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

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26 **Abstract**

- 27 1. Fig trees (*Ficus* spp.) and their host specific pollinator fig wasps (Agaonidae) are
28 partners in an obligate mutualism. Receptive phase figs release specific volatiles to
29 attract their pollinators and this is generally effective in preventing pollinator species
30 from entering figs of the wrong hosts.
- 31 2. If entry is attempted into atypical host figs then ostiole size and shape and style length
32 may also prevent reproduction. Despite these barriers, there is increasing evidence
33 that fig wasps enter atypical hosts, and that this can result in hybrid seed and fig wasp
34 offspring.
- 35 3. This study examines the basis of pollinator specificity in two dioecious fig species
36 from different geographical areas. *Kradibiatentacularis* pollinates *Ficus montana* in
37 Asia. *F. asperifolia* from East Africa is closely related, but is pollinated by a different
38 species of *Kradibia*.
- 39 4. In glasshouses, *K. tentacularis* was attracted to its normal host, F1s and backcrosses,
40 but only rarely entered figs of *F. asperifolia*. Foundresses were able to lay eggs in
41 hybrids, backcrosses and *F. asperifolia*, although flower occupancy was lowest in *F.*
42 *asperifolia* figs and intermediate in hybrids.
- 43 5. The fig wasp failed to reproduce in female *F. montana*, male *F. asperifolia* and male
44 F1s, and most but not all backcrosses to *F. montana*. This was a result of the failure to
45 initiate gall production.
- 46 6. Host specificity in this fig wasp is strongly influenced by host volatiles, but ability to
47 gall may be the ultimate determinant of whether it can reproduce.

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

49 **Running title:** Pollinator specificity in dioecious figs

50

51 **Introduction**

52 *Ficus* is one of the most diverse genera of plants (Harrison, 2005) with approximately 800
53 described species (Berg and Corner, 2005). Fig trees and fig wasps have an obligatory
54 mutualistic relationship that had persisted for over 75 million years (Compton *et al.*,
55 2010; Cruadet *et al.*, 2012). Fig wasps transport pollen from their natal figs to receptive figs (figs
56 that are ready to be pollinated) while wasps only lay their eggs in fig flowers, where the
57 larvae develop in and feed on galled ovaries (Cook and Segar, 2010). There are two types of
58 pollination in fig trees, passive and active. Passive pollination is where the pollinators
59 haphazardly pollinate receptive flowers by carrying the pollen on their bodies (Cook and
60 Rasplus, 2003) and do not display any behaviour to collect the pollen (Jousselin *et al.*, 2001).
61 Fig wasps with this inefficient type of pollination lack coxal combs and their host figs have
62 high anther to ovary ratios (Kjellberg *et al.*, 2001). Active pollination, where female insects
63 collect, store and then release the pollen, has been recorded in at least three pollinator-plant
64 mutualisms: yuccas and yucca moths (Pellmyr and Huth, 2002), senita cacti and senita moths
65 (Fleming and Holland, 1998) and figs and fig wasps (Janzen, 1979). Fig wasps display
66 morphological and behavioural adaptations for collecting and depositing pollen with pollen
67 pockets and coxal combs that help in collecting and depositing the pollen (Jousselin *et al.*,
68 2003). Active pollination may be a way to provide their larvae with an additional food
69 source, and can reduce larval mortality rates (Jousselin and Kjellberg, 2001; Tarachai *et al.*,
70 2008). The ratio of male to female flowers is low in figs with active pollination (Kjellberg *et*
71 *al.*, 2001).

72 It was believed that each fig tree species is pollinated by one specific fig wasp species
73 (Ramirez, 1970; Bronstein, 1987) but there is increasing evidence that many *Ficus* species are
74 pollinated by more than one fig wasp species (Ware and Compton, 1992; Molbo *et al.*, 2003;
75 Marussich and Machado, 2007; Compton *et al.*, 2009; Lin *et al.*, 2010). Floral scents are

76 often important signals from flowering plants to their pollinators and in obligate mutualisms
77 chemicals released by one partner can be crucial for attraction of the other (Grison-Pige *et al.*,
78 2002; Raguso, 2008; Soler *et al.*, 2011). Specificity between fig trees and their pollinators is
79 maintained by a combination of chemical barriers (stage and host plant specific attractant
80 volatiles) and physical barriers determined by ostiole diameter and style length (Ware and
81 Compton, 1994; van Noort and Compton, 1986). Figs of each species emit a species-specific
82 blend of volatile chemical compounds during their receptive phase that differs from that
83 released by younger and older figs (van Noort *et al.*, 1989; Grison-Pige *et al.*, 2001). Changes
84 in the floral scent after figs are pollinated result in pollinators avoiding these figs (Proffitt *et*
85 *al.*, 2008) and the figs can become repellent by the time that the next generation of pollinators
86 emerges (Guet *al.*, 2012). Once the female wasps have landed on the surface of the receptive
87 fig there are further physical or chemical cues that can influence whether they attempt entry
88 (Wang *et al.*, 2013). The females then have to gain entry through the ostiole, which acts as a
89 physical barrier. The ostiolar bracts become looser at the receptive phase to make penetration
90 easier (Verkerke, 1986), but a proportion of pollinator females fail to pass successfully
91 through the ostiole (Liu *et al.*, 2013) despite their morphological adaptations to aid entry
92 (Ramirez, 1974; Verkerke, 1989; van Noort *et al.*, 1989).

93 In monoecious fig trees, seeds and fig wasp offspring are produced in the same figs.
94 Inside the figs there are both male and female flowers. Male flowers produce pollen and
95 female flowers can develop seeds or support wasp offspring. Female flowers vary in their
96 style lengths and pedicels, with flowers with shorter styles having longer pedicels and vice
97 versa (Verkerke, 1989). This allows all the stigmas to be at the same level at the time that
98 pollinators enter, providing a synstigma over which the wasp females can walk and probe the
99 styles. It was initially believed that female wasps only lay their eggs inside female flowers
100 that have short styles and those with long style produce seeds, but most fig wasps have

101 ovipositors that can reach most or all of the female flowers in their host figs, and mean style
102 and ovipositor lengths among species pairs are strongly correlated (Nefdt and Compton,
103 1996). In dioecious fig trees, wasps and seeds are produced on different plants. Figs borne on
104 male trees have male and female flowers, but are functionally male. Male flowers produce
105 pollen and the female flowers all have short styles that are easily accessible to female fig
106 wasps for oviposition and do not produce seeds. Female trees have flowers with longer styles
107 and stigmas adapted for pollen collection. They only develop seeds rather than fig wasp
108 offspring (Corlett *et al.*, 1990), but it is unknown whether they oviposit into those flowers (if
109 any) that are accessible.

110 There are over 13,000 species of insects recorded as being able to induce plants to
111 make galls (Dreger-Jauffret and Shorthouse, 1992; Roskam, 1992). Galls can develop on
112 leaves, stems, buds, petioles, fruits and roots (Weis *et al.*, 1988; Dreger-Jauffret and
113 Shorthouse, 1992; Raman *et al.*, 2007). They provide a food resource and shelter to the
114 inducing insects or their offspring (Sanver and Hawkins, 2000; Raman, 2007; Hardy and
115 Cook, 2010). Gall inducers are more host specific than most other guilds of herbivorous
116 insects (Hardy and Cook, 2010). The galls can be induced during feeding or
117 oviposition (Miles, 1968; Raman, 2007; Matsukura *et al.*, 2009). Galls that are initiated by
118 oviposition are believed to be responding to compounds secreted with the eggs or coming the
119 eggs themselves, but larvae can also continue to stimulate gall development (Miles, 1968;
120 Stone *et al.*, 2002). The nature of these compounds and the mechanisms involved however, are
121 still unclear (Stone and Schonrogge, 2003; Tooker and De Moraes, 2008). Most gall inducers
122 are specific to particular plant organs and specific host plants (Shorthouse *et al.*, 2005) and
123 attraction to the wrong host can lead to failures in gall formation and progeny
124 development (Weis *et al.*, 1988; Wool, 2005).

125 Female fig wasps are able to induce galls in the ovules where they lay their eggs
126 (Jansen-González *et al.*, 2014). After they enter receptive figs they insert their ovipositors into
127 the ovaries through the styles and lay a single egg in each ovary together with a secretion that
128 stimulates the ovaries to enlarge. There are several reported cases when fig wasps pollinators
129 enter atypical hosts (Janzen, 1979; Ware and Compton, 1992). This can lead to viable hybrid
130 seeds (Ramirez, 1970; Ramirez and Montero, 1988; Ware and Compton, 1992) and hybrid
131 plants (Parrish *et al.*, 2003; Moe and Weiblen, 2012). Viable hybrid fig trees represent
132 potential routes for introgression between fig tree species, and a mechanism that facilitates
133 speciation (Kasumi *et al.*, 2012).

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

141

142 **Materials and Methods**

143 **Study site and species**

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

149 *asperifolia* branches less frequently, tends to grow taller and produces figs that grow slightly
150 larger (Berg and Wiebes, 1992).

151 Glasshouse populations of *F. montana* and its active pollinator, *Kradibia* (= *Liporrhopalum*)
152 *tentacularis* (Grandi) were maintained at the experimental gardens of Leeds
153 University from 1996 (Raja *et al.*, 2008b; Suleman *et al.*, 2012, Suleman *et al.*, 2013c). They
154 originated from Bogor (Java, Indonesia), and the Krakatau Islands (Indonesia). Only the
155 green-leaved form of *F. montana* (Tarachai *et al.*, 2012) was used. The *F.*
156 *asperifolia* originated from seed collected Kibale Forest, Uganda, in August
157 2004. *Kradibia hillii* Wiebes is the pollinator of *F. asperifolia* in East Africa (Berg and Wiebes,
158 1992), but it was not available and all experiments and crosses involved *K. tentacularis*. Most
159 *F. asperifolia* figs in the general (mixed-species) glasshouse population remained un-
160 pollinated, but small numbers were entered by *K. tentacularis* females. Both species develop
161 rapidly from seed and experimentally-generated offspring started to produce figs from as
162 little as nine months after germination.

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

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

174

175 **Crosses**

176 The developmental stages of the figs were reclassified according to the terminology of Galil
177 and Eiskowitch (1968) as modified for dioecious figs by Valdeyron and Lloyd (1979).
178 Hybrids of *F. montana* (F1s) were generated in 2005 from *F. montana* males and *F.*
179 *asperifolia* females by introducing adult female *K. tentacularis* from D phase male figs into B
180 (receptive) phase female figs of *F. asperifolia* (Ghana, 2012). Reciprocal crosses were not
181 possible because only *K. tentacularis* was available. Backcrosses to *F. montana* were
182 generated from male *F. montana* and female F1s by introducing *F. montana* pollinators into
183 female F1s in 2006.

184

185 **Attraction to receptive figs and pollinator entry**

186 Six individuals (mixed sexes) of *F. montana*, *F. asperifolia*, F1s and backcrosses were chosen
187 haphazardly from the glasshouse populations and seven pre-receptive figs on each tree were
188 selected. The plants were organised in a row in an alternating sequence with approximately 50
189 cm spaces between each other. The figs on each plant were not close to each other and their
190 branches were surrounded by Vaseline petroleum jelly[®] to prevent any wingless *K.*
191 *tentacularis* from re-emerging from figs elsewhere and entering the focal figs (Raja *et al.*,
192 2008b). These were checked regularly and entry by winged pollinators was detected by the
193 presence of detached wings at their ostioles. The figs were removed after seven days and
194 opened under a binocular microscope to score how many *K. tentacularis* had entered, based
195 on the numbers of wings and antennae left in the ostiolar area.

196

197 **Ovipositor length constraints**

198 Thirty oneimmature A phase figs from three male plants of *F. montana* were chosen
199 haphazardlyandindividual netting bags (pore diameter 0.19 mm) were placed around them to
200 exclude pollinators. Receptivity was tested each day by introducing wasps to the ostiolar
201 area. Any attempt to enter the figs was taken as indicative that the figs were receptive. These
202 figs were then collected and dissected under a binocular microscope. All the flowers in the
203 figs were picked and positioned horizontally on slides under a compound microscope to
204 measure the lengths of the styles using an ocular scale. The measurements followed
205 thosedescribed by Nefdt and Compton (1996), where style length was taken as the distance
206 between the top of the stigma and the end of style whereit connected with the ovary.The
207 procedure was repeated for male F1s, backcrosses and *F. asperifolia* (three plants with five
208 figs from each group), but with a modification for *F. asperifolia* figs, because *K. tentacularis*
209 females were generally unwilling to enter them. To stimulate them to attempt entry, receptive
210 figs of *F. montana* were rubbedacross the ostioles of the *F. asperifolia*figs and if the females
211 could then enter the figs through the ostiole, they were considered to be receptive. Figs of*F.*
212 *asperifolia*, F1s and backcross are larger than those of *F. montana* and were dissected
213 longitudinally through the ostioleinto four pieces and flowers from one quarter were
214 measured, rather than all the flowers.

215 Ovipositor lengths were measured by collecting *K. tentacularis* females from 25 late
216 phase D figs from five male trees of *F. montana* (five figs from each plant). The figs were
217 kept in plastic containers until the wasps emerged, usually on the next day. About ten wasps
218 from each fig were collected, slide mounted and measured under a compound microscope.
219

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

221 Five male *F. montana*, F1s and backcrosses and three *F. asperifolia*plants were chosen
222 haphazardly from those with figs in the general population. FiveA phase figs from each plant

223 were bagged to prevent fig wasps from entering. Adult female *K. tentacularis* were obtained as
224 before and introduced into receptive figs of *F. montana*, F1s and backcrosses by putting one
225 or two on the ostiole area of each fig. If a wasp entered, the figs were re-bagged to prevent any
226 more wasps from entering. The wasps were introduced similarly into male figs of *F.*
227 *Asperifolia* using the technique described previously. The figs were left for 24 hours, then
228 collected and stained to reveal whether eggs had been laid.

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

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

243

244 **Ability to gall**

245 Relative ovary diameters in phases B and C male figs (representing pre- and post-oviposition
246 periods) were used as indicators of the ability of the wasps to initiate galls. Three *F. montana*,
247 F1s, backcrosses and *F. asperifolia* plants were chosen haphazardly. Ten figs on each plant

248 were covered with mesh bags as before. Half had their ovaries measured once they became
249 receptive and the remainder had single pollinator females introduced and were then re-bagged.
250 They were then collected seven days later. All the figs were dissected longitudinally into four
251 pieces under a dissected microscope and the diameter of ovaries from all the flowers in one
252 quarter were recorded (Fig. 1).

253

254 **Data analysis**

255 A generalized linear mixed-effect regression model (Lmer) was used with a Poisson error for
256 count data to determine whether there were significant differences in the numbers of
257 pollinators entering figs of *F. asperifolia* and *F. montana*, F1s and backcrosses to *F.*
258 *montana*. The fixed effect variables in the model were the different fig groups (*F. montana*, *F.*
259 *asperifolia*, F1s and backcrosses) with fig number and plant number as random effects.
260 GLMs, Wilcoxon rank-sum tests, t tests and nested ANOVA were all performed in R
261 (2.12.2).

262

263 **Results**

264 **Attraction to receptive figs and pollinator entry**

265 Fig wasp foundresses are typically attracted to receptive figs of their host plants by volatiles
266 released during B phase, but not by receptive figs belonging to other species. In the
267 greenhouse environment, where plants with receptive figs were positioned close to each other
268 (Table 1), up to six *K. tentacularis* females entered male and female figs on F1 plants, in
269 similar numbers as in *F. montana* figs. Similarly, up to nine females entered backcross figs.
270 These results were in contrast to *F. asperifolia* figs, which at most were entered by a single
271 pollinator, and often remained un-entered (Table 1). Although the number of foundresses
272 entering the receptive figs ranged from 0 to 9, most figs were entered by one foundress in all

273 groups (Table 1). Lmer detected no significant difference in foundress numbers between F1s
274 and backcrosses with *F. montana*, but fig wasp densities were significantly lower in *F.*
275 *asperifolia* figs (Table 2). There was a significant difference after removing the plant sex
276 interaction term from the model and comparing the new model to the previous one, showing
277 that sex influenced the number of fig wasps that entered the figs (Lmer: $X^2 = 9.53$, $df = 3$, $P <$
278 0.05).

279 All the receptive figs of *F. montana* were entered by fig wasps, compared with less
280 than 10% of the *F. asperifolia* figs ($X^2 = 65.79$, $df = 1$, $P < 0.001$). Around 85 % of the F1 figs
281 were entered (Table 3), which was a significantly lower than for the *F. montana* figs ($X^2 =$
282 4.49 , $df = 1$, $P < 0.05$). There was no significant difference between backcrosses and *F.*
283 *montana* ($X^2 = 1.38$, $df = 1$, $P = 0.24$).

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

288

289 **Ovipositor length constraints**

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

297

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

299 *F. asperifolia*, F1s and backcrosses had a higher number of female flowers in their male figs
300 than *F. montana*, with means \pm SE of 340.47 ± 27.98 , 209.88 ± 14.54 and 167.28 ± 6.61
301 respectively, compared with 86.64 ± 4.63 in *F. montana* (Fig. 3). There was a significant
302 difference in female flower numbers between the groups (nested ANOVA, $F(3,14) = 10.3$, P
303 < 0.001). Pairwise t tests that compared female flowers number between groups showed that
304 differences between all groups were significant ($P < 0.05$ between F1s and backcrosses and P
305 < 0.001 for other combinations).

306 Around 28200 fig flowers were examined for the presence of eggs. No *K.*
307 *tentacularis* adult offspring had ever emerged from male *F. asperifolia* and F1 figs in the
308 greenhouse populations, but the foundresses were found to have laid eggs in F1s, backcrosses
309 and *F. asperifolia*, as well as *F. montana*. Although figs of *F. asperifolia*, F1s and
310 backcrosses contained more female flowers than *F. montana* figs, the numbers of eggs laid by
311 a single foundress were about the same, with means of 53.47 ± 10.21 , 72 ± 8.66 and $95.60 \pm$
312 6.87 respectively, compared with 83.16 ± 4.25 eggs in *F. montana* figs. There was no
313 significant difference in the numbers of eggs laid (nested ANOVA, $F(3, 14) = 1.99$, $P =$
314 0.16).

315 The egg occupancy rates in F1 male figs entered by a single female ranged between
316 17.32% and 50.59% of the female flowers (Table 4), compared with 36.71% to 86.91% in
317 backcrosses, 7.65% to 20.41% in *F. asperifolia* and 94.8 to 98.2% in *F. montana*. When two
318 female fig wasps were introduced into the receptive figs, the lowest occupancy rate was in
319 F1s (41.41 to 66.61%) followed by backcrosses (66.71 to 88.24%) and *F. montana* (77.2 to
320 96.9%). No two-foundress data are available for *F. asperifolia*.

321 There were no significant differences in the style lengths of flowers that contained
322 eggs or were free of eggs in all groups: *F. montana* (Wilcoxon RS, $W = 8427.5$, $P = 0.44$), *F.*

323 *asperifolia* (Wilcoxon RS, $W = 8504.5$, $P = 0.57$), F1s (Wilcoxon RS, $W = 29437.5$, $P =$
324 0.64) and backcrosses (Wilcoxon RS, $W = 30153.5$, $P = 0.73$, Fig. 4a). Eggs numbers per
325 ovary ranged from 0 to 2, but most ovaries contained only one egg. In *F. asperifolia*(when
326 one wasp was introduced),most flowers were empty (84%) and no flowers were recorded
327 with double oviposition (Table 4). In F1s, about halfthe flowers contained one egg or were
328 without eggs when either one or two femaleswere introduced,and only three ovaries were
329 recorded with two eggs (Table 4). In backcrosses the proportion of flowers containing eggs
330 was higher than in F1 figs,with around 60%utilized when one wasp was introduced and 80%
331 when two wasps were introduced, but only four flowers were recorded with two eggs (Table
332 4). In *F. montana*, the normal host for *K tentacularis*, double oviposition was again rare with
333 only 24 examples among the 4432 female flowers examined (Table 4). The positions of the
334 eggs inside the ovaries were similar in all groups (Fig. 1)with no significant differences in
335 location between the groups. The positions rangedfrom 0.12 to 0.32mm in *F. montana*, 0.17
336 to 0.25mm in *F. asperifolia*, 0.15 to 0.27 mm in F1s and 0.15 to 0.25 mm in backcrosses.
337 There was no significant interaction effect between style length, plant group and egg position
338 (Mixed effect modelsall $P > 0.05$) (Fig. 4b).

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

342

343 **Ability to gall**

344 Ovaries in B phase figs of *F. montana*were $0.48 \text{ mm} \pm 0.001$ in diameter ($n = 440$), compared
345 with $0.76 \text{ mm} \pm 0.007$ in C phase figs ($n = 393$), after the ovaries had responded to
346 oviposition and galling. In F1 figs, the mean diameter of the ovaries shrank over time ($0.50 \pm$
347 0.001 mm in B phase ($n = 720$) compared with $0.48 \pm 0.002 \text{ mm}$ in C phase ($n = 678$, ~~Fig.~~

348 | [Supporting Information Figure 1](#))), but a small number of ovaries had started to expand after
349 | pollinator entry and had reached between 0.6 and 0.7 mm in diameter (Fig. [56](#)). These
350 | enlarged ovaries were only detected in two figs, both collected from the same plant. In
351 | backcrosses, the overall measurements of the ovaries in B phase were $0.46 \text{ mm} \pm 0.001$ ($n =$
352 | 643) and $0.51 \text{ mm} \pm 0.005$ in C phase ($n = 714$). Many ovaries in these figs had started to
353 | develop after pollinator entry and were larger than 0.45 mm, the maximum ovary diameter
354 | recorded in B phase backcross figs. In contrast, there was no evidence of ovary expansion
355 | after oviposition in *F. asperifolia* figs, where the ovary diameters were $0.50 \text{ mm} \pm 0.001$ ($n =$
356 | 712) in B phase figs and $0.49 \text{ mm} \pm 0.003$ at C phase ($n = 746$).

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

363

364 | **Discussion**

365 | The floral scents emitted by receptive figs vary sufficiently between stages and between
366 | species for pollinators to be attracted to their specific host species at the particular time when
367 | the figs are ready to be pollinated (Raguso, 2008; Soleret *et al.*, 2010, but see Zhang *et al.*, 2014
368 | for an apparent exception). *K. tentacularis* routinely pollinates *F. montana* and flying females
369 | of this species are attracted to its receptive figs, but probably not at all to receptive figs of
370 | the closely related *F. asperifolia*. Under greenhouse conditions small numbers of *K.*
371 | *tentacularis* females nonetheless do land on *F. asperifolia* figs and attempt to enter them.
372 | These occasional encounters were probably the result of the high densities and intermixing of

373 the two *Ficus* species in the closed environment of the glasshouses. In nature, even if the
374 species were sympatric and showed similar habitat preferences, the differences in volatiles
375 released by the figs would be sufficient to make chance encounters between *K.*
376 *tentacularis* and *F. asperifolia* figs extremely rare. A proportion of these rare encounters may
377 nonetheless result in *K. tentacularis* entering the figs, as has been documented with other fig
378 wasp species (Ware and Compton 1992; van Noort *et al.*, 2013), and small numbers of *K.*
379 *tentacularis* also entered figs of another related fig tree (*F. sandanakana* Berg) in the
380 glasshouses (S. Ghana, personal observations). The specific chemical compounds released by
381 the receptive figs of the two species (Proffit *et al.*, 2009; Soler *et al.*, 2011) are therefore
382 highly effective at preventing pollen flow from *F. montana* to *F. asperifolia*, but would not
383 represent an absolute barrier in situations where the two plant species were sympatric. Given
384 the evident difference in volatile profiles of the two species, it is likely that pollen flow in the
385 other direction, mediated by the pollinator of *F. asperifolia*, would be similarly restricted.

386 The willingness of pollinator females to attempt entry into figs can be influenced by
387 cues on the surface of receptive figs that are independent of the volatiles responsible for long-
388 distance attraction (Wang *et al.*, 2013). *K. tentacularis* females placed experimentally on the
389 surface of receptive *F. asperifolia* figs were generally disinterested in entering them, but some
390 entries did nonetheless take place when receptive figs were available for long periods in the
391 glasshouses. Rubbing receptive *F. montana* figs on the surface of the *F. asperifolia* figs had
392 a strong effect on pollinator entry, by stimulating the females to seek out the ostiole. This
393 involved an increase in speed of walking and antennal drumming on the fig surface while
394 they were apparently seeking out the narrow entrance provided by the ostiole. The stimuli
395 provided by the *F. montana* figs may have been the same volatiles that are responsible for
396 long distance attraction, less volatile compounds emanating from the fig surface, or a
397 combination of the two.

398 The ostiole of figs is often regarded as a physical filter that can trap female pollinators
399 that are associated with different species of fig trees (van Noortand Compton, 1996), but *K.*
400 *tentacularis* females that attempted entry into *F. asperifolia* figs apparently had no problems
401 passing through the ostiole, because no dead females were found in the ostiole facing
402 inwards. The females routinely re-emerge from figs of *F. montana* and the same behaviour
403 was observed among females that had entered *F. asperifolia* figs.

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

417 Artificial hybrid crosses have been generated between some fig tree species and an
418 increasing number of examples of putative hybrid fig trees are being recorded from natural
419 environments (Condit, 1950; Parrish *et al.*, 2003; Kusumiet *al.*, 2012). F1 hybrids between
420 *F. montana* and *F. asperifolia*, and backcrosses to *F. montana*, provided indications of how
421 some of the plant characters associated with pollination can be inherited in hybrid offspring
422 and how the pollinator of one of the parents responds to the novel characters generated by

423 hybridisation. F1s between plant species often have volatile profiles intermediate between
424 those of their parents (Schnitzler *et al.*, 2004; Salzmann *et al.*, 2007; Shuttleworth and
425 Johnson, 2010). Chemical analysis of the volatiles released from receptive F1s and
426 backcrosses suggests that the overall volatile profiles were more similar to those of *F.*
427 *asperifolia* than *F. montana* (C. Soler, personal communication), but clearly the hybrids
428 inherited the key compounds that made the figs attractive to *K. tentacularis*. The F1 hybrids
429 were also closer to the female parents in terms of mean style lengths, but the number of
430 flowers in the figs was more intermediate. As expected, the floral characters of backcrosses
431 tended to be closer to *F. montana*.

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

441 Species specific volatiles released from receptive figs and the responses of pollinator
442 fig wasps to them are clearly the major source of host specificity in the fig tree: fig wasp
443 mutualism, because they greatly reduce the chances that fig wasp females will land on figs
444 belonging to species other than their routine hosts. Chance encounters result in fig wasps
445 having the opportunity to attempt fig entry. Whether or not they do so may depend on short-
446 range stimuli from the surface of the figs, but the list of examples where pollinators are
447 known to enter non-host figs is increasing (Harrison, 2006; Janzen, 1979; Ware and Compton,

448 1992).After negotiating the ostiole, so long as the style lengths of the flowers in the male figs
449 are not too long (Nefdt and Compton, 1996), then our results suggest that egg deposition is
450 likely to occur, but for reproduction to be successful the female must also be able to
451 successfully induce gall formation in the ovules where she lays her eggs. Gall-forming insects
452 are often highly host specific, with unpredictable abilities to gall hybrids with their usual
453 hosts (Skuhravy *et al.*, 1997). If the inability the pollinator of *F. montana* to induce galls in
454 even a closely related species is typical of most fig wasps, then the ability to form galls could
455 be considered as the ultimate factor limiting the host range of these pollinators. However,
456 results from another fig tree suggest that it is not always the case. *F. microcarpa* L. f. is an
457 Asian fig tree introduced to South Africa, where it has no very close relatives. Despite this,
458 small numbers of two native African pollinator fig wasps, belonging to a different genus from
459 its usual pollinator, have been reared from its figs, showing that they can successfully form
460 galls (van Noort *et al.*, 2013).

461

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469

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

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

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

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

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

697 Fig. 56 Ovary diameters in female flowers from male figs of *F. montana*, *F. asperifolia*, F1s
698 and backcrosses. a) B phase b) C phase (after pollinator entry).

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704 Table 1. The numbers of *K. tentacularis* females entering male and female figs in the general
 705 glasshouse population.

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	Figs (n)	Wasp (n)	Mean	SE	Range
<i>F. montana</i>					
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
<i>F. asperifolia</i>					
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

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715 Table 2. The minimal adequate generalized linear mixed-effects model with Poisson errors
 716 for the number of wasps attracted to receptive figs and its interaction with plant sex when
 717 compared with *F. montana* (intercept).

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	β Value	SE	t-value	P
Intercept	0.32996	0.23566	1.4	0.161
<i>F. asperifolia</i>	-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
<i>F. asperifolia</i> :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

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

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Group	Total figs	Total figs entered	Mean	SE	Range
<i>F. montana</i>	42	42	1.64	0.18	1-6
<i>F. asperifolia</i>	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

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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.

Species	Tree	One foundress					Two foundresses				
		N ovaries	Occupancy (%)	Eggs per flower			N ovaries	Occupancy (%)	Eggs per flower		
				Mean	Variance	Range			Mean	Variance	Range
<i>F. montana</i>	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				
<i>F. asperifolia</i>	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					

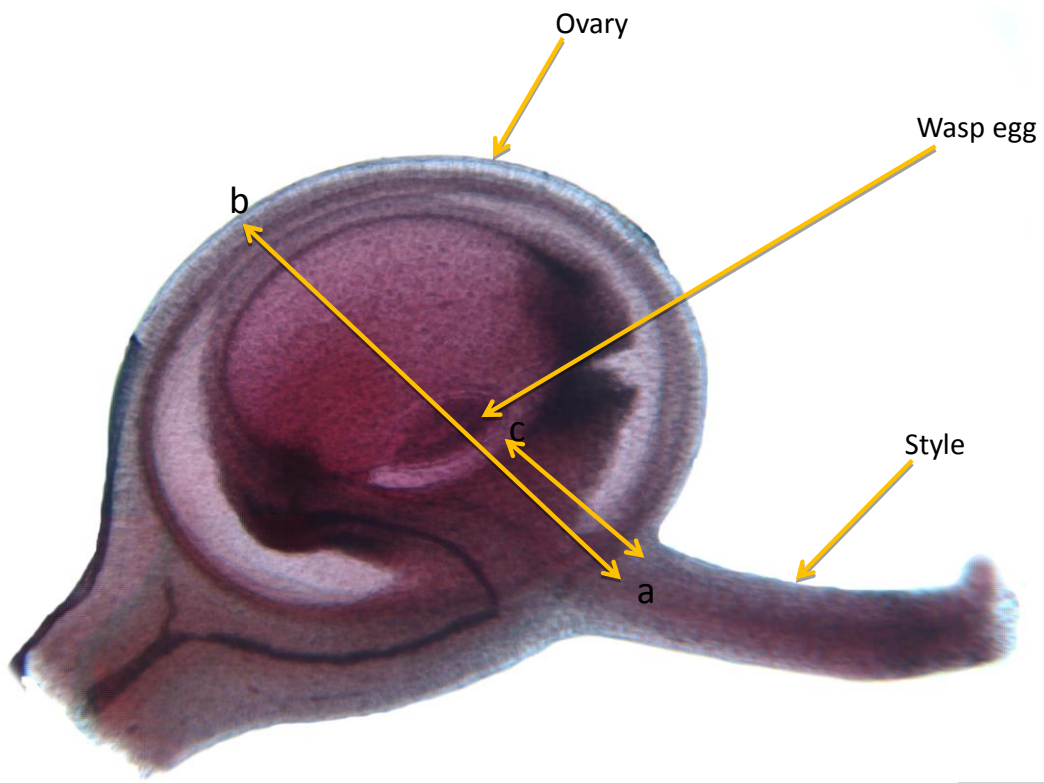


Fig. 1

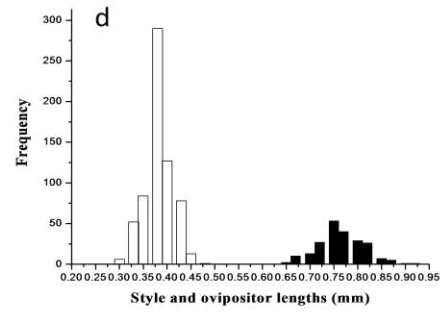
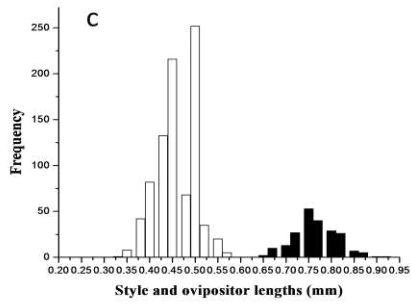
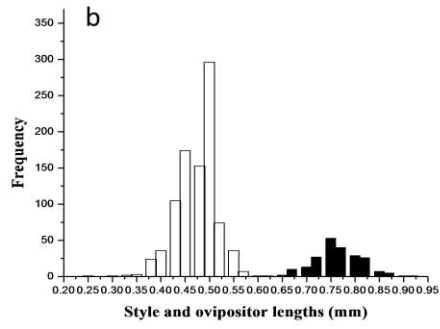
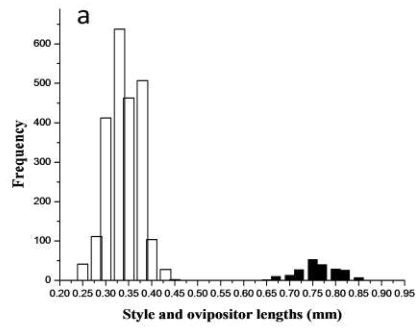


Fig. 2

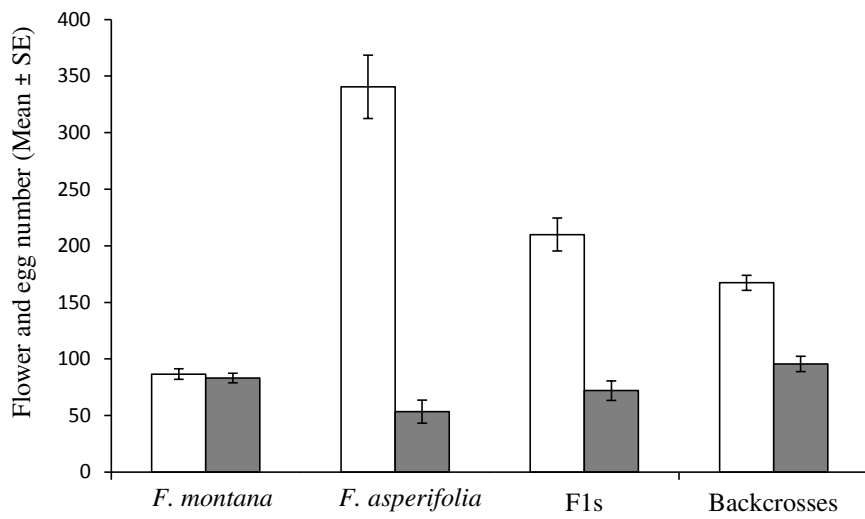


Fig. 3

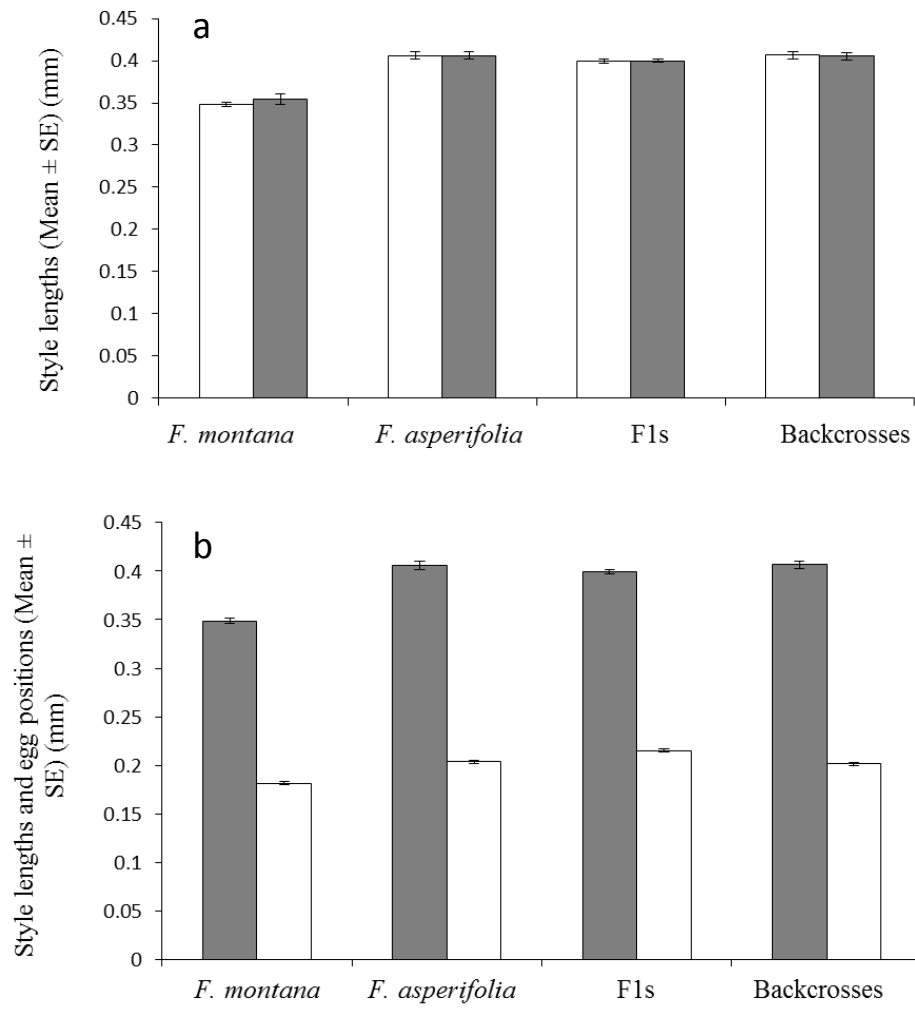
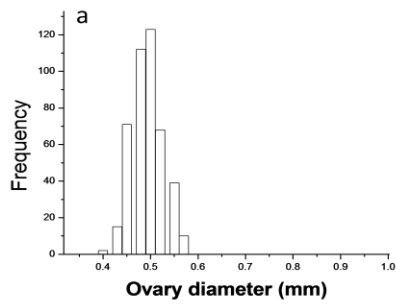
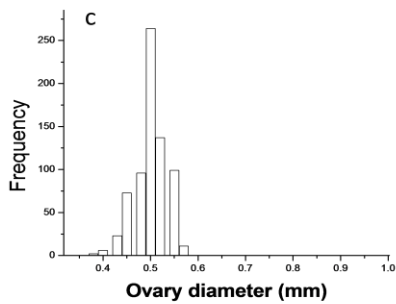
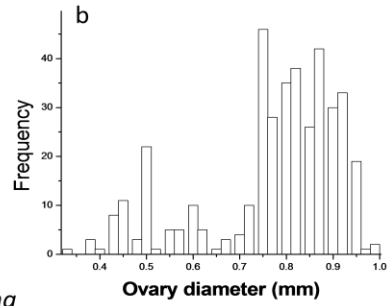


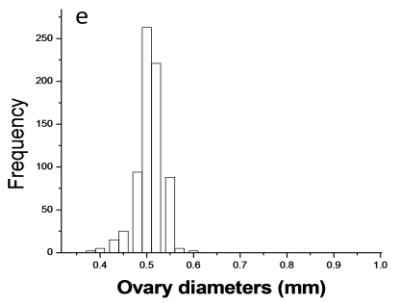
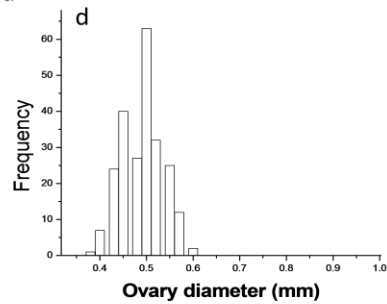
Fig. 4



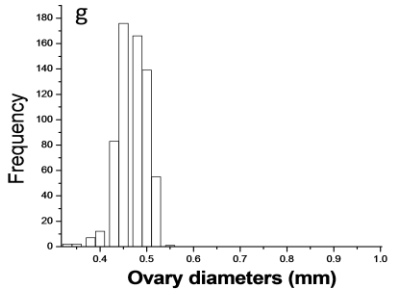
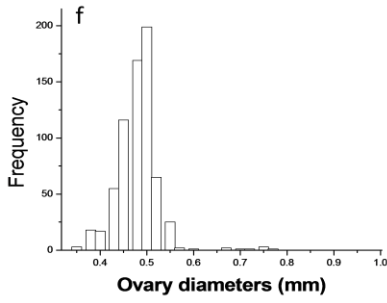
F. montana



F. asperifolia



F1s



Backcrosses

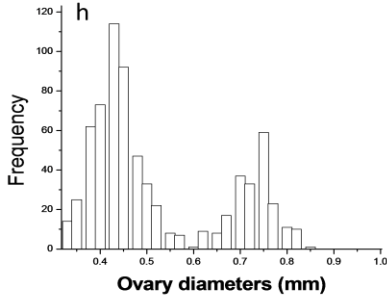


Fig. 5

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Supporting Information

Fig. S1 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.

