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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 waspis 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 obligatory 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 manyFicusspecies 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 ofthe 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 entrythrough the ostiole, which acts asa physicalbarrier. 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 offspringare 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 involvedhowever,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-Gonzálezet 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 studywere (I) to determine iffemales 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 ifK. tentacularisthat 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.montanaBlumeis a dioecious fig treenative 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; Rajaet al., 2008a). FicusasperifoliaMiqisdistributed 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 andWiebes, 1992).

Glasshouse populations of F.montana and its active pollinator, Kradibia (= Liporrhopalum) tentacularis(Grandi) were maintained at the experimental gardens of Leeds Universityfrom 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 (Tarachaiet al., 2012) was used. TheF. asperifoliaoriginated from seed collected Kibale Forest, Uganda, in August 2004.KradibiahilliWiebesis 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 un-pollinated, but small numbers were entered by K. tentacularisfemales.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. montanahad an asynchronous all-year fruiting pattern on individual plants and among the population as a whole, but with fewer figs produced in winter (Sulemanet 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 (Sulemanet al., 2011a).

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

Crosses

The developmental stages of the figs wereclassified according to the terminology of Galil andEiskowitch (1968) as modified for dioecious figs by Valdeyronand Lloyd (1979). Hybrids of F. montana (F1s) were generated in 2005from F. montana males and F. asperifolia females by introducing adult female K. tentacularisfrom 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. montanawere 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

Sixindividuals (mixed sexes) ofF. montana,F. asperifolia, F1s and backcrosses were chosen haphazardlyfrom the glasshouse populationsand seven pre-receptive figs on each tree were selected. The plants were organised in a row in analternating 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. tentacularishad entered, based on the numbers of wings and antennae left in the ostiolar area.

Ovipositor length constraints

Thirty oneimmature A phase figs from three male plants of F. montana were chosen haphazardlyandindividual 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 thosedescribed by Nefdt and Compton (1996), where style length was taken as the distance between the top of the stigma and the end of style whereit 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 rubbedacross the ostioles of the F. asperifoliafigs and if the females could then enter the figs through the ostiole, they were considered to be receptive. Figs ofF. asperifolia, F1s and backcross are larger than those of F. montana and were dissected longitudinally through the ostioleinto 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. asperifoliaplants were chosen haphazardly from those with figs in the general population. FiveA phase figs from each plant were bagged to prevent fig wasps from entering. Adult female *K. tentacularis* were obtained as before 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. Asperifolia* 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 receptiveand the remainder had single pollinator females introduced and werethen re-bagged. Theywere 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). Lmerdetected 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 theF. montana figs (X^2= 4.49, df = 1, $P < 0.05$). There was no significantdifference between backcrosses andF. 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 teststhat compared female flowers 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. tentacularisadult 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 mean s of 53.47 ± 10.21, 72 ± 8.66 and 95.60 ± 6.87 respectively, compared with 83.16 ± 4.25 eggs in F. montanafigs. 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-foundressdata 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.

asperifolia (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. asperifolia(when one wasp was introduced),most flowers were empty (84%) and no flowers were recorded with double oviposition (Table 4). In F1s, about halfthe flowers contained one egg or were without eggs when either one or two femaleswere 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 rangedfrom 0.12 to 0.32mm in F. montana, 0.17 to 0.25mm in F. asperifolia, 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 modelsall P > 0.05) (Fig. 4b).

There were no typical eggs found in the 2723 female flowers from female figs ofF. 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. montanawere 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, ~~Fig.~~

~~5~~Supporting Information Figure 1)), but a small number of ovaries had started to expandafter pollinator entry and had reached between 0.6and 0.7mm in diameter (Fig. 5~~6~~). 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; Soleret 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 at all to receptive figs of the closely related F. asperifolia. Under greenhouse conditions small numbers of K. tentacularisfemales 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. tentacularisand 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 Noortet al.,2013), and small numbers of K. tentacularis also entered figs of another related fig tree (F. sandanakanaBerg) 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. montanato 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. tentacularisfemales placed experimentally on the surface of receptive F. asperifoliafigs 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. montanafigs 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 Noortand 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. asperifoliawere 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 utilisedbecauseof 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. asperifoliacontrasts 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 crosseshave 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 inheritedin 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 thanF. montana (C. Soler, personal communication), but clearly the hybrids inherited the key compounds that made the figs attractive to K. tentacularis.The F1hybrids were also closer to the female parents in terms of mean style lengths, but the number of flowers in thefigs 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 showedgall 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 andCompton, 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. microcarpaL. 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 Noortet al.,2013).

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Fig.1A female flower from a male F1hybrid 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. 3Female flower numbers in male figs of F. asperifolia, F. montana and their hybrids, and the numbers of eggs laid by K. tentacularisfoundresses. 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. 5Ovary 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. 5~~6~~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.

	Figs (n)	Wasp (n)	Mean	SE	Range
F. montana					
Total figs	42	69	1.64	0.18	1-6
Male figs	21	39	1.86	0.32	1-6
Female figs	21	30	1.43	0.16	1-3
F. asperifolia					
Total figs	42	4	0.09	0.05	0-1
Male figs	21	0	0.00	0.00	0
Female figs	21	4	0.19	0.09	0-1
F1s					
Total figs	42	57	1.36	0.16	0-6
Male figs	21	19	0.91	0.17	0-3
Female figs	21	38	1.81	0.25	1-6
Backcrosses					
Total figs	42	84	2.00	0.27	0-9
Male figs	21	48	2.29	0.35	0-6
Female figs	21	36	1.71	0.41	0-9

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).

	β Value	SE	t-value	P
Intercept	0.32996	0.23566	1.4	0.161
F. asperifolia	-2.03332	0.57855	-3.514	0.001 ***
F1s	0.2319	0.32251	0.719	0.472
Backcrosses	0.18752	0.32447	0.578	0.563
Sex male	0.24087	0.32188	0.748	0.454
F. asperifolia:Sex male	-16.9067	2126.735	-0.008	0.993
F1s:Sex male	-0.93693	0.47681	-1.965	0.049 *
Backcrosses:Sex male	0.03083	0.443	0.07	0.944

Table 3. The numbers of figs entered by pollinator females and their densities in figs where they were present.

Group	Total figs	Total figs entered	Mean	SE	Range
F. montana	42	42	1.64	0.18	1-6
F. asperifolia	42	4	1.00	0.05	0-1
F1s	42	36	1.58	0.16	0-6
Backcrosses	42	39	2.15	0.27	0-9

Table 4. The distribution of K. tentacularis eggs within ovaries of Ficusmontana, F. asperifolia and their hybrids. Five figs with one ortwo foundresses were examined from each tree. The contents of all the female flowers within these figs were recorded.

Species	Tree	One foundress					Two foundresses				
		N ovaries	Occupancy (%)	Eggs per flower			N ovaries	Occupancy (%)	Eggs per flower		
				Mean	Variance	Range			Mean	Variance	Range
F. montana	1	281	95.73	0.96	0.04	0-1	306	94.77	0.97	0.07	0-2
	2	521	96.35	0.97	0.04	0-2	477	77.15	0.78	0.19	0-2
	3	498	95.18	0.96	0.05	0-2	467	94.65	0.95	0.06	0-2
	4	386	98.19	0.98	0.02	0-2	363	96.69	0.98	0.05	0-2
	5	480	94.79	0.95	0.05	0-2	653	96.94	0.97	0.03	0-1
	Total	2166					2266				
F. asperifolia	1	2347	19.13	0.19	0.15	0-1	-	-	-	-	-
	2	1112	20.41	0.2	0.16	0-1	-	-	-	-	-
	3	1648	7.65	0.07	0.07	0-1	-	-	-	-	-
	Total	5107									
F1s	1	579	49.91	0.5	0.25	0-1	602	66.61	0.67	0.22	0-1
	2	1020	50.59	0.5	0.25	0-1	884	52.04	0.52	0.25	0-1
	3	1149	17.32	0.17	0.14	0-1	1473	41.41	0.41	0.24	0-1
	4	1578	27.76	0.28	0.2	0-1	1330	44.89	0.45	0.25	0-1
	5	921	38.87	0.39	0.24	0-2	842	44.42	0.44	0.25	0-2
	Total	5247					5131				
Backcrosses	1	999	58.16	0.58	0.24	0-1	1110	74.41	0.77	0.18	0-1
	2	948	36.71	0.37	0.23	0-1	780	79.10	0.79	0.17	0-1
	3	868	38.25	0.38	0.24	0-1	790	66.71	0.66	0.22	0-1
	4	710	78.59	0.79	0.17	0-1	769	77.50	0.77	0.17	0-1
	5	657	86.91	0.87	0.11	0-2	663	88.24	0.88	0.1	0-2
	Total	4182					4112				

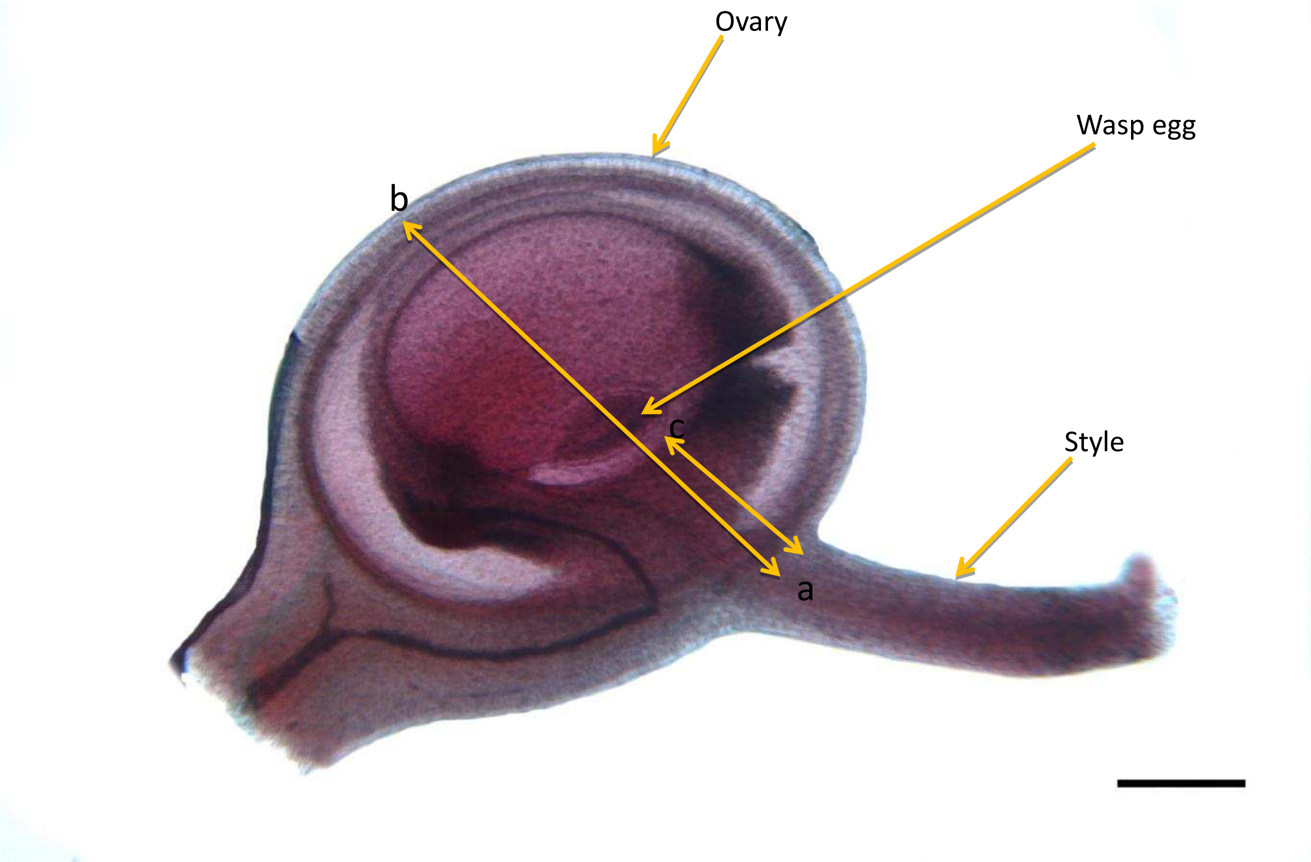

Fig. 1

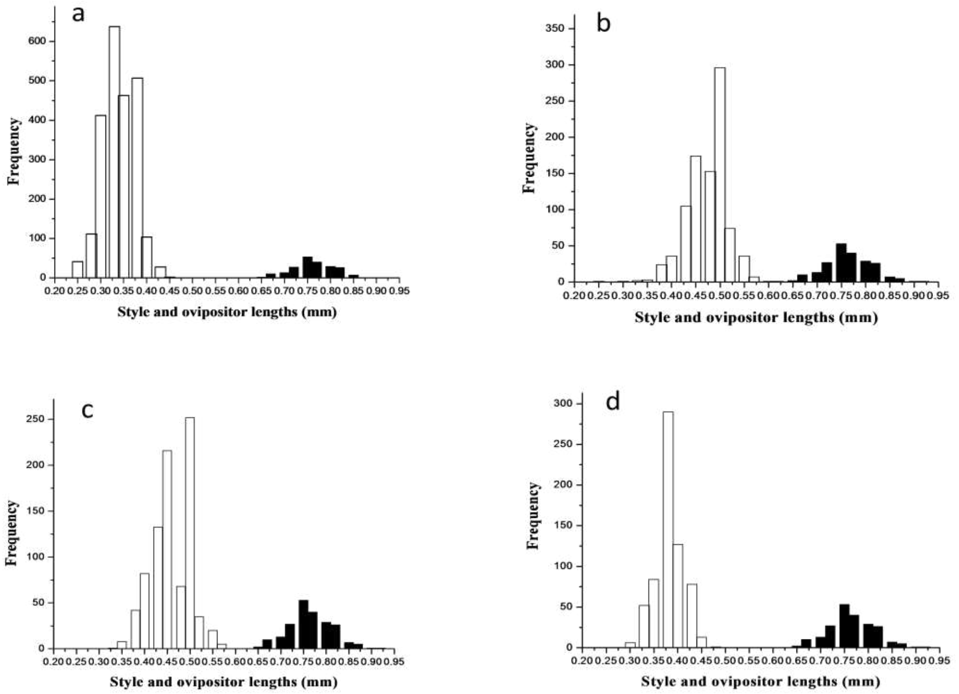
a
Frequency
0
100
200
300
400
500
600
0.20 0.25 0.30 0.35 0.40 0.45 0.50 0.55 0.60 0.65 0.70 0.75 0.80 0.85 0.90 0.95
Style and ovipositor lengths (mm)
b
Frequency
0
50
100
150
200
250
300
350
0.20 0.25 0.30 0.35 0.40 0.45 0.50 0.55 0.60 0.65 0.70 0.75 0.80 0.85 0.90 0.95
Style and ovipositor lengths (mm)
c
Frequency
0
50
100
150
200
250
0.20 0.25 0.30 0.35 0.40 0.45 0.50 0.55 0.60 0.65 0.70 0.75 0.80 0.85 0.90 0.95
Style and ovipositor lengths (mm)
d
Frequency
0
50
100
150
200
250
300
0.20 0.25 0.30 0.35 0.40 0.45 0.50 0.55 0.60 0.65 0.70 0.75 0.80 0.85 0.90 0.95
Style and ovipositor lengths (mm)

Fig. 2

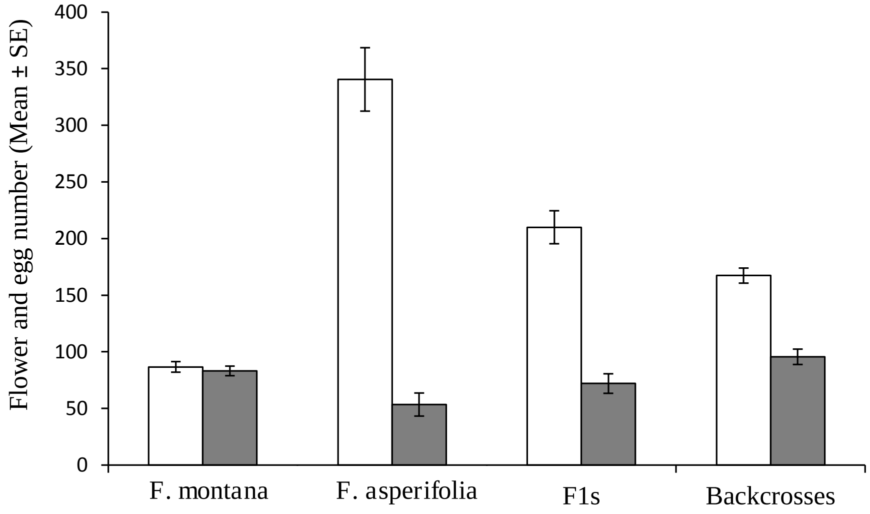
Flower and egg number (Mean ± SE)
400
350
300
250
200
150
100
50
0
F. montana
F. asperifolia
F1s
Backcrosses

Fig. 3

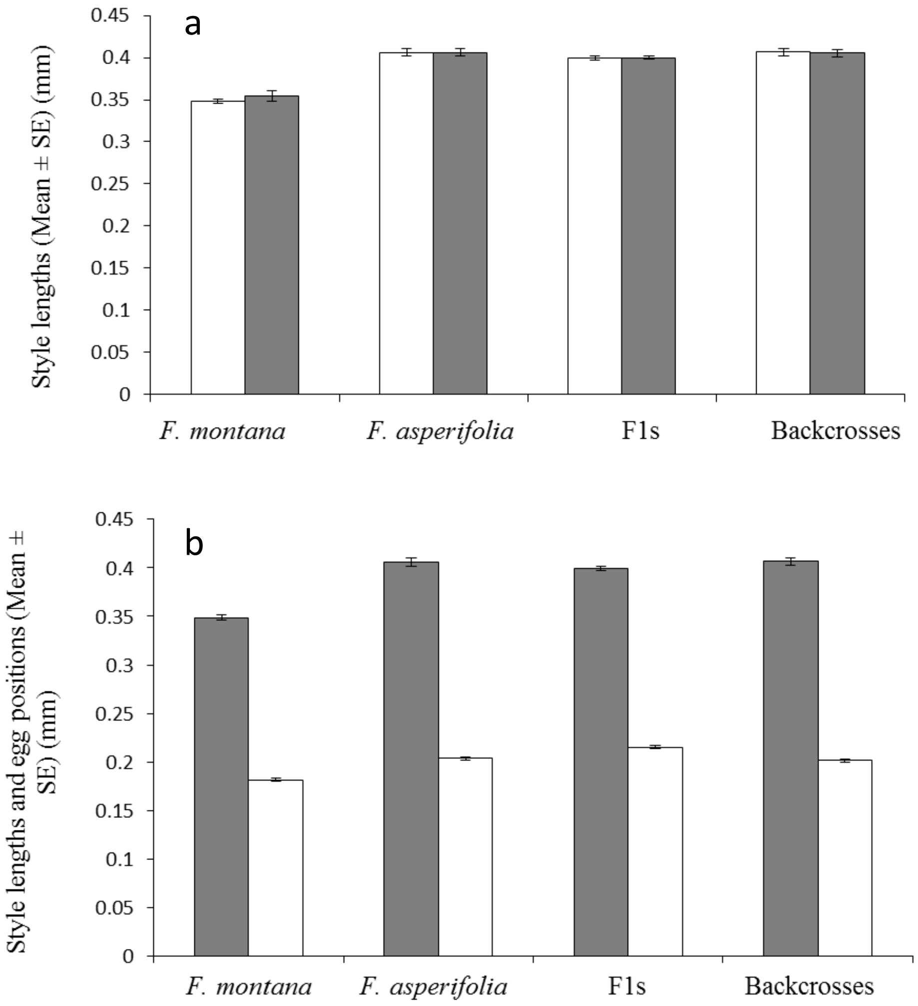
a
Style lengths (Mean ± SE) (mm)
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4
0.45
F. montana
F. asperifolia
F1s
Backcrosses
b
Style lengths and egg positions (Mean ± SE) (mm)
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4
0.45
F. montana
F. asperifolia
F1s
Backcrosses

Fig. 4

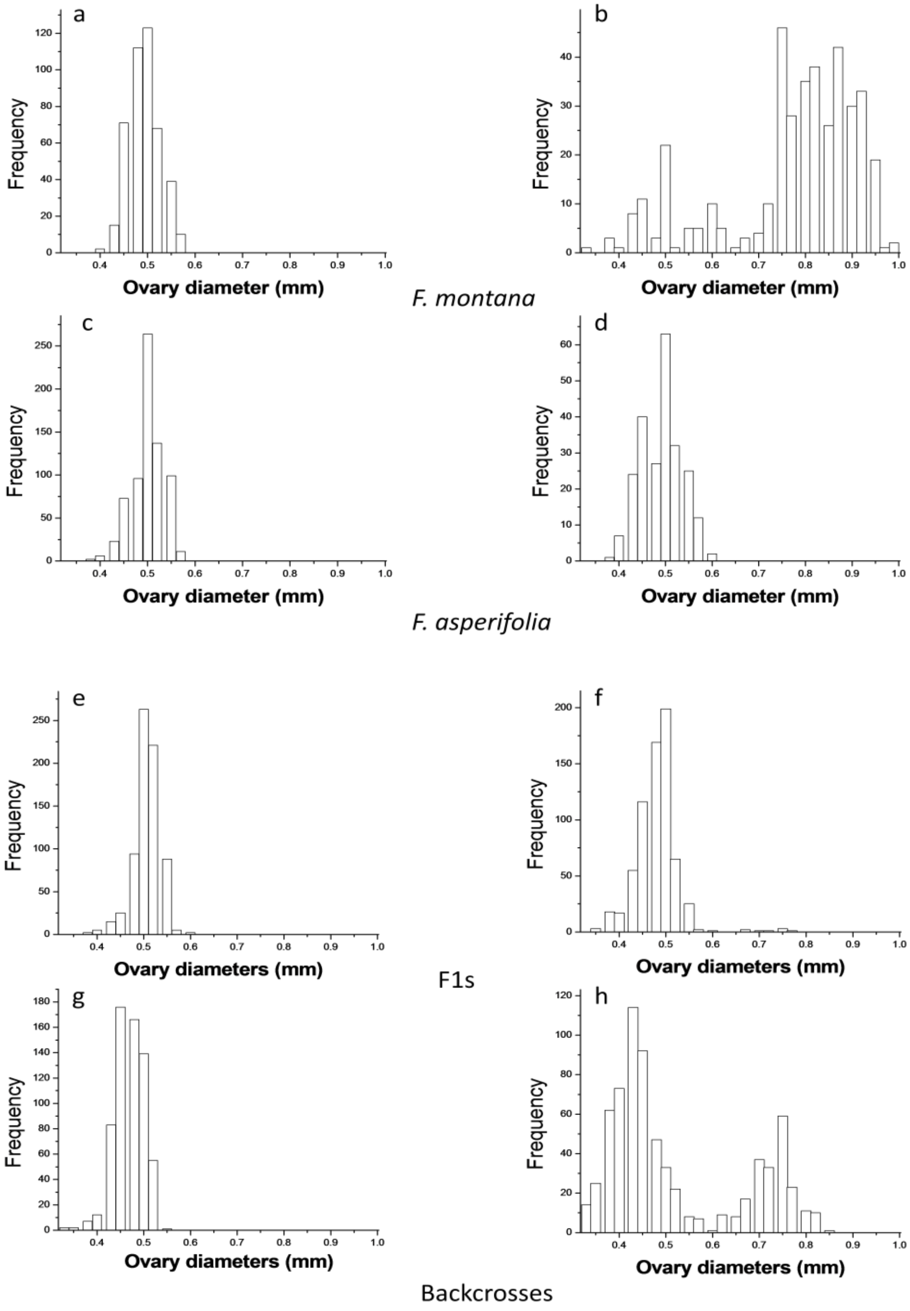

Fig. 5

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Supporting Information

Fig. ~~5~~1Ovary 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.