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Ability to gall: the ultimate basis of host specificity in fig wasps?

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Abstract

1. Fig trees (Ficus spp.) and their host specific pollinator fig wasps (Agaonidae) are partners in an obligate mutualism. Receptive phase figs release specific volatiles to attract their pollinators and this is generally effective in preventing pollinator species from entering figs of the wrong hosts.

2. If entry is attempted into atypical host figs then ostiole size and shape and style length may also prevent reproduction. Despite these barriers, there is increasing evidence that fig wasps enter atypical hosts, and that this can result in hybrid seed and fig wasp offspring.

3. This study examines the basis of pollinator specificity in two dioecious fig species from different geographical areas. Kradibiatentacularispollinates Ficusmontana in Asia. F. asperifolia from East Africa is closely related, but is pollinated by a different species of Kradibia.

4. In glasshouses, K. tentacularis was attracted to its normal host, F1s and backcrosses, but only rarely entered figs of F. asperifolia. Foundresses were able to lay eggs in hybrids, backcrosses and F. asperifolia, although flower occupancy was lowest in F. asperifoliafigs and intermediate in hybrids.

5. The fig wasp failed to reproduce in female F. montana, male F. asperifolia and male F1s, and most but not all backcrosses to F. montana. This was a result of the failure to initiate gall production.

6. Host specificity in this fig wasp is strongly influenced by host volatiles, but ability to gall may be the ultimate determinant of whether it can reproduce.

Key words: Agaonidae, dioecy, Ficus, hybrids, Kradibia, volatiles

Running title: Pollinator specificity in dioecious figs
Introduction

Ficus is one of the most diverse genera of plants (Harrison, 2005) with approximately 800 described species (Berg and Corner, 2005). Fig trees and fig wasps have an obligate mutualistic relationship that had persisted for over 75 million years (Compton et al., 2010; Cruadet al., 2012). Fig wasps transport pollen from their natal figs to receptive figs (figs that are ready to be pollinated) while wasps only lay their eggs in fig flowers, where the larvae develop in and feed on galled ovaries (Cook and Segar, 2010). There are two types of pollination in fig trees, passive and active. Passive pollination is where the pollinators haphazardly pollinate receptive flowers by carrying the pollen on their bodies (Cook and Rasplus, 2003) and do not display any behaviour to collect the pollen (Jousselin et al., 2001). Fig wasps with this inefficient type of pollination lack coxal combs and their host figs have high anther to ovary ratios (Kjellberg et al., 2001). Active pollination, where female insects collect, store and then release the pollen, has been recorded in at least three pollinator-plant mutualisms: yuccas and yucca moths (Pellmyr and Huth, 2002), senita cacti and senitamoths (Fleming and Holland, 1998) and figs and fig wasps (Janzen, 1979). Fig wasps display morphological and behavioural adaptations for collecting and depositing pollen with pollen pockets and coxal combs that help in collecting and depositing the pollen (Jousselin et al., 2003). Active pollination may be a way to provide their larvae with an additional food source, and can reduce larval mortality rates (Jousselin and Kjellberg, 2001; Tarachai et al., 2008). The ratio of male to female flowers is low in figs with active pollination (Kjellberg et al., 2001).

It was believed that each fig tree species is pollinated by one specific fig wasp species (Ramirez, 1970; Bronstein, 1987) but there is increasing evidence that many Ficus species are pollinated by more than one fig wasp species (Ware and Compton, 1992; Molbo et al., 2003; Marussich and Machado, 2007; Compton et al., 2009; Lin et al., 2010). Floral scents are
often important signals from flowering plants to their pollinators and in obligate mutualisms chemicals released by one partner can be crucial for attraction of the other (Grison-Pige et al., 2002; Raguso, 2008; Soler et al., 2011). Specificity between fig trees and their pollinators is maintained by a combination of chemical barriers (stage and host plant specific attractant volatiles) and physical barriers determined by ostiole diameter and style length (Ware and Compton, 1994; van Noort and Compton, 1986). Figs of each species emit a species-specific blend of volatile chemical compounds during their receptive phase that differs from that released by younger and older figs (van Noort et al., 1989; Grison-Pige et al., 2001). Changes in the floral scent after figs are pollinated result in pollinators avoiding these figs (Proffit et al., 2008) and the figs can become repellent by the time that the next generation of pollinators emerges (Guet al., 2012). Once the female wasps have landed on the surface of the receptive fig there are further physical or chemical cues that can influence whether they attempt entry (Wang et al., 2013). The females then have to gain entry through the ostiole, which acts as a physical barrier. The ostiolar bracts become looser at the receptive phase to make penetration easier (Verkerke, 1986), but a proportion of pollinator females fail to pass successfully through the ostiole (Liu et al., 2013) despite their morphological adaptations to aid entry (Ramirez, 1974; Verkerke, 1989; van Noort et al., 1989).

In monoecious fig trees, seeds and fig wasp offspring are produced in the same figs. Inside the figs there are both male and female flowers. Male flowers produce pollen and female flowers can develop seeds or support wasp offspring. Female flowers vary in their style lengths and pedicels, with flowers with shorter styles having longer pedicels and vice versa (Verkerke, 1989). This allows all the stigmas to be at the same level at the time that pollinators enter, providing a synstigma over which the wasp females can walk and probe the styles. It was initially believed that female wasps only lay their eggs inside female flowers that have short styles and those with long style produce seeds, but most fig wasps have
ovipositors that can reach most or all of the female flowers in their host figs, and mean style
and ovipositor lengths among species pairs are strongly correlated (Nefdt and Compton,
1996). In dioecious fig trees, wasps and seeds are produced on different plants. Figs borne on
male trees have male and female flowers, but are functionally male. Male flowers produce
pollen and the female flowers all have short styles that are easily accessible to female fig
wasps for oviposition and do not produce seeds. Female trees have flowers with longer styles
and stigmas adapted for pollen collection. They only develop seeds rather than fig wasp
offspring (Corlett et al., 1990), but it is unknown whether they oviposit into those flowers (if
any) that are accessible.

There are over 13,000 species of insects recorded as being able to induce plants to
make galls (Dreger-Jauffret and Shorthouse, 1992; Roskam, 1992). Galls can develop on
leaves, stems, buds, petioles, fruits and roots (Weis et al., 1988; Dreger-Jauffret and
Shorthouse, 1992; Raman et al., 2007). They provide a food resource and shelter to the
inducing insects or their offspring (Sanver and Hawkins, 2000; Raman, 2007; Hardy and
Cook, 2010). Gall inducers are more host specific than most other guilds of herbivorous
insects (Hardy and Cook, 2010). The galls can be induced during feeding or
oviposition (Miles, 1968; Raman, 2007; Matsukura et al., 2009). Galls that are initiated by
oviposition are believed to be responding to compounds secreted with the eggs or coming the
eggs themselves, but larvae can also continue to stimulate gall development (Miles, 1968;
Stone et al., 2002). The nature of these compounds and the mechanisms involved however, are
still unclear (Stone and Schonrogge, 2003; Tooker and De Moraes, 2008). Most gall inducers
are specific to particular plant organs and specific host plants (Shorthouse et al., 2005) and
attraction to the wrong host can lead to failures in gall formation and progeny
development (Weis et al., 1988; Wool, 2005).
Female fig wasps are able to induce galls in the ovules where they lay their eggs (Jansen-González et al., 2014). After they enter receptive figs they insert their ovipositors into the ovaries through the styles and lay a single egg in each ovary together with a secretion that stimulates the ovaries to enlarge. There are several reported cases when fig wasps pollinators enter atypical hosts (Janzen, 1979; Ware and Compton, 1992). This can lead to viable hybrid seeds (Ramirez, 1970; Ramirez and Montero, 1988; Ware and Compton, 1992) and hybrid plants (Parrish et al., 2003; Moe and Weiblen, 2012). Viable hybrid fig trees represent potential routes for introgression between fig trees species, and a mechanism that facilitates speciation (Kasumi et al., 2012).

We investigated whether fig wasps were willing and able to lay eggs in experimentally-generated male hybrid figs. The aims of this study were (I) to determine if females of Kradibia (= Liporrhopalum) tentacularis, the pollinator of the dioecious fig tree F. montana, are attracted to and can enter figs of another closely-related species and their hybrids, (II) to determine if K. tentacularis that enter these figs can lay eggs in these atypical hosts and if so whether their offspring develop successfully, and (III) whether they also lay eggs into accessible flowers in female figs of F. montana, but the eggs fail to develop.

**Materials and Methods**

**Study site and species**

F. montana Blume is a dioecious fig tree native to SE Asia (Berg and Corner, 2005). It is a branched understory shrub with figs that develop in the leaf axils or clustered on spurs from the older wood (Suleman, 2007; Raja et al., 2008a). Ficus asperifolia Miq is distributed across tropical Africa. It is closely related to F. montana (both belong to subgenus Sycidium) and the two species are superficially similar, with figs produced in the same locations, but F.
asperifolia branches less frequently, tends to grow taller and produces figs that grow slightly larger (Berg and Wiebes, 1992).

Glasshouse populations of F. montana and its active pollinator, Kradibia (= Liporrhopalum) tentacularis (Grandi) were maintained at the experimental gardens of Leeds University from 1996 (Raja et al., 2008b; Suleman et al., 2012, Suleman et al., 2013c). They originated from Bogor (Java, Indonesia), and the Krakatau Islands (Indonesia). Only the green-leaved form of F. montana (Tarachai et al., 2012) was used. The F. asperifolia originated from seed collected Kibale Forest, Uganda, in August 2004. Kradibiahilli Wiebes is the pollinator of F. asperifolia in East Africa (Berg and Wiebes, 1992), but it was not available and all experiments and crosses involved K. tentacularis. Most F. asperifolia figs in the general (mixed-species) glasshouse population remained unpollinated, but small numbers were entered by K. tentacularis females. Both species develop rapidly from seed and experimentally-generated offspring started to produce figs from as little as nine months after germination.

The glasshouses were provided with heating to maintain temperatures and with artificial lights to maintain a minimum day length to 14 hours during the winter period. Under glasshouse conditions, both sexes of F. montana had an asynchronous all-year fruiting pattern on individual plants and among the population as a whole, but with fewer figs produced in winter (Suleman et al., 2011a). F. asperifolia displayed similar fruiting patterns, but with a more extreme seasonal response, and few figs were present on the trees during the winter months (Suleman et al., 2011a).

Foundress females of K. tentacularis routinely re-emerge from the first F. montana figs they enter, after losing their wings and part of their antennae, and are capable of utilising up to four additional figs nearby (Suleman, 2007; Suleman et al., 2013b). Because they lose their wings, re-emerged foundresses cannot fly to other trees (Suleman et al., 2011b).
Crosses

The developmental stages of the figs were classified according to the terminology of Galil and Eiskowitch (1968) as modified for dioecious figs by Valdeyron and Lloyd (1979).

Hybrids of F. montana (F1s) were generated in 2005 from F. montana males and F. asperifolia females by introducing adult female K. tentacularis from D phase male figs into B (receptive) phase female figs of F. asperifolia (Ghana, 2012). Reciprocal crosses were not possible because only K. tentacularis was available. Backcrosses to F. montana were generated from male F. montana and female F1s by introducing F. montana pollinators into female F1s in 2006.

Attraction to receptive figs and pollinator entry

Six individuals (mixed sexes) of F. montana, F. asperifolia, F1s and backcrosses were chosen haphazardly from the glasshouse populations and seven pre-receptive figs on each tree were selected. The plants were organised in a row in an alternating sequence with approximately 50 cm spaces between each other. The figs on each plant were not close to each other and their branches were surrounded by Vaseline petroleum jelly® to prevent any wingless K. tentacularis from re-emerging from figs elsewhere and entering the focal figs (Raja et al., 2008b). These were checked regularly and entry by winged pollinators was detected by the presence of detached wings at their ostioles. The figs were removed after seven days and opened under a binocular microscope to score how many K. tentacularis had entered, based on the numbers of wings and antennae left in the ostiolar area.

Ovipositor length constraints
Thirty one immature A phase figs from three male plants of F. montana were chosen haphazardly and individual netting bags (pore diameter 0.19 mm) were placed around them to exclude pollinators. Receptivity was tested each day by introducing wasps to the ostiolar area. Any attempt to enter the figs was taken as indicative that the figs were receptive. These figs were then collected and dissected under a binocular microscope. All the flowers in the figs were picked and positioned horizontally on slides under a compound microscope to measure the lengths of the styles using an ocular scale. The measurements followed those described by Nefdt and Compton (1996), where style length was taken as the distance between the top of the stigma and the end of style where it connected with the ovary. The procedure was repeated for male F1s, backcrosses and F. asperifolia (three plants with five figs from each group), but with a modification for F. asperifolia figs, because K. tentacularis females were generally unwilling to enter them. To stimulate them to attempt entry, receptive figs of F. montana were rubbed across the ostioles of the F. asperifolia figs and if the females could then enter the figs through the ostiole, they were considered to be receptive. Figs of F. asperifolia, F1s and backcross are larger than those of F. montana and were dissected longitudinally through the ostiole into four pieces and flowers from one quarter were measured, rather than all the flowers.

Ovipositor lengths were measured by collecting K. tentacularis females from 25 late phase D figs from five male trees of F. montana (five figs from each plant). The figs were kept in plastic containers until the wasps emerged, usually on the next day. About ten wasps from each fig were collected, slide mounted and measured under a compound microscope.

**Ability to lay eggs in atypical hosts and in female figs**

Five male F. montana, F1s and backcrosses and three F. asperifolia plants were chosen haphazardly from those with figs in the general population. Five A phase figs from each plant
were bagged to prevent fig wasps from entering. Adult female *K. tentacularis* were obtained and introduced into receptive figs of *F. montana*, *F1s* and backcrosses by putting one or two on the ostiole area of each fig. If a wasp entered, the figs were re-bagged to prevent any more wasps from entering. The wasps were introduced similarly into male figs of *F. A sperifolia* using the technique described previously. The figs were left for 24 hours, then collected and stained to reveal whether eggs had been laid.

The staining protocol was modified from Khan and Saxena (1986). Figs were dissected into several pieces and immersed into hot (recently boiling) water for one minute. This step was repeated three times with gaps of less than one minute. The figs were then immersed in 1% aqueous acid fuchsin for 4-6 hours and finally washed in water. Individual flowers were removed using a dissecting microscope and placed on glass slides under a cover slip. The ovary contents could then be examined using a compound microscope. Preliminary experiments had shown that this technique was effective at finding eggs until about 48 hours after oviposition, after which growth of the ovary wall makes subsequent observations more difficult. From each fig, ten styles from flowers containing eggs and a similar number from flowers without eggs were measured to compare style lengths and to determine whether the absence of eggs was related to style length.

To study the ability of *K. tentacularis* to lay its eggs in female flowers from female figs of *F. montana*, five plants and five figs from each plant were selected and the same methods as with male figs were applied.

**Ability to gall**

Relative ovary diameters in phases B and C male figs (representing pre- and post-oviposition periods) were used as indicators of the ability of the wasps to initiate galls. Three *F. montana*, *F1s*, backcrosses and *F. asperifolia* plants were chosen haphazardly. Ten figs on each plant
were covered with mesh bags as before. Half had their ovaries measured once they became receptive and the remainder had single pollinator females introduced and were then re-bagged. They were then collected seven days later. All the figs were dissected longitudinally into four pieces under a dissected microscope and the diameter of ovaries from all the flowers in one quarter were recorded (Fig. 1).

**Data analysis**

A generalized linear mixed-effect regression model (Lmer) was used with a Poisson error for count data to determine whether there were significant differences in the numbers of pollinators entering figs of F. asperifolia and F. montana, F1s and backcrosses to F. montana. The fixed effect variables in the model were the different fig groups (F. montana, F. asperifolia, F1s and backcrosses) with fig number and plant number as random effects.

GLMs, Wilcoxon rank-sum tests, t tests and nested ANOVA were all performed in R (2.12.2).

**Results**

**Attraction to receptive figs and pollinator entry**

Fig wasp foundresses are typically attracted to receptive figs of their host plants by volatiles released during B phase, but not by receptive figs belonging to other species. In the greenhouse environment, where plants with receptive figs were positioned close to each other (Table 1), up to six K. tentacularis females entered male and female figs on F1 plants, in similar numbers as in F. montana figs. Similarly, up to nine females entered backcross figs. These results were in contrast to F. asperifolia figs, which at most were entered by a single pollinator, and often remained un-entered (Table 1). Although the number of foundresses entering the receptive figs ranged from 0 to 9, most figs were entered by one foundress in all
groups (Table 1). Lmer detected no significant difference in foundress numbers between F1s and backcrosses with F. montana, but fig wasp densities were significantly lower in F. asperifolia figs (Table 2). There was a significant difference after removing the plant sex interaction term from the model and comparing the new model to the previous one, showing that sex influenced the number of fig wasps that entered the figs (Lmer: $X^2 = 9.53$, df = 3, $P < 0.05$).

All the receptive figs of F. montana were entered by fig wasps, compared with less than 10% of the F. asperifolia figs ($X^2 = 65.79$, df = 1, $P < 0.001$). Around 85% of the F1 figs were entered (Table 3), which was a significantly lower than for the F. montana figs ($X^2 = 4.49$, df = 1, $P < 0.05$). There was no significant difference between backcrosses and F. montana ($X^2 = 1.38$, df = 1, $P = 0.24$).

No female K. tentacularis were found trapped in the ostioles while facing inwards, suggesting that all those pollinators that attempted to enter the figs did so successfully. Often there were more pairs of wings at the entrance to the ostioles than wasps dead inside, because K. tentacularis routinely re-emerge from the figs.

**Ovipositor length constraints**

After female fig wasps have managed to enter a receptive male fig they start laying eggs in the female flowers by inserting their ovipositor along the length of their styles. Ovipositor lengths of K. tentacularis ranged from 0.65 to 0.92 mm (Fig. 2). Comparisons of the ovipositor lengths with the style lengths of female flowers in male figs of F. montana (its natural host), F. asperifolia, F1s and backcrosses showed that the ovipositor of K. tentacularis can potentially reach all the flowers in F1s, backcrosses and F. asperifolia, as well as those of F. montana (Fig. 2).
Ability to lay eggs in atypical hosts and in female figs

F. asperifolia, F1s and backcrosses had a higher number of female flowers in their male figs than F. montana, with means ± SE of 340.47 ± 27.98, 209.88 ± 14.54 and 167.28 ± 6.61 respectively, compared with 86.64 ± 4.63 in F. montana (Fig. 3). There was a significant difference in female flower numbers between the groups (nested ANOVA, F (3,14) = 10.3, P < 0.001). Pairwise t tests that compared female flower number between groups showed that differences between all groups were significant (P < 0.05 between F1s and backcrosses and P < 0.001 for other combinations).

Around 28200 fig flowers were examined for the presence of eggs. No K. tentacularis adult offspring had ever emerged from male F. asperifolia and F1 figs in the greenhouse populations, but the foundresses were found to have laid eggs in F1s, backcrosses and F. asperifolia, as well as F. montana. Although figs of F. asperifolia, F1s and backcrosses contained more female flowers than F. montana figs, the numbers of eggs laid by a single foundress were about the same, with means of 53.47 ± 10.21, 72 ± 8.66 and 95.60 ± 6.87 respectively, compared with 83.16 ± 4.25 eggs in F. montana figs. There was no significant difference in the numbers of eggs laid (nested ANOVA, F (3, 14) = 1.99, P = 0.16).

The egg occupancy rates in F1 male figs entered by a single female ranged between 17.32% and 50.59% of the female flowers (Table 4), compared with 36.71% to 86.91% in backcrosses, 7.65% to 20.41% in F. asperifolia and 94.8 to 98.2% in F. montana. When two female fig wasps were introduced into the receptive figs, the lowest occupancy rate was in F1s (41.41 to 66.61%) followed by backcrosses (66.71 to 88.24%) and F. montana (77.2 to 96.9%). No two-foundress data are available for F. asperifolia.

There were no significant differences in the style lengths of flowers that contained eggs or were free of eggs in all groups: F. montana (Wilcoxon RS, W = 8427.5, P = 0.44), F.
asparifolia (Wilcoxon RS, W = 8504.5, P = 0.57), F1s (Wilcoxon RS, W = 29437.5, P = 0.64) and backcrosses (Wilcoxon RS, W = 30153.5, P = 0.73, Fig. 4a). Eggs numbers per ovary ranged from 0 to 2, but most ovaries contained only one egg. In F. asparifolia (when one wasp was introduced), most flowers were empty (84%) and no flowers were recorded with double oviposition (Table 4). In F1s, about half the flowers contained one egg or were without eggs when either one or two females were introduced, and only three ovaries were recorded with two eggs (Table 4). In backcrosses the proportion of flowers containing eggs was higher than in F1 figs, with around 60% utilized when one wasp was introduced and 80% when two wasps were introduced, but only four flowers were recorded with two eggs (Table 4). In F. montana, the normal host for K tentacularis, double oviposition was again rare with only 24 examples among the 4432 female flowers examined (Table 4). The positions of the eggs inside the ovaries were similar in all groups (Fig. 1) with no significant differences in location between the groups. The positions ranged from 0.12 to 0.32 mm in F. montana, 0.17 to 0.25 mm in F. asparifolia, 0.15 to 0.27 mm in F1s and 0.15 to 0.25 mm in backcrosses. There was no significant interaction effect between style length, plant group and egg position (Mixed effect models all P > 0.05) (Fig. 4b).

There were no typical eggs found in the 2723 female flowers from female figs of F. montana, that were examined, but a single egg of atypical shape and position was found in one ovary.

**Ability to gall**

Ovaries in B phase figs of F. montana were 0.48 mm ± 0.001 in diameter (n = 440), compared with 0.76 mm ± 0.007 in C phase figs (n = 393), after the ovaries had responded to oviposition and galling. In F1 figs, the mean diameter of the ovaries shrank over time (0.50 ± 0.001 mm in B phase (n = 720) compared with 0.48 ± 0.002 mm in C phase (n = 678).
Supporting Information Figure 1), but a small number of ovaries had started to expand after pollinator entry and had reached between 0.6 and 0.7 mm in diameter (Fig. 56). These enlarged ovaries were only detected in two figs, both collected from the same plant. In backcrosses, the overall measurements of the ovaries in B phase were 0.46 mm ± 0.001 (n = 643) and 0.51 mm ± 0.005 in C phase (n = 714). Many ovaries in these figs had started to develop after pollinator entry and were larger than 0.45 mm, the maximum ovary diameter recorded in B phase backcross figs. In contrast, there was no evidence of ovary expansion after oviposition in F. asperifolia figs, where the ovary diameters were 0.50 mm ± 0.001 (n = 712) in B phase figs and 0.49 mm ± 0.003 at C phase (n = 746).

In F. montana there was a significant increase in the ovary diameters of female flowers between B and C phases (Wilcoxon RS, W = 14976, P < 0.001), whereas there was a significant decline in ovary diameters from B to C phases in F1 figs (W = 355075, P < 0.001). In backcrosses and F. asperifolia there was no significant difference between ovary diameters of female flowers in B and C phase figs (W = 240326.5, P = 0.13 and W = 274183, P = 0.27, respectively).

**Discussion**

The floral scents emitted by receptive figs vary sufficiently between stages and between species for pollinators to be attracted to their specific host species at the particular time when the figs are ready to be pollinated (Raguso, 2008; Soler et al., 2010, but see Zhang et al., 2014 for an apparent exception). K. tentacularis routinely pollinates F. montana and flying females of this species are attracted to its receptive figs, but probably not to all to receptive figs of the closely related F. asperifolia. Under greenhouse conditions small numbers of K. tentacularis females nonetheless do land on F. asperifolia figs and attempt to enter them. These occasional encounters were probably the result of the high densities and intermixing of
the two Ficus species in the closed environment of the glasshouses. In nature, even if the
species were sympatric and showed similar habitat preferences, the differences in volatiles
released by the figs would be sufficient to make chance encounters between K.
tentacularis and F. asperifolia figs extremely rare. A proportion of these rare encounters may
nonetheless result in K. tentacularis entering the figs, as has been documented with other fig
wasp species (Ware and Compton 1992; van Noort et al., 2013), and small numbers of K.
tentacularis also entered figs of another related fig tree (F. sandanakana Berg) in the
glasshouses (S. Ghana, personal observations). The specific chemical compounds released by
the receptive figs of the two species (Proffit et al., 2009; Soler et al., 2011) are therefore
highly effective at preventing pollen flow from F. montana to F. asperifolia, but would not
represent an absolute barrier in situations where the two plant species were sympatric. Given
the evident difference in volatile profiles of the two species, it is likely that pollen flow in the
other direction, mediated by the pollinator of F. asperifolia, would be similarly restricted.
The willingness of pollinator females to attempt entry into figs can be influenced by
cues on the surface of receptive figs that are independent of the volatiles responsible for long-
distance attraction (Wang et al., 2013). K. tentacularis females placed experimentally on the
surface of receptive F. asperifolia figs were generally disinterested in entering them, but some
entries did nonetheless take place when receptive figs were available for long periods in the
glasshouses. Rubbing receptive F. montana figs on the surface of the F. asperifolia figs had
a strong effect on pollinator entry, by stimulating the females to seek out the ostiole. This
involved an increase in speed of walking and antennal drumming on the fig surface while
they were apparently seeking out the narrow entrance provided by the ostiole. The stimuli
provided by the F. montana figs may have been the same volatiles that are responsible for
long distance attraction, less volatile compounds emanating from the fig surface, or a
combination of the two.
The ostiole of figs is often regarded as a physical filter that can trap female pollinators that are associated with different species of fig trees (van Noort and Compton, 1996), but K. tentacularis females that attempted entry into F. asperifolia figs apparently had no problems passing through the ostiole, because no dead females were found in the ostiole facing inwards. The females routinely re-emerge from figs of F. montana and the same behaviour was observed among females that had entered F. asperifolia figs.

Female K. tentacularis that entered male figs of F. asperifolia were able to lay as many eggs as those that entered F. montana figs. The former contained more flowers, all of which were accessible to the females based on their style lengths, but these additional resources probably could not be utilised because of the limited egg loads of the single pollinators that were introduced. Any differences between the species in terms of ease of oviposition were apparently unimportant. The ease with which K. tentacularis laid eggs in figs of F. asperifolia contrasts strongly with its ability to induce growth in the ovaries where the eggs were laid. Whereas galled ovules in F. montana figs rapidly expanded, there was no apparent response from the ovules of F. asperifolia, and no successful development of fig wasp larvae. In contrast to this inability to generate galls, the lack of K. tentacularis offspring in female figs of its routine host species, F. montana results from an almost total inability to deposit eggs in the ovules of female figs, reflecting their flowers’ longer styles and different stigma structure (Shi et al., 2006).

Artificial hybrid crosses have been generated between some fig tree species and an increasing number of examples of putative hybrid fig trees are being recorded from natural environments (Condit, 1950; Parrish et al., 2003; Kusumiet al., 2012). F1 hybrids between F. montana and F. asperifolia, and backcrosses to F. montana, provided indications of how some of the plant characters associated with pollination can be inherited in hybrid offspring and how the pollinator of one of the parents responds to the novel characters generated by
hybridisation. F1s between plant species often have volatile profiles intermediate between those of their parents (Schnitzler et al., 2004; Salzmann et al., 2007; Shuttleworth and Johnson, 2010). Chemical analysis of the volatiles released from receptive F1s and backcrosses suggests that the overall volatile profiles were more similar to those of F. asperifolia than F. montana (C. Soler, personal communication), but clearly the hybrids inherited the key compounds that made the figs attractive to K. tentacularis. The F1 hybrids were also closer to the female parents in terms of mean style lengths, but the number of flowers in the figs was more intermediate. As expected, the floral characters of backcrosses tended to be closer to F. montana.

Hybrid figs were just as attractive as F. montana to K. tentacularis females and just as many eggs were laid in the hybrid figs, but no fig wasp offspring have been seen to develop in any of the F1 figs maintained in the glasshouses, despite small numbers of ovaries on one of the experimental plants showing signs of expansion in response to galling. Premature abortion of any F1 figs that contained the very small numbers of galls appears to have been responsible. In contrast to F1 plants, ovules in figs of backcrosses to F. montana showed gall development, though more rarely and not to the same extent as ovules inside figs of F. montana, and some of the backcross plants were able to support the successful development of pollinator offspring.

Species specific volatiles released from receptive figs and the responses of pollinator fig wasps to them are clearly the major source of host specificity in the fig tree: fig wasp mutualism, because they greatly reduce the chances that fig wasp females will land on figs belonging to species other than their routine hosts. Chance encounters result in fig wasps having the opportunity to attempt fig entry. Whether or not they do so may depend on short-range stimuli from the surface of the figs, but the list of examples where pollinators are known to enter non-host figs is increasing (Harrison, 2006; Janzen, 1979; Ware and Compton,
1992). After negotiating the ostiole, so long as the style lengths of the flowers in the male figs are not too long (Nefdt and Compton, 1996), then our results suggest that egg deposition is likely to occur, but for reproduction to be successful the female must also be able to successfully induce gall formation in the ovules where she lays her eggs. Gall-forming insects are often highly host specific, with unpredictable abilities to gall hybrids with their usual hosts (Skuhravy et al., 1997). If the inability the pollinator of F. montana to induce galls in even a closely related species is typical of most fig wasps, then the ability to form galls could be considered as the ultimate factor limiting the host range of these pollinators. However, results from another fig tree suggest that it is not always the case. F. microcarpa L. f. is an Asian fig tree introduced to South Africa, where it has no very close relatives. Despite this, small numbers of two native African pollinator fig wasps, belonging to a different genus from its usual pollinator, have been reared from its figs, showing that they can successfully form galls (van Noort et al., 2013).

Acknowledgements

Dr. Finn Kjellberg (CNRS, Montpellier, France) suggested the technique for stimulating pollinator entry in atypical host figs. This research was funded by the Libyan Higher Education Ministry. The admirable service of maintaining fig and fig wasp populations at the Leeds University Experimental Gardens was provided by Martin Lappage. We also acknowledge anonymous referees, Associate Editor and the Editor in Chief of the journal for their valuable suggestions in improving the manuscript.

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associated with Populus tremula, P. tremuloides and their hybrid (Salicaceae).

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Fig. 1A female flower from a male F1 hybrid fig, showing the presence of an egg laid by K. tentacularis. Ovary diameter was measured from a to b. Egg position distance was measured from a to c. The scale bar represents 0.1 mm.

Fig. 2 The relationship between the ovipositor lengths of F. montana’s pollinator (solid bars) and style lengths in male figs of a) F. montana b) F. asperifolia c) F1s and d) backcrosses to F. montana.

Fig. 3 Female flower numbers in male figs of F. asperifolia, F. montana and their hybrids, and the numbers of eggs laid by K. tentacularis foundresses. Open bars = flower numbers, solid bars = egg numbers.

Fig. 4 (a) Style lengths from flowers with and without eggs. Open bars = flowers that contained eggs, solid bars = flowers that contained no eggs (b) Style lengths and egg positions in F. montana, F. asperifolia and their hybrids. Solid bars = style lengths, open bars = egg positions.

Fig. 5 Ovary diameters in B phase and C phase (post pollinator entry) male figs of F. montana, F. asperifolia, F1s and backcrosses. Open bars = B phase figs, solid bars = C phase figs.

Fig. 56 Ovary diameters in female flowers from male figs of F. montana, F. asperifolia, F1s and backcrosses. a) B phase b) C phase (after pollinator entry).
Table 1. The numbers of K. tentacularis females entering male and female figs in the general glasshouse population.

<table>
<thead>
<tr>
<th></th>
<th>Figs (n)</th>
<th>Wasp (n)</th>
<th>Mean</th>
<th>SE</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>F. montana</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total figs</td>
<td>42</td>
<td>69</td>
<td>1.64</td>
<td>0.18</td>
<td>1-6</td>
</tr>
<tr>
<td>Male figs</td>
<td>21</td>
<td>39</td>
<td>1.86</td>
<td>0.32</td>
<td>1-6</td>
</tr>
<tr>
<td>Female figs</td>
<td>21</td>
<td>30</td>
<td>1.43</td>
<td>0.16</td>
<td>1-3</td>
</tr>
<tr>
<td><strong>F. asperifolia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total figs</td>
<td>42</td>
<td>4</td>
<td>0.09</td>
<td>0.05</td>
<td>0-1</td>
</tr>
<tr>
<td>Male figs</td>
<td>21</td>
<td>0</td>
<td>0.00</td>
<td>0.00</td>
<td>0</td>
</tr>
<tr>
<td>Female figs</td>
<td>21</td>
<td>4</td>
<td>0.19</td>
<td>0.09</td>
<td>0-1</td>
</tr>
<tr>
<td><strong>F1s</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total figs</td>
<td>42</td>
<td>57</td>
<td>1.36</td>
<td>0.16</td>
<td>0-6</td>
</tr>
<tr>
<td>Male figs</td>
<td>21</td>
<td>19</td>
<td>0.91</td>
<td>0.17</td>
<td>0-3</td>
</tr>
<tr>
<td>Female figs</td>
<td>21</td>
<td>38</td>
<td>1.81</td>
<td>0.25</td>
<td>1-6</td>
</tr>
<tr>
<td><strong>Backcrosses</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total figs</td>
<td>42</td>
<td>84</td>
<td>2.00</td>
<td>0.27</td>
<td>0-9</td>
</tr>
<tr>
<td>Male figs</td>
<td>21</td>
<td>48</td>
<td>2.29</td>
<td>0.35</td>
<td>0-6</td>
</tr>
<tr>
<td>Female figs</td>
<td>21</td>
<td>36</td>
<td>1.71</td>
<td>0.41</td>
<td>0-9</td>
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</table>
Table 2. The minimal adequate generalized linear mixed-effects model with Poisson errors for the number of wasps attracted to receptive figs and its interaction with plant sex when compared with F. montana (intercept).

<table>
<thead>
<tr>
<th></th>
<th>β Value</th>
<th>SE</th>
<th>t-value</th>
<th>P</th>
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<tr>
<td>Intercept</td>
<td>0.32996</td>
<td>0.23566</td>
<td>1.4</td>
<td>0.161</td>
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<tr>
<td>F. asperifolia</td>
<td>-2.03332</td>
<td>0.57855</td>
<td>-3.514</td>
<td>0.001 ***</td>
</tr>
<tr>
<td>F1s</td>
<td>0.2319</td>
<td>0.32251</td>
<td>0.719</td>
<td>0.472</td>
</tr>
<tr>
<td>Backcrosses</td>
<td>0.18752</td>
<td>0.32447</td>
<td>0.578</td>
<td>0.563</td>
</tr>
<tr>
<td>Sex male</td>
<td>0.24087</td>
<td>0.32188</td>
<td>0.748</td>
<td>0.454</td>
</tr>
<tr>
<td>F. asperifolia:Sex male</td>
<td>-16.9067</td>
<td>2126.735</td>
<td>-0.008</td>
<td>0.993</td>
</tr>
<tr>
<td>F1s:Sex male</td>
<td>-0.93693</td>
<td>0.47681</td>
<td>-1.965</td>
<td>0.049 *</td>
</tr>
<tr>
<td>Backcrosses:Sex male</td>
<td>0.03083</td>
<td>0.443</td>
<td>0.07</td>
<td>0.944</td>
</tr>
</tbody>
</table>
Table 3. The numbers of figs entered by pollinator females and their densities in figs where they were present.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total figs</th>
<th>Total figs entered</th>
<th>Mean</th>
<th>SE</th>
<th>Range</th>
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<tbody>
<tr>
<td>F. montana</td>
<td>42</td>
<td>42</td>
<td>1.64</td>
<td>0.18</td>
<td>1-6</td>
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<td>F. asperifolia</td>
<td>42</td>
<td>4</td>
<td>1.00</td>
<td>0.05</td>
<td>0-1</td>
</tr>
<tr>
<td>F1s</td>
<td>42</td>
<td>36</td>
<td>1.58</td>
<td>0.16</td>
<td>0-6</td>
</tr>
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<td>Backcrosses</td>
<td>42</td>
<td>39</td>
<td>2.15</td>
<td>0.27</td>
<td>0-9</td>
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</table>
Table 4. The distribution of K. tentacularis eggs within ovaries of Ficus montana, F. asperifolia and their hybrids. Five figs with one or two foundresses were examined from each tree. The contents of all the female flowers within these figs were recorded.

<table>
<thead>
<tr>
<th>Species</th>
<th>Tree</th>
<th>N ovaries</th>
<th>Occupancy (%)</th>
<th>Eggs per flower</th>
<th>N ovaries</th>
<th>Occupancy (%)</th>
<th>Eggs per flower</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>one foundress</td>
<td></td>
<td>two foundresses</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>mean</td>
<td>variance</td>
<td>range</td>
<td></td>
<td>mean</td>
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<tr>
<td>F. montana</td>
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<td>281</td>
<td>95.73</td>
<td>0.96</td>
<td>0.04</td>
<td>0-1</td>
<td>306</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>521</td>
<td>96.35</td>
<td>0.97</td>
<td>0.04</td>
<td>0-2</td>
<td>477</td>
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<tr>
<td></td>
<td>3</td>
<td>498</td>
<td>95.18</td>
<td>0.96</td>
<td>0.05</td>
<td>0-2</td>
<td>467</td>
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<tr>
<td></td>
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<td>386</td>
<td>98.19</td>
<td>0.98</td>
<td>0.02</td>
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<td></td>
<td>5</td>
<td>480</td>
<td>94.79</td>
<td>0.95</td>
<td>0.05</td>
<td>0-2</td>
<td>653</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>2166</td>
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<td></td>
<td></td>
<td></td>
<td>2266</td>
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<tr>
<td>F. asperifolia</td>
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<td>2347</td>
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<td>0.19</td>
<td>0.15</td>
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<td>1112</td>
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<td>-</td>
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<tr>
<td>Total</td>
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<td>5107</td>
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<td>-</td>
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<td>F1s</td>
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<td>Total</td>
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<td></td>
<td></td>
<td>5131</td>
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<td>0-1</td>
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<td>780</td>
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<td>0.38</td>
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<td>0.17</td>
<td>0-1</td>
<td>769</td>
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<tr>
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<td>657</td>
<td>86.91</td>
<td>0.87</td>
<td>0.11</td>
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<td>663</td>
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<tr>
<td>Total</td>
<td></td>
<td>4182</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4112</td>
</tr>
</tbody>
</table>
Fig. 1
Fig. 2
Fig. 3

Flower and egg number (Mean ± SE)

- F. montana
- F. asperifolia
- F1s
- Backcrosses
Fig. 4

(a) Style lengths (Mean ± SE, mm)

(b) Style lengths and egg positions (Mean ± SE, mm)
Fig. 5

F. montana

F. asperifolia

F1s

Backcrosses

Fig. 5
Supporting Information

Fig. 5. Ovary diameters in B phase and C phase (post pollinator entry) male figs of *F. montana*, *F. asperifolia*, F1s and backcrosses. Open bars = B phase figs, solid bars = C phase figs.