sub-optimal conditions? What are the consequences of this behavior for the fitness of pollinators? And most importantly, why does such behavior not result in the breakdown of species-specific mutualisms?

Among plants, barriers promoting reproductive isolation are typically classified as either pre- or post-pollination (Baack et al. 2015). In species-specific pollination systems, most studies have found that pre-pollination barriers were more prevalent often featuring plants emitting distinct pollinator specific volatile organic compound (VOC) attractants (Althoff 2014; Okamoto et al. 2007; Scopece et al. 2013; Whitehead & Peakall 2014). However, post-pollination barriers (including pre- and post-zygotic mechanisms) do occur in some cases and are typically mediated by pollen-stigma incompatibility, pollen competition, embryo abortion and hybrid sterility (Scopece et al. 2013).

Ficus, feature a unique globular inflorescence usually called a syconium or fig (Janzen 1979), and are one of the largest genera of terrestrial plants and are often considered keystone species in tropical biomes (Nason et al. 1998). Their pollinating wasps are mostly species-specific (Cook & Rasplus 2003) with larvae developing within the fig's ovules (Weiblen 2002). In dioecious species, figs of male trees produce wasps and pollen in female flowers and seeds in female figs. When figs reach receptive phase, they emit floral scents comprising a mix of VOCs, which constitute the main signal used by a typically exclusive pollinator to locate its host plant. Floral scents exhibit quantitative and qualitative variation in composition of VOCs among host plant species (Souto-Vilarós et al. 2018). Pollinating insects can therefore rely on these specific signals as semiochemicals to locate their typical host, thus ensuring species-specific interactions (Raguso 2008). A key determinant of oviposition success is relative ovipositor to style length. In male figs, styles of female flowers are shorter than the ovipositor, so that all female flowers receive a pollinator egg while the styles of flowers in female figs are much longer, so wasps only pollinate but cannot oviposit (Shi et al. 2006).

Incomplete specificity of fig-wasp mutualisms has been documented in around 30-40% of cases (Cook & Segar 2010; Machado et al. 2005; Rasplus 1996; Segar et al. 2014; Yang et al. 2015). In most of these, multiple pollinators are associated with a widely distributed *Ficus* species in different parts of its range (Bain et al. 2016; Cornille et al. 2012; Darwell et al. 2014; Rasplus 1996; Rodriguez et al. 2017; Yu et al. 2021). However, in some cases, figs are entered not only by the typical pollinator but also by a pollinator from asymparically occurring *Ficus* sp. (Machado et al. 2005; McLeish & van Noort 2012; Moe et al. 2011; Ramírez 1970; Souto-Vilarós et al. 2018; Yu et al. 2021). Fig wasps may enter a non-typical host because it produces floral scents similar to those of its regular host. However, little is known about how pollinating fig wasps perceive the odours of receptive figs and may be induced to visit them as studies have typically focused on variation in VOC composition of floral odours among host plant species (Ackerman 1983; Starr et al. 2013; Sutton et al. 2017; Wang et al. 2016; Zhang et al. 2012) rather than on their similarities.

The evolutionary consequences of non-typical pairings for figs and for wasps is also unknown. Limited recent gene exchange among *Ficus* species has been observed in cases where pollinators of one species regularly visit (normally at low frequencies) an alternative host (Machado et al. 2005; Wang et al. 2016; Wei et al. 2014; Yang et al. 2015). This finding is consistent with data from studies of controlled pollinator introductions into non-typical hosts. Introduction of four fig wasp species from other *Ficus* species into *F. turbinata* in Venezuela showed that while these wasps produced offspring, no viable seeds were produced (Ramírez 1970). In dioecious *F. montana*, its pollinator*, Kradibia tentacularis*, produced no progeny when introduced into male figs of *F. asperifolia*, but female figs of *F. asperifolia* produced viable seeds after introduction of *K. tentacularis* bearing *F. montana* pollen (Ghana et al. 2015). Few experimental studies of pollinating wasps entering non-typical hosts have examined the fates of resulting pollinator offspring and fig seeds. Where wasp offspring are produced in non-typical hosts, no information exists on resultant morphological traits and whether fitnesses are affected. It is also unknown whether pollinators and plants might also hybridize.

The fig-wasp pairs, *F. auriculata-Ceratosolen emarginatus* and *F. hainanensis-Ceratosolen* sp., provide an ideal study system in which the pollinators occasionally visit figs of the alternate host (Fig. 1). Here we address the following questions: i) What are the frequencies of pollinator visitation on non-typical hosts in natural populations? ii) What are the differences and the similarities in profiles of the VOCs produced by receptive figs of the two host species? iii) Do receptive figs of both hosts attract *C. emarginatus*? iv) Do the two fig species share semiochemicals that are electro-physiologically active in *C. emarginatus*? v) Does relative ovipositor length of *C. emarginatus* among both typical and non-typical hosts vary? vi) What are the potential evolutionary consequences of entry by *C. emarginatus* into a non-typical host, for both wasps and figs?

Materials & Methods

Study sites

This study was conducted in Xishuangbanna, Yunnan, southwestern China. *Ficus auriculate* is distributed in moist valleys in rain forests, whereas *F. hainanensis* is distributed in limestone areas along rivers. We chose the natural habitats of the two species as study sites, the former in the rain forest in XTBG (Xishuangbanna Tropical Botanical Garden) in Menglun town, and the latter along the Mengxing River in Mengxing town. All study trees are non-cultivated. The two study sites are 10km apart.

Study species

F. auriculata and *F. hainanensis* are dioecious species (subsection Neomorphe, section *Sycomorus*, subgenus *Sycomorus*). Alongside *F. oligodon*, they form a species group, the *F. auriculata* complex, whose taxonomic status is still under debate. Here we follow the assignation to species based on morphology and corroborated by genetic data proposed by Wei et al. (2014) . The species group comprises three species of pollinating wasps all belonging to the species group *Ceratosolen emarginatus*, which may be found on any of the three host species (Wang et al. 2016; Wei et al. 2014). *F. auriculata* is mainly pollinated by the species traditionally identified as *Ceratosolen emarginatus* Mayr (Clade 1 in Wang et al., 2016) and occasionally by what could be another species (Clade 3 in Wang et al., 2016), while *F. hainanensis* is almost exclusively pollinated by a closely related species, *Ceratosolen* sp. (Clade 2 in Wang et al., 2016). We further identified morphological traits distinguishing *C. emarginatus* and *Ceratosolen* sp. (Fig. 2): most obviously, that the appendages of the mandibulae are notably closer to the maxilla in *C. emarginatus* compared to *Ceratosolen* sp.. To control which wasp species were used in laboratory experiments, we introduced a single female wasp of known species into each fig so that we could use the resultant offspring for experimentation without need for species delimitation.

Investigation of pollinator host use in natural populations

We investigated the species and numbers of pollinating wasps that had entered figs of *F. auriculata* and *F. hainanensis* by collecting figs just after the period of receptivity. Adult wasps emerge from male figs about two months later when figs are ripe (male phase). We collected wasps emerging from male phase figs, identified their species and counted them. For F. auriculata, we collected at least 20 post-receptive figs from each of four male and four female trees. In addition, for male trees, we collected 20 male-phase figs per tree. For *F. hainanensis*, we collected 20 post-receptive figs from each of three male and three female trees, and 20 male phase figs from male trees.

Each post-receptive fig was cut open and foundresses were collected from the cavity and preserved in 75% alcohol for morphological species identification and counting. Every male-phase fig was put into a nylon bag (120 mesh; to prevent escape) shortly before wasp emergence. Once wasps had emerged into the bag, 10 wasps per bag were randomly selected and preserved in 75% alcohol for subsequent species identification.

Behavioral bioassays

To test whether *C. emarginatus* was preferentially attracted to its usual over non-typical figs, behavioral choice experiments were performed using a Y-tube olfactometer (ID:1.5 cm, length of each arm 9 cm, stem 8 cm). Two arms of the Y-tube were each connected to a polyethylene terephthalate bag (Toppits® GmbH, Germany) containing a source of odour. Airflow was pumped by a mini-vacuum pump (Xinweicheng® Xinweicheng Machinery & Electric Co., Ltd, Chengdu, China) through the bags into the arms of the Y-tube, after being purified by passing through an activated charcoal filter. The flow rate through each arm was maintained at 100 ml/min. A wasp was deposited in the third arm of the olfactometer for behavioral observation. To prepare the odour sources, pre-receptive stage figs of *F. auriculata* and *F. hainanensis* were enclosed in nylon bags to protect them from oviposition by wasps until the figs became receptive. Male figs were collected just before pollinator emergence to obtain freshly emerged fig wasps, and only one wasp was selected from each fig. To test the response of *C. emarginatus* to receptive figs of typical and non-typical host, three treatments were carried out: receptive figs of F. auriculata versus air, receptive figs of *F. hainanensis* versus air, and receptive figs of F. auriculata versus receptive figs of *F. hainanensis*. Because of differences in fig size between species, equal weights of figs of the two hosts were used (F. auriculata: 5-6 figs, *F. hainanensis*: 22-28 figs). All bioassays were performed in a darkened room, between 10:00-12:00 hours, within three hours after collection of fresh figs from trees. Each of the female fig wasps tested was positioned at the entrance to the stem of the olfactometer, and the arm it selected was recorded as well as the time to decision. If the wasp did not make a decision within 5 minutes, it was excluded from the total number counted and from statistical analysis. After testing five successive wasps, Y-tube arms were reversed to cancel out any orientation bias effect between. Each wasp individual was tested only once, and, after 10 successive wasps, the Y-tube was replaced with a new one to avoid any influence of residual materials remaining in the apparatus. Three treatments were repeated with 31, 38 and 44 fig wasps that made a decision. Preliminary experiments showed that *C. emarginatus* was equally attracted by male and female figs of *F. auriculata*, so we used only male figs as a source of odours.

Comparison of VOCs emitted by focal *Ficus* species

VOCs were collected using the dynamic headspace technique (Chen et al. 2009). Small pre-receptive figs for each gender of *F. auriculata* and *F. hainanensis* were enclosed in nylon bags on trees to prevent wasps from entering and ovipositing. When the figs had reached receptive phase, they were enclosed in polyethylene terephthalate bags for the collection of VOCs. Airflow, purified by passing through a filter of activated charcoal (20-40 mesh, Supelco® Sigma-Aldrich, USA), was maintained through the bag by a mini-vacuum pump connected to the entrance by flow-meters with a flow rate of 300 ml/min, while a VOC trap containing 80mg Porapak® Q adsorbent (80-100 mesh, Supelco® Sigma-Aldrich, USA) was connected to the exit of the bag at a flow rate of 300 ml/min. To check for possible contaminant compounds sampled during collection, empty bags were used as blanks for extraction by means of the same dynamic headspace technique and equipment. VOC collection was performed for four hours from 10:00 to 14:00, the period of the day when fig wasps are most active. Three repeats were performed for each gender of each tree species. After VOC collection, the adsorbents were eluted three times with a total of 500 µl of dichloromethane and concentrated down to 100 µl with 99.99% N2.

Then, two internal standards (octane and dodecane, at 200 ng/μl) were added to every sample prior to gas chromatography.

All VOC samples were analysed using gas chromatography-mass spectrometry (GC-MS, Agilent Technologies 7890A-5975C, USA) with an HP-5MS column (30 m, ID: 250 μm, film thickness 0.25 μm). Helium was used as carrier gas at a flow rate of 1ml/min. The injector split vent was set at a ratio of 1:4 and the injector temperature was 250 °C. Oven temperature was set at 40 °C, and then programmed to rise to 150 °C at a rate of 3 °C/min, then at 10 °C/min to 260 °C, and finally temperature was maintained at 260 °C for 5 minutes. Compound identification was based on comparison of retention times (RT), matching of the mass spectra with the NIST 08 MS library, and Kovats retention indices (RI) taken from both the NIST Chemistry Web Book (http://webbook.nist.gov) and the RI database (Adams 2007). Where available, we used synthetic compounds as a more precise reference (see Supplementary table 1).

Electrophysiological responses of *C. emarginatus* to VOCs of the two host species

To identify which VOCs of the receptive fig odours were detected by the wasps and thus constituted candidate semiochemicals, we performed electrophysiological tests. The responses of *C. emarginatus* antennae to odours from receptive figs of *F. auriculata* and *F. hainanensis* were recorded using gas chromatography-electroantennography (GC-EAD, Agilent, USA, Syntech, Netherlands). VOCs were collected for injection in the GC-EAD as for the VOC analysis procedures except that we extended the collection duration to six hours in order to extract larger quantities of VOCs. The GC program was the same as that used for the analysis of VOCs presented above. A head with an antenna was placed on a micro-operating platform (MP-15, Syntech, Netherlands), and two glass electrodes filled with saline solution (NaCl, 4 g; Na_2HPO_4 0.57 g; KH_2PO_4, 0.1 g; KCl, 0.1 g in 500 ml distilled water; pH 7.4) were connected to the distal tip of the antenna and to the antennal scape. Antenna depolarization was recorded using the Electroantennography version 2.5 software package (Syntech, Netherlands). Three antennae from *C. emarginatus* were tested for *F. auriculata* VOCs and three for *F. hainanensis* VOCs.

Relative ovipositor to style length

As pollinating wasps oviposit by inserting their ovipositors into styles, we investigated style length in both host *Ficus* species and ovipositor length in both pollinating wasp species. We enclosed young figs of *F. auriculata* and *F. hainanensis* in nylon bags (120 mesh) to prevent wasp entrance. When the figs became receptive, we collected 32 figs from four *F. auriculata* male trees and 30 figs from three female trees; we collected 30 figs from three *F. hainanensis* male trees and 31 figs from three female trees. Then 20 flowers per fig were randomly selected to measure style length. 90 male figs of *F. auriculata* were collected and placed in separate bags before female wasp emergence. After emergence we selected one *C. emarginatus* from every fig and measured their ovipositor lengths. The same method was applied to *F. hainanensis* using 90 wasps emerged from 90 male figs. All measurements of style length of figs and ovipositor length of pollinators were conducted using a micrometer in a dissection microscope (Olympus SZX12-3141, Tokyo, Japan).

C. emarginatus introduction experiments

Young figs of *F. auriculata* and *F. hainanensis* were enclosed in large nylon bags (120 mesh) to prevent wasp oviposition. When these figs reached receptive phase, one *C. emarginatus* emerging from *F. auriculata* was introduced into each fig. Wasps were introduced into at least 20 figs for each tree. We chose three trees of each gender for each host species. After introduction, figs were re-enclosed in large nylon bags until just before wasps emerged from the figs. The figs were then removed from the tree and enclosed in individual nylon bags. All emerging wasps were preserved in 75% alcohol for subsequent counting and measurements. Three traits representing fig wasp size (head width, thorax width and ovipositor length, see Liu et al., 2011 were measured on the offspring for comparison with the foundresses. At least 40 wasps from each fig were measured. All measurements were carried out under a stereomicroscope.

Data analysis

Data analyses were mostly performed in R version 4.0.5 (R Development Core Team; URL http://www.R-project.org). For VOC analyses, non-metric multi-dimensional scaling (NMDS) methods were conducted using the Vegan package and the Bray-Curtis distance was used to find the best two-dimensional representation of the distance matrix. A Permutational Multivariate Analysis of Variance (PERMANOVA) was used to compare the VOC composition between *F. auriculata* and *F. hainanensis*. Chi-square tests were used to determine whether pollinators showed preferences for their typical or non-typical host. Mann-Whitney U tests were used to test the time that pollinators took to make a choice in the behavior-choice experiment. ANOVAs were used to examine differences in head width, thorax width and ovipositor length between the foundresses of *C. emarginatus* and that of *Ceratosolen* sp., and between foundresses and offspring in the two treatments. Style length, ovipositor length and number of offspring in the two treatments were also compared using Mann-Whitney U tests.

Results

Frequency of pollinators entering a non-usual host

We collected a total of 2251 wasps that had entered 175 receptive figs of *F. auriculata* (1036 wasps in 86 male figs and 1215 in 88 female figs). These were mostly *C. emarginatus* (94.31% in male figs and 98.02% in female figs, Fig. 3). Among these, 23 out of 86 male figs (2.57±1.31 per fig) and 18 out of 88 female figs (1.33±0.59 per fig) contained *Ceratosolen* sp.. We collected a total of 931 wasps (757 in 60 male figs and 174 in 60 female figs) that had entered 120 receptive *F. hainanensis* figs. These were predominantly *Ceratosolen* sp. (88.11% in male figs and 93.68% in female figs; Fig. 3). Among these, 24 out of 60 male figs (3.75±3.35 per fig) and 11 out of 60 female figs (1.00±0.00 per fig) contained *C. emarginatus*. Results for offspring production were similar to those for fig visitation, but the differences between typical and non-typical hosts were more extreme. In *F. auriculata*, out of 800 fig wasp offspring from 80 male figs, 7 *Ceratosolen* sp. individuals (0.88% of the total) were found while from 600 fig wasp offspring from 60 male *F. hainanensis* figs, there were 30 *C. emarginatus* individuals (5.00% of the total) (Fig. 3).

Pollinator bioassays

Female *C. emarginatus* individuals were strongly attracted by receptive figs of their usual host, *F. auriculata*, when confronted with a choice between it and air or the non-typical host *F. hainanensis*. The wasps preferred *F. hainanensis* receptive figs over air (Fig. 4). When the wasps were given a choice between *F. auriculata* and *F. hainanensis*, there was a reduction in the proportion of wasps choosing *F auriculata* figs when compared to the *F. auriculata* versus air choice (χ^2=8.656, P=0.003).

When given a choice between *F. auriculata* odour and air, *C. emarginatus* spent the shortest mean recorded time before entering a branch of the olfactometer (39.91±22.35s, P=0.036). *C. emarginatus* preferred *F. auriculata* odour when given a choice between it and *F. hainanensis*, with the time taken independent of final arm choice (*F. auriculata*: 54.78±36.41s, *F. hainanensis*: 56.75±46.60s, Z=-0.078, P=0.938). In the *F. hainanensis* versus air treatment, *C. emarginatus* took longer before entering an arm (63.99±38.29s) in comparison to the *F. auriculata* versus air treatment (Z=-2.641, P=0.008).

Comparison of the VOCs emitted by the two *Ficus* species

We identified a total of 78 VOCs in scents emitted by receptive figs of *F. auriculata* and *F. hainanensis*. VOCs emitted by receptive figs did not differ significantly between sexes within species (Fig. 5, PERMANOVA; for *F. auriculata*, F=2.68, P=0.062, for *F. hainanensis*, F=3.95, P=0.100; electronic supplementary material Table S1). Thirty-four VOCs were shared between the odours produced by the two fig species, and among these, ten VOCs were abundant (> 5%) in one or both species. In particular, the relative ratios of β-funebrene were high in both species (*F. auriculata*: 12.22±5.50, *F. hainanensis*: 26.83±5.48). Nevertheless, the complements of VOCs produced by *F. auriculata* and *F. hainanensis* were distinguishable (PERMANOVA, F=11.297, P=0.002).

Electrophysiological responses of *C. emarginatus* to VOCs of host species

C. emarginatus presented electroantennographic responses to nine compounds in the odours from *F. auriculata* and to six in the odours from *F. hainanensis* (Fig. 6). Among these compounds, α-copaene and β-funebrene were produced by both host trees. These two compounds represented 38% and 31% of the scents emitted by male and female *F. hainanensis* figs respectively.

Matching of ovipositor length and style length

Style lengths were bimodally distributed in both *F. auriculata* and *F. hainanensis* (Fig.7). Style lengths of figs of female trees (*F. auriculata*: 2.03±0.75mm, *F. hainanensis*: 1.54±0.18mm) were much longer than in figs of male trees in both *Ficus* species (*F. auriculata*: Z=-25.534, p<0.001, *F. hainanensis*: Z=-30.292, p<0.001). Ovipositors of *both C. emarginatus* and *Ceratosolen* sp. were somewhat longer than the styles of male figs from their respective hosts (Fig. 7). Style length in figs of male trees of *F. auriculata* (0.97±0.11 mm) was around 0.05 mm longer than that for figs of male trees of *F. hainanensis* (0.92±0.05mm) (Z=9.295, p<0.001). The ovipositor length of *C. emarginatus* (1.19±0.09mm, n=90) was 0.17 mm longer than that of *Ceratosolen* sp. (1.03±0.07, n=90) (Z=9.486, P<0.001).

Consequences of introduction of *C. emarginatus*

Single *C. emarginatus* females produced significantly fewer offspring in *F. auriculata* figs than in *F. hainanensis* figs (472.85±18.68 versus 618.49±27.31) (Z=-4.490, p<0.001, Table 1). When *C. emarginatus* was introduced into female figs of *F. hainanensis*, all treated figs aborted (Table 1). Female *C. emarginatus* reared from *F. hainanensis* were similar in size to *Ceratosolen* sp. (head width: P=0.925; thorax width: P=0.999) and significantly smaller than those reared from their usual host *F. auriculata* (head width: P=0.018; thorax width: P= 0.0002). Their ovipositor was shorter than those of both *Ceratosolen* sp. (P=0.034) and of *C. emarginatus* raised on its typical host (p<0.001; Table 2).

Discussion

The proximate factors that mediate non-typical host-use among co-evolved, highly species-specific mutualists are unknown. This is especially intriguing when we consider that such interactions likely incur a fitness cost for both partners (e.g. Janzen 1979, Ghana et al. 2015). Moreover, given that these phenomena naturally occur, it is also unknown what mechanisms impede either the formation of hybrid swarms among closely-related species or the evolution of hybridization-induced speciation events – both of which may cause breakdown of species-specificity with resultant fluxes in extant biodiversity patterns. Here we present a novel study that incorporates comprehensive sampling alongside detailed ecological data to investigate both the mechanisms and potential evolutionary outcomes of non-typical host-use events. We show that while promoting mechanisms may facilitate non-typical host-use, vetoing mechanisms may regularly operate that select against sustained introgression and help maintain species-specificity patterns.

Pollinator visits to non-typical hosts

Frequent use of several host species by a pollinator species has only been reported among American and African monoecious *Ficus* lineages (Cornille et al. 2012; Machado et al. 2005; McLeish & van Noort 2012) were host-species recognition mechanisms appear to lack precision and the ecological conditions under which multiple host use is observed is generally not reported. In our study, we investigated visitation of two host species' populations, situated several kilometers apart, by their respective pollinating wasps in their natural habitat. We identified to species 3182 wasps that had entered 295 receptive phase figs and 1400 offspring wasps emerging from 140 figs. Our results reveal strong specialization with only a low frequency of individuals colonizing the non-typical host species, as seen in other dioecious *Ficus* species (Moe et al. 2011; Silvieus 2006; Weiblen et al. 2001). Further, both host species were only visited by their two closely-related pollinators, *Ceratosolen emarginatus* and *Ceratosolen* sp..

We document a reduction in the proportion of non-typical pollinator offspring relative to the proportion of initial foundresses in natural populations. Moreover, female *C. emarginatus* experimentally introduced into figs of *F. hainanensis* produced larger broods than on its typical host and more than is produced by *F. hainanensis*'s typical pollinator, *Ceratosolen* sp. (Yang et al. 2012). However, our results suggest that *C. emarginatus* individuals visiting figs of *F. hainanensis* do not attain optimal fitness due to trait mismatching resulting from reduced offspring body size that is likely to compromise oviposition abilities. It is possible that individuals of *C. emarginatus* visiting figs of *F. hainanensis* are wasps that have failed to locate receptive figs of *F. auriculata* (Liu et al. 2015), and have become less choosy towards the end of their life spans. In some cases, wasps entering non-typical hosts failed to reproduce.

Our results show that 23.74% of receptive male *F. auriculata* and 41.67% of receptive male *F. hainanensis* figs harbored both pollinator species. Co-occurrence of these pollinator species across two fig species indicates potential for hybridization (although we did not observe any wasps possessing morphological characters suggestive of hybridization). In *F. rubiginosa*, three cryptic species of *Pleistodontes imperialis* coexist in the same localities while retaining reproductive isolation (Sutton et al. 2017), with Wolbachia identified as the most likely candidate of post-zygotic reproductive isolation (Haine & Cook 2005).

Host volatile semiochemicals attracting pollinators

It has been suggested that closely related sympatric figs species may emit similar floral scents to attract pollinators and this could result in pollinators confusing typical and non-typical host species (Moe et al. 2011; Wang et al. 2013). However, VOC composition of the receptive fig odours of *F. auriculata* and *F. hainanensis* were clearly differentiated, consistent with different VOC profiles between sister species in Papua New Guinea (Souto-Vilarós et al. 2018). They shared 34 VOCs including 10 quantitatively important compounds (i.e. >5%), but only two that were shown to elicit wasp antennal response as semiochemicals. The shared semiochemicals eliciting antennal response represented 19% of receptive male fig odour and 13% of receptive female fig odour in *F. auriculata* and 38% and 31% for male and female *F. hainanensis* figs respectively, indicating a large degree of chemical attractant overlap. Wasp attraction to receptive figs may be from long and short distance alongside contact attractants. In *F. curtipes* it has been shown that one VOC is mainly responsible for long distance attraction while another is more important for fig entry (Gu & Yang 2013). When *C. emarginatus* were given a choice between *F. auriculata* and *F. hainanensis* receptive figs, 30% chose *F. hainanensis* figs, a figure much higher than the observed frequency of non-typical pollinators on our wild figs and suggesting that initial contact with the typical host may result in an immediate drop in choosiness.

Offspring of pollinating fig wasps in non-typical host species

C. emarginatus produced a greater number of smaller offspring in *F. hainanensis* than in its typical host. *F. auriculata* and *F. hainanensis* fit the general pattern by which style lengths in male figs are shorter than the ovipositor of pollinators, allowing wasps to oviposit into male fig ovules (Ganeshaiah et al. 1995; Shi et al. 2006; Weiblen 2002). *C. emarginatus* ovipositors are longer than the styles in male figs of *F. hainanensis* which have shorter styles than *F. auriculata*. This may explain why *C. emarginatus* produced more offspring in *F. hainanensis* than in F. auriculata, as oviposition can be more rapid in flowers with shorter styles. However, offspring body size was reduced in comparison with *C. emarginatus* developing in *F. auriculata*. This probably results from the developing larva consuming the endosperm of the smaller plant ovules of *F. hainanensis* (0.61±0.01mm, n=400), compared with those of *F. auriculata* (0.88±0.01mm, n=420), which may cause a concomitant reduction in fitness due to reduced body size featuring shorter ovipositors which should impinge subsequent oviposition attempts.

Nevertheless, *C. emarginatus* offspring will leave male *F. hainanensis* figs carrying pollen. These wasps are likely to search for *F. auriculata* figs and may subsequently initiate host hybridization (Machado et al. 2005). However, small wasps will probably have reduced capacity to reach receptive figs and will therefore be poor pollen vectors. In comparison, introductions of *Ceratosolen* sp. into *F. auriculata* resulted in most figs aborting and the few remaining figs producing few offspring (Yang et al. 2012). This may be due to the shorter ovipositor of *Ceratosolen* sp. which may limit oviposition. Hence, for both wasp species, visiting non-typical host figs may constitute a genetic dead-end.

In experimental introductions into receptive figs of *F. hispidioides* of wasps originating from four other *Ficus* species, no wasps developed, although oviposition attempts led to ovule development in male figs (Moe et al. 2011). Beyond the *F. auriculata* species complex, successful emergence of offspring wasps from non-typical host figs has, to our knowledge, never been reported, while development to imago stage has rarely been reported (Compton & Nefdt 1990). Hence, despite reports of fig pollinating wasps visiting several host species the outcome of these visits should be ascertained before drawing conclusions on their evolutionary significance (Moe et al. 2011). Mechanisms can therefore exist that both permit atypical interactions while simultaneously preventing introgression and species-specificity break-down. However, it can be further envisaged that this system could be easily reconfigured to favour hybridization events if vetoing mechanisms fail or are selectively removed, potentially due to changes in abiotic conditions that may further vary at the population level.

Hybridization implied by pollinator's presence in a non-typical host

With respect to potential plant hybridization, our data show that all female *F. hainanensis* figs visited by *C. emarginatus* undergo selective abortion. High abortion levels have also been documented for *F. auriculata* figs visited by wasps (most probably *Ceratosolen* sp.) emerging from *F. hainanensis* (Yang et al. 2012). As viable hybrids have been observed in more phylogenetically distant *Ficus* species (e.g.(Condit 1950)), we suggest that the lack of seed production results from variation between wasp species in pollination behavior and trait matching as artificial pollination leading to the production of viable offspring

Four genetic studies based on RFLPs (Parrish et al. 2003) and on microsatellite data (Moe et al. 2011; Wang et al. 2016; Wei et al. 2014) have suggested the presence of limited gene flow between closely related Ficus species. In two of those studies the genetic results confirmed that morphologically intermediate individuals were interspecific hybrids (Parrish et al. 2003; Wei et al. 2014). We cut open all aborted female figs to inspect the ovaries in every cavity containing a *C. emarginatus* foundresses to reveal that ovaries did not develop. This indicates that strong post-zygotic isolation mechanisms prevent the hybridization of *F. auriculata* and *F. hainanensis*. Interestingly, although our experiments suggest that *F. auriculata* and *F. hainanensis* do not typically hybridize, gene flow has been documented between them (Wang et al. 2016). *F. oligodon* which is sympatric with both species hybridized with *F. auriculata* and produced a normal number of seeds (Yang et al. 2012), but hybridization events with *F. hainanensis* produced few seeds and a high fig abortion ratio. This suggests that *F. oligodon* may act as a bridge species facilitating introgression between *F. auriculata* and *F. hainanensis*. Therefore, in addition to sharing pollinators, such a bridge species may play a pivotal role in diversification and speciation events via reticulate evolution (Cornille et al. 2012).

Conclusions

Our findings show that a low frequency of both pollinating wasp species enter the alternate, non-typical host in natural populations, and that a small proportion of these complete development into wasp offspring. The pollinating wasps associated with each fig appear to enter receptive figs of the alternate host because emitted signal scents share some of the main semiochemicals, although their overall VOC compositions are different. However, mismatches between the length of ovipositors of fig wasps and of styles in male figs appear to limit successful reproduction of pollinators in the non-typical host, *F. hainanensis*, and further reduce the fitness of progeny that are produced. Our findings also show that no seeds were produced when *C. emarginatus* with *F. auriculata* pollen were introduced into female figs of *F. hainanensis*, owing either to mismatches between wasp behavior and plant anatomy, interspecific incompatibility, or both. Thus, we show that despite an ongoing potential for introgression, specificity in sympatric fig-wasp pollination mutualisms can be maintained. Moreover, our data indicate that suites of mechanisms either promoting or hindering hybridization may co-exist, with eventual outcomes contingent on dominant vetoing mechanisms. However, over evolutionary timescales, occasional use of non-typical hosts may provide opportunities for host shifts, hybridization or other events leading to diversification of both figs and wasps if vetoing mechanisms fail or are selectively removed.

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

Xie Hua performed field experiments and laboratory work, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.

Pei Yang conceived and designed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.

Yan Xia performed field experiments, authored or reviewed drafts of the paper, and approved the final draft.

Finn Kjellberg analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.

Clive T. Darwell analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.

Zong-Bo Li conceived and designed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.

Field Study Permissions

The following information was supplied relating to field study approvals (i.e., approving body and any reference numbers):

The agonid wasps, *C. emarginatus* and *Ceratosolen* sp., are common insects and collection permitted by the leader of Xishuangbanna Tropical Botanical Garden, Chinese Academy of Science.

Data Availability Statement

The following information was supplied regarding data availability:

The raw data is provided in the Supplemental Files.

Supplemental Information

Table S1: Occurrence (Occ) and relative amounts of tentatively identified volatile organic compounds emitted by receptive figs of *Ficus auriculata* and *F. hainanensis*. a compound with their identity confirmed by mass spectrometry analysis; b compounds with their identity confirmed by comparison of Kovats retention index with the NIST chemistry Web Book (http://webbook.nist.gov) and literature; c compounds with their identity confirmed by comparison of MS and retention time with laboratory standards (Sigma-Aldrich, USA).

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Figure 1

Illustration of sharing the pollinator between two distinct host *Ficus* species.

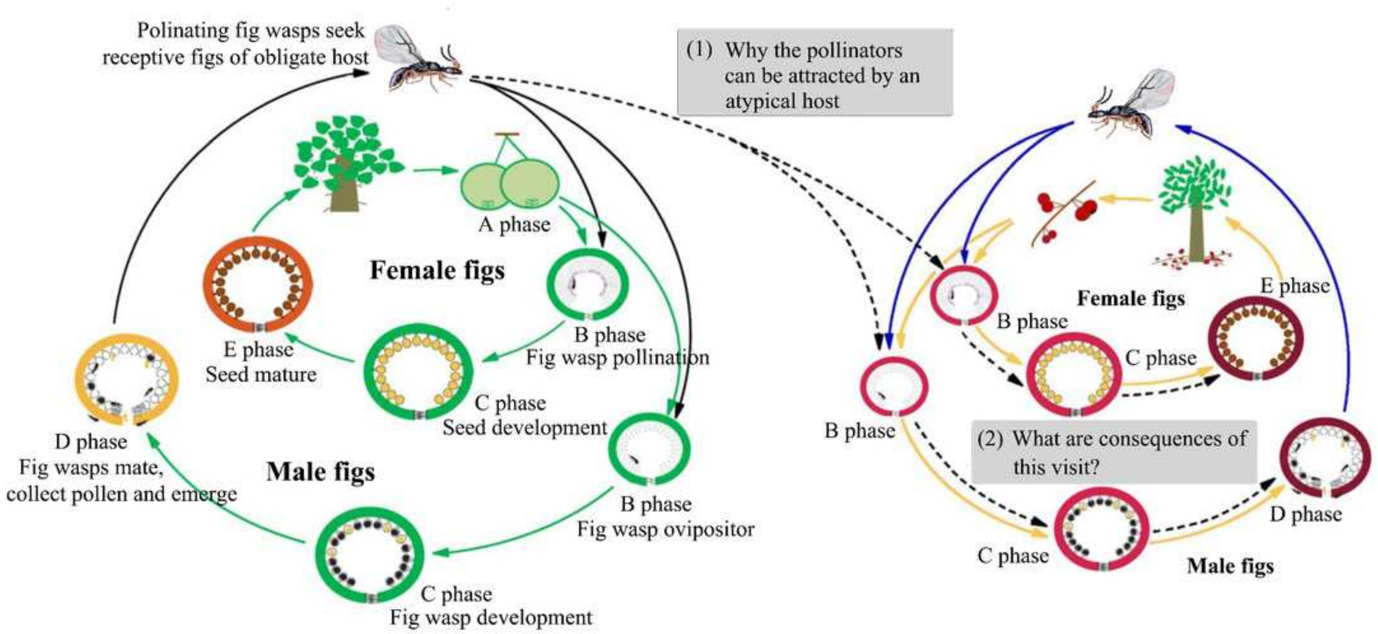

Figure 2

Characteristics of *Ceratosolen emarginatus* and *Ceratosolen* sp.

(A) dorsal side of head of *C. emarginatus*; (B) ventral side of head of *C. emarginatus*; (C) dorsal side of head of *Ceratosolen* sp.; (D) ventral side of head of *Ceratosolen* sp.; (E) dorsal side of hind leg of *C. emarginatus*; (F) ventral side of hind leg of *C. emarginatus*; (G) dorsal side of hind leg of *Ceratosolen* sp.; *(H)* ventral side of hind leg of *Ceratosolen* sp.. Scale: 200 µm. Note that, when the mandibulae are closed, the appendages of the female mandibulae are close to the maxilla in *C. emarginatus* while the mandibular appendages are more divergent from each other and hence more separated from the maxilla in *Ceratosolen* sp.. Furthermore, the hind legs of *C. emarginatus* have a large ventral tooth in the coxa and a sharp tooth in the femur, thus differing from those of *Ceratosolen* sp..

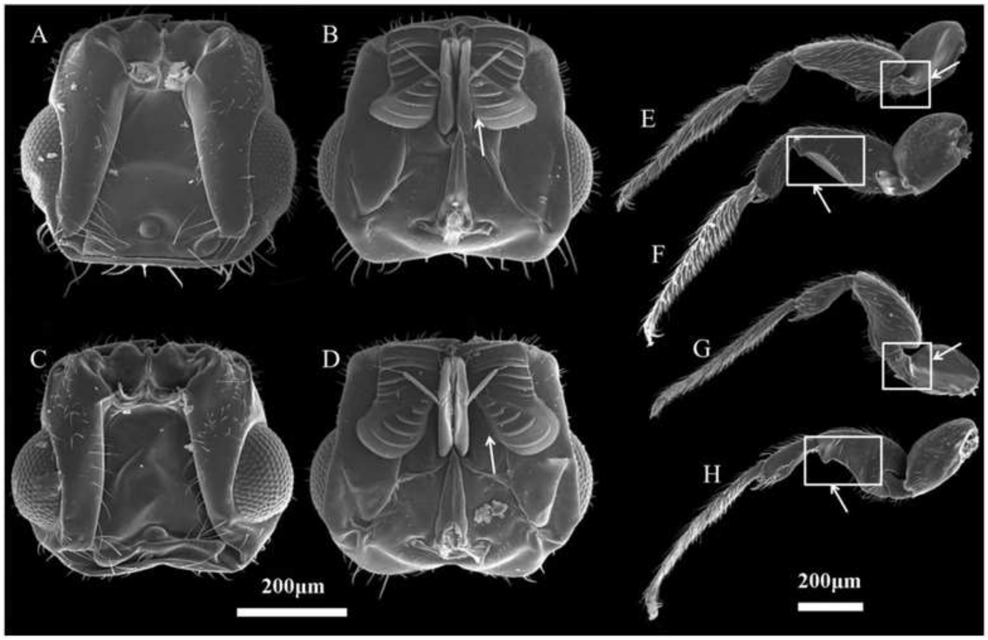
A
B
C
D
E
F
G
H
200μm
200μm

Figure 3

Proportions of pollinator species entering figs of *F. auriculata* and *F. hainanensis* to oviposit and proportions of offspring of the two species emerging from the figs.

The two pollinator species visited both host figs and produced viable offspring. However, for both pollinator species the frequency of visitation was much lower for the non-typical host than for the typical host.

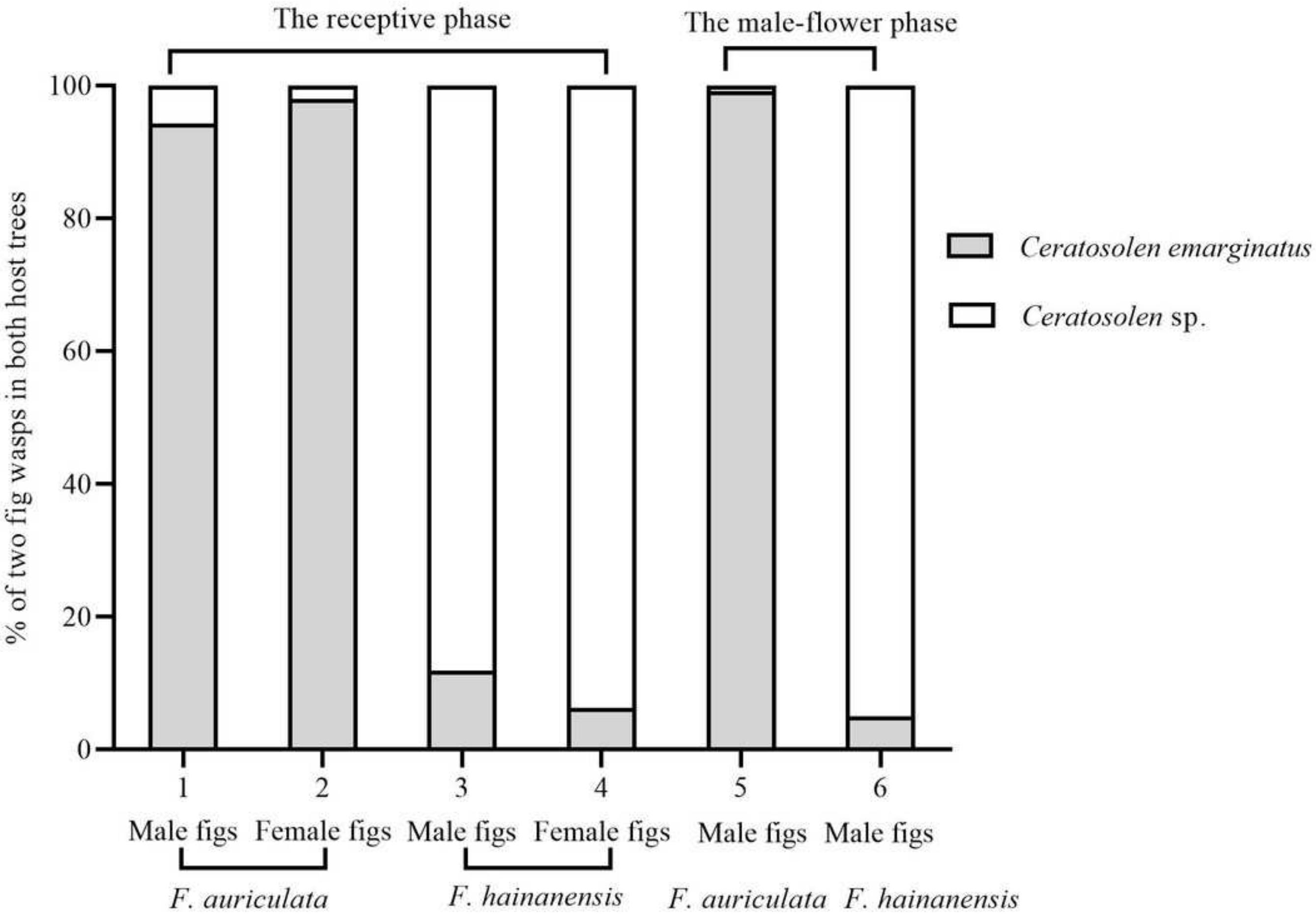

Figure 4

Bioassays of female *Ceratosolen emarginatus* responses to receptive figs of *Ficus auriculata* and *F. hainanensis* performed using Y-tube olfactometer tests

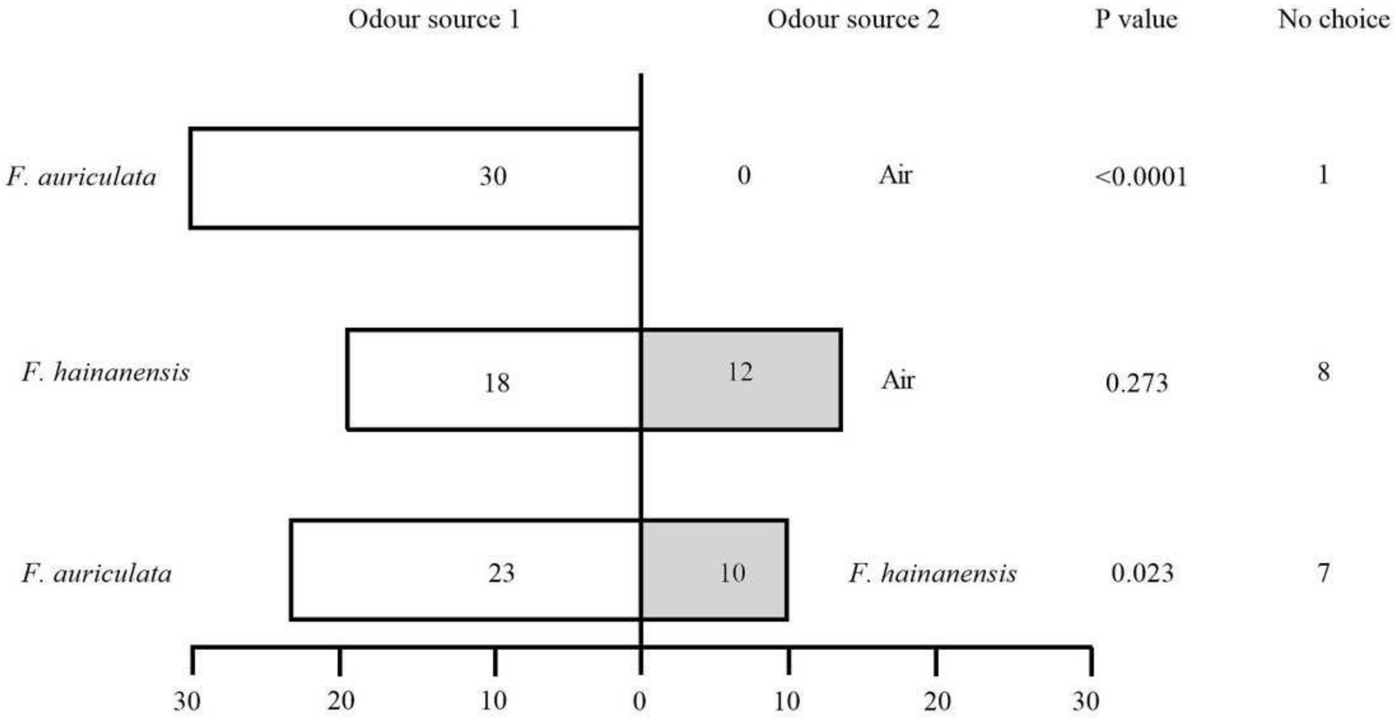

Figure 5

Non-metric multi-dimensional scaling of the relative proportions of VOCs emitted by receptive figs of *Ficus auriculata* and *F. hainanensis* based on Bray-Curtis dissimilarity index (stress=0.062).

The tendency for a slight difference in receptive fig odour between sexes is non-significant whereas the difference between species is significant.

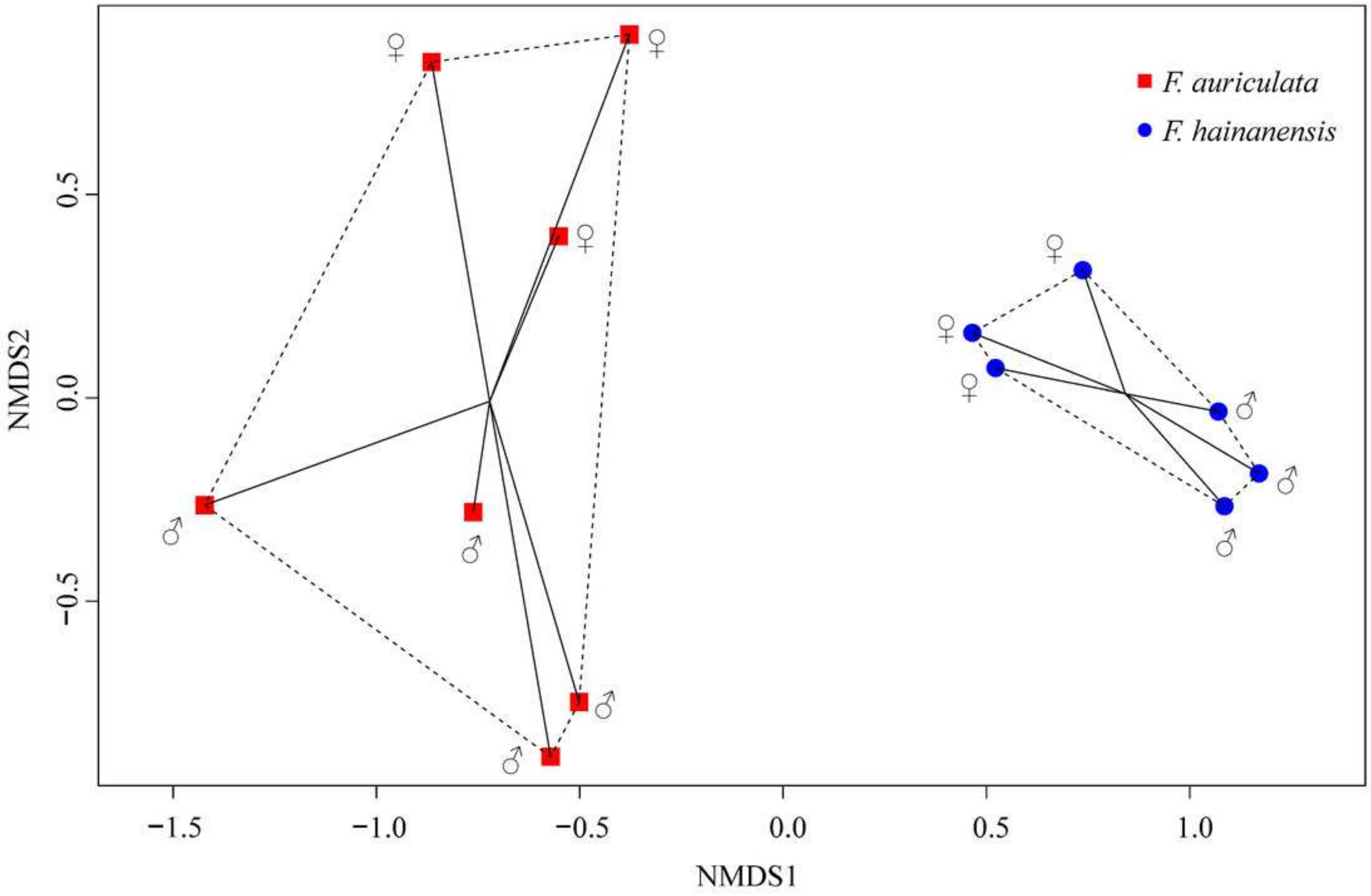

Figure 6

Electroantennographic responses of *Ceratosolen emarginatus* to receptive fig scent extracts of (A) *Ficus auriculata* and (B) *F. hainanensis*.

GC-FID (black line), and GC-EAD responses of *C. emarginatus* antennae (inverted blue line). VOC identification: 1: 2-Heptanone; 2: Ylangene; 3 and 1’: α-Copaene; 4: α-Funebrene; 5: α-Gurgujene; 6 and 3’: β-Funebrene; 7: trans-β-Farnesene; 8: α-Patchoulene; 9: β-Cadinene; 2’: α-Cedrene; 4’: β-Cedrene; 5’: α-Guaiaene; 6’: Aromadendrene).

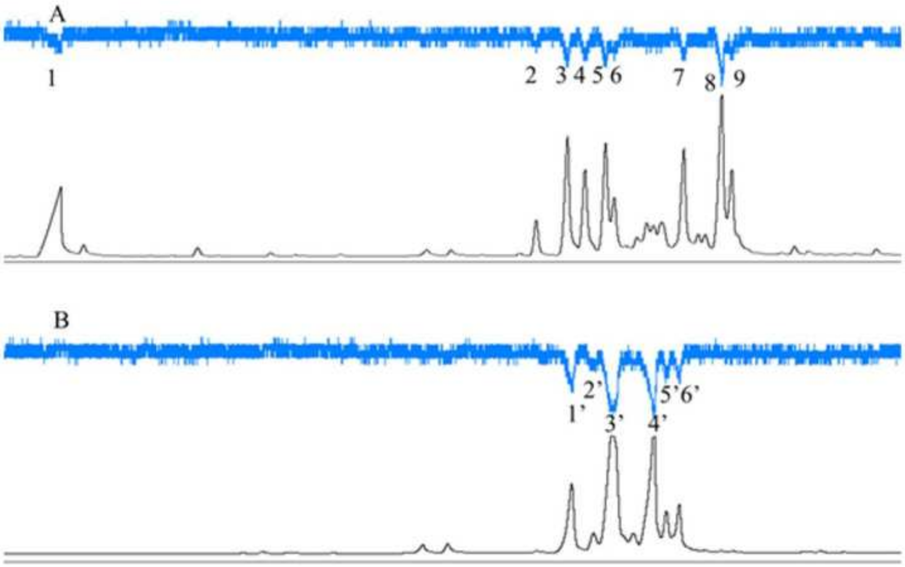
A
1
2
3
4
5
6
7
8
9
B
1'
2'
3'
4'
5'
6'

Figure 7

The distribution of fig style length and ovipositor length of the corresponding typical wasp pollinator. (A): Style length of receptive figs from *Ficus auriculata*; (B): Style length of receptive figs from *F. hainanensis*.

(A): Style length of receptive figs from *Ficus auriculata*; (B): Style length of receptive figs from *F. hainanensis*.

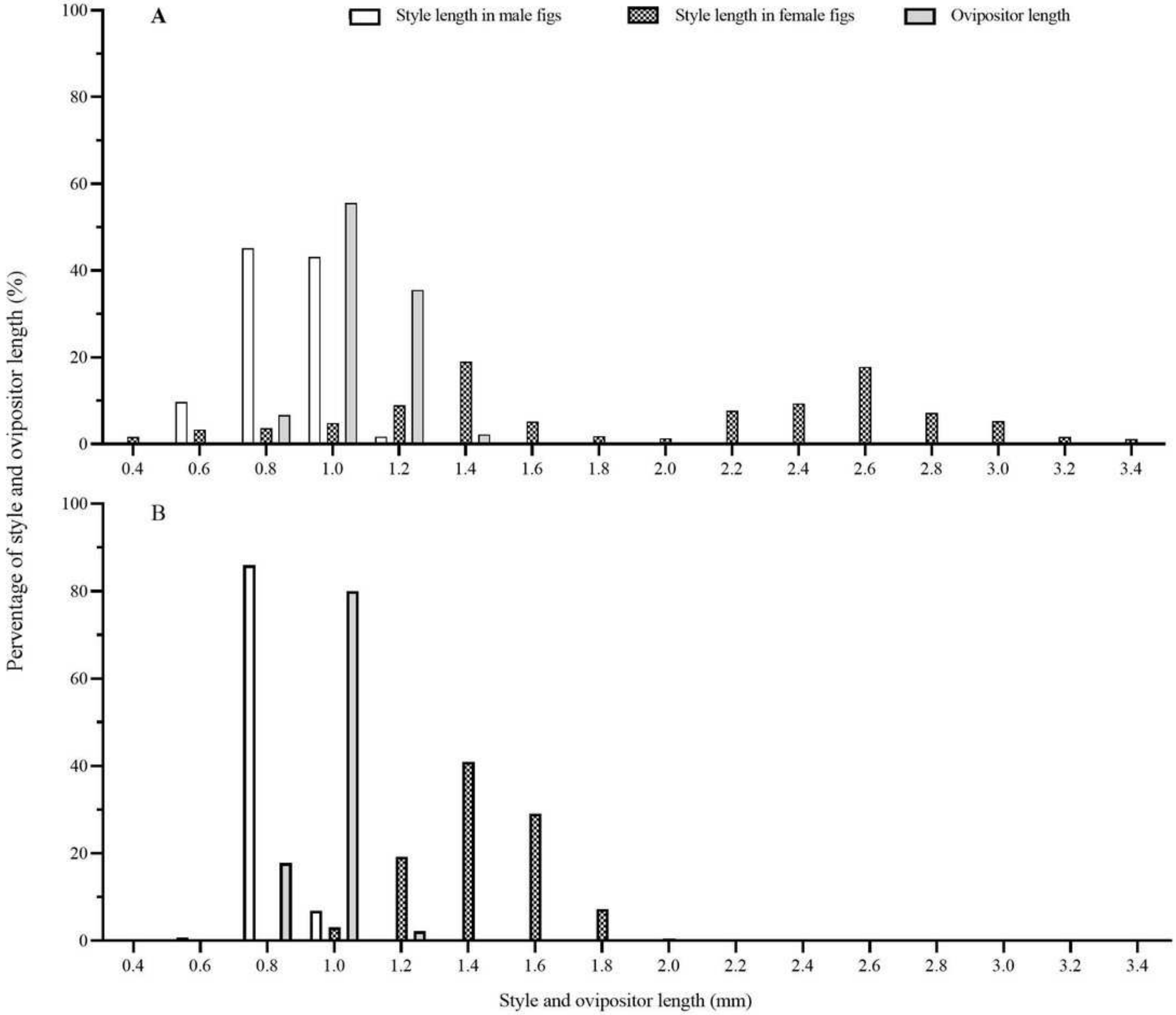

Table 1(on next page)

Numbers of offspring and seeds produced by single foundress of *Ceratosolen emarginatus* in *Ficus auriculata* and *F. hainanensis*

Ceratosolen emarginatus, Fa: *Ficus auriculata*, Fh: *F. hainanensis*

Table 1.

Ce:

Treatments	Sample size	No. of offspring (mean±SE)	No. of seeds (mean±SE)
Ce–Fa, male	60	472.85 ± 18.68	
Ce–Fh, male	60	618.49 ± 27.31	
Ce–Fa, female	60	-	974.21 ± 82.94
Ce–Fh, female	60 (all aborted)	-	

Table 2(on next page)

Body size and ovipositor length of *Ceratosolen emarginatus* emerging from *F. auriculata* figs, and of *Ceratosolen* sp. and *C.emarginatus* emerging from *F. hainanensis* figs.

Ce: *Ceratosolen emarginatus*, Fa: *Ficus auriculata*, Fh: *F. hainanensis*

Table 2.

Species	Sample size	Head width (mm) (mean±SD)	Thorax width (mm) (mean±SD)	Ovipositor length (mm) (mean ± SD)
Ce	77	0.48±0.05	0.59±0.05	1.15±0.11
Cs	69	0.46±0.04	0.55±0.04	1.07±0.11
Ce from Fh figs	114	0.46±0.04	0.55±0.07	1.03±0.06