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2 Running title: Active pollination in a dioecious *Ficus* species

3 Active pollination of in a functionally dioecious *Ficus* species: an interplay between
4 pollinator behaviour and floral morphology

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27 Submit:

28 **Special volume Ecology and evolution of plant-pollinator interactions: the importance of**
29 **natural history**

30 **Highlights 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet**
31 **point**

- 32 ● In male figs Agaonid wasps deposit a single pollen grain each time they oviposit.
- 33 ● Stigma shape and pollen transfer to the hypopygium ensure very precise pollination.
- 34 ● In female figs wasp behaviour is less precise than in male figs.
- 35 ● In female figs exposed stigmatic papillae and synstigma ensure efficient seed set.
- 36 ● Plant morphology stabilises the mutualism.

37 **ABSTRACT**

38 Active pollination has evolved four times in brood site pollination mutualisms. When
39 pollination is active, the pollinators present behaviours specifically evolved to ensure flower
40 fertilisation. In functionally dioecious *Ficus* species, the inflorescences of functionally male
41 trees (male figs) present pistillate flowers that host pollinating wasp larvae instead of seeds.
42 The wasps cannot breed in the inflorescences of female trees (female figs), because the styles
43 are there longer than the wasp ovipositor and no egg is laid. Here we investigate the dioecious
44 *Ficus hispida*. We show that in functionally male figs, every time the wasp has laid an egg into
45 a pistillate flower, it removes one pollen grain from its pollen pockets and places it on the
46 hypopygium. When the wasp inserts its ovipositor into the next flower, the pollen grain is
47 deposited deep within the style. Each ovipositor insertion results in flower pollination and insect
48 oviposition. Because of systematic pollination of the flowers into which the eggs are laid, the
49 wasp larvae benefit from feeding on fertilized endosperm while no seed are produced. In female
50 figs, after probing a flower, the wasp presents pollination behaviour only every five visits.
51 However, if it does occur, this behaviour lasts longer than in male figs and results in the
52 deposition of on average 10 pollen grains on the hypopygium. The exposed sticky papillae on
53 the stigmatic surface collect pollen from the hypopygium and pollen tubes may grow to
54 neighbouring stigmas, ensuring secondary dispersal and efficient ovule fertilisation. Overall,
55 our study demonstrates that the floral morphology of male figs facilitates precise pollen
56 deposition, beneficial for the wasp progeny, while the floral morphology of female figs
57 compensates for wasp pollination behaviour that is not selected in those figs. We conclude that

58 the morphology of the arena in which interactions with its pollinator are played out is the result
59 of selection on the plant to maximize its male and female fitness. Incidentally this morphology
60 stabilises the mutualistic interaction.

61 **Keywords:** Mutualism, Active pollination, *Ficus hispida*, Coevolution, Dioecy

62 1. Introduction

63 In brood site pollination mutualisms *sensu stricto*, pollinating insects breed in plant
64 reproductive structures and their offspring feed on developing ovules or seeds (Hembry and
65 Althoff, 2016). Female insect traits and behaviours that facilitate the formation of the seeds on
66 which its offspring will feed should be favoured by natural selection. Nevertheless, active
67 pollination, defined as a set of behaviours of female insects specifically evolved to ensure plant
68 ovule fertilisation (Galil and Eisikowitch, 1974), has only been reported in five insect lineages
69 (Nunes et al., 2018; Pellmyr et al. 2020). Each of these cases of active pollination presents
70 distinctive natural history features. A fine understanding of variation in natural history among
71 cases may help uncover why they present contrasted patterns of diversification.

72 One of these five cases is parasitic. The larva of the pollinating *Montella* weevil
73 (Curculionidae, Baridinae) consumes all the seeds the female weevil has fertilised in the flowers
74 of the orchid *Diachaea cogniauxiana* (Orchidaceae) (Nunes et al., 2018). The four other cases
75 of active pollination are mutualistic. One, between the host specific moth *Upiga virescens*
76 (Pyralidae, Lepidoptera) and the Senita cactus *Lophocereus schottii* (Cactaceae), is facultative
77 for the plant. It involves specialist and non-specialist plant individuals (Holland and Flemming,
78 1999). This facultative association has not diversified.

79 The three other associations are obligate and involve specialised insects and specialised
80 plants. They constitute species-rich radiations (Pellmyr et al., 2020). They include species of
81 *Tegeticula* and *Parategeticula* moths (Prodoxidae) pollinating *Yucca* and *Hesperoyucca*

82 (Agavaceae) (Pellmyr, 2003); *Epicephala* moths (Gracillariidae) that have established
83 mutualistic but also antagonistic interactions with several lineages of Phyllantaceae
84 (Euphorbiaceae) (Kawakita and Kato, 2009); and Agaonidae wasps (Chalcidoidea) that have
85 codiversified with *Ficus* (Moraceae) (Cruaud et al., 2012). In these specialised mutualistic
86 interactions, pollination involves pollinator morphological adaptation and behaviour, but also
87 plant adaptations such as pollen presentation (Galil and Eisikowitch, 1974; Pellmyr, 2003),
88 hidden stigmas (Jousselin and Kjellberg, 2001; Kato et al., 2003; Pellmyr, 2003) and reduced
89 pollen/ovule ratios (Pellmyr et al., 2020). These plant adaptations preclude pollination by
90 generalist pollinators.

91 Actively pollinating insects are often presented as parasitic on seeds, and active pollination
92 as a behaviour evolved by the insects to fertilise the seeds their offspring will consume (e.g.
93 Kato et al., 2003). While this is the case for the parasitic *Montella* weevil, selection to fertilise
94 more seeds than the pollinator larvae will consume is pervasive in the mutualistic interactions.
95 For instance, in *Glochidion acuminatum* Müll.Arg., an *Epicephala* larva usually consumes two
96 seeds, while the moth deposits 8 pollen grains fertilising on average 5 of the flower's 6 ovules
97 (Kato et al., 2003). This observation suggests that there may be selection on female moths to
98 fertilise more ovules than their larva will consume. In what is probably a similar biological
99 situation, *Yucca* abort many developing flowers, selectively retaining those that contain the
100 most seeds. To reduce flower abortion, individual moths are selected to fertilise more seeds than
101 their offspring will consume (Huth and Pellmyr, 2000).

102 In the four active pollination systems just described, an individual plant does not benefit

103 from breeding pollinator larvae. Indeed, the insects do not preferentially disperse pollen from
104 the plant on which they developed (e.g. Addicot et al., 1990). Therefore, an individual plant
105 that would kill pollinator larvae would preserve some of its seeds from larval consumption
106 without affecting its pollen dispersal. The case of Agaonid wasps and *Ficus* is strikingly
107 different. Agaonid wasp larvae develop in galled ovules of pistillate flowers inside figs (the
108 closed urnshaped inflorescences of *Ficus*). The wasps become adult in the fig and get loaded
109 with pollen before leaving their natal fig. The wasps borne within a fig are the sole dispersers
110 of its pollen. Hence, a pistillate flower may contribute to the plant's male function by hosting a
111 wasp larvae, *i.e.* a pollen disperser, or, it may contribute to the plant's female function by
112 producing a seed (Anstett et al., 1997). Therefore, fig trees are selected to favour Agaonid
113 wasp development in some of their pistillate flowers.

114 In about half of *Ficus* species, every fig contains both pistillate flowers that develop into
115 seeds and pistillate flowers that develop into galls from which pollen dispersing wasps will
116 emerge. These species are monoecious.

117 In other *Ficus* species, some trees bear figs whose pistillate flowers never host a pollinator
118 larva and those figs do not produce pollen. These trees are female. The other trees bear figs
119 whose pistillate flowers either develop into a gall containing a wasp larva or remain sterile.
120 Such figs also contain staminate flowers. These plants produce pollen vectors and pollen, but
121 no or almost no seed. Hence, these plants, despite containing numerous pistillate flowers, are
122 functionally male. While morphologically gynodioecious, these species are functionally
123 dioecious (Jousselin and Kjellberg, 2001). Active pollination of functionally dioecious plant

124 species is unique to the Agaonid wasp-*Ficus* interaction.

125 Agaonid wasps oviposit by inserting their ovipositor through the style of a pistillate flower.
126 They deposit a single egg within the plant ovule between the inner integument and the nucellus.
127 In functionally male figs the styles of the pistillate flowers are shorter than the wasp ovipositors
128 allowing access to the locus of egg deposition. In female figs the styles are longer than the wasp
129 ovipositors and no egg is laid (Jousselin and Kjellberg, 2001). In monoecious *Ficus* species,
130 style lengths are variable and the wasps preferentially oviposit in shorter styled pistillate flowers
131 (Nefdt and Compton, 1996). The agaonid wasp larva develops within the ovule, feeding first
132 on the nucellus and later on galled endosperm (Jansen-González et al., 2012). The development
133 of the endosperm may be initiated parthenogenetically by the wasp larva, or by double
134 fertilisation (Borges and Kjellberg, 2014). If wasp larvae develop better in fertilised ovules,
135 ovipositing females may be selected to pollinate actively (Jousselin et al., 2003). Nevertheless,
136 a major selective force favouring the maintenance of active pollination in monoecious *Ficus*
137 species, is that actively pollinated figs that contain numerous seeds are less prone to abortion
138 and provide more nutrients to the wasps than figs containing few or no seeds (Jandér et al.,
139 2012; Jandér and Herre, 2016; Jandér et al., 2016; Sun and Wang, 2019). Hence, in all
140 specialised monoecious mutualistic active pollination systems, pollinator offspring seem to
141 benefit from the initiation by their mother of seeds that their larvae will not feed on.

142 Within this general context, active pollination of functionally dioecious *Ficus* species
143 stands apart. Indeed, Agaonid wasp larvae develop in galled ovules in male figs which produce
144 no seeds (Anstett et al., 1997). Therefore, they cannot benefit from seed development within

145 the fig. Data on three actively pollinated dioecious species, *F. hispida* L.f., *F. fistulosa* Reinw.
146 ex Blume, and *F. fulva* Reinw. ex Blume, show that wasp larvae develop better when their
147 mother carried pollen (Jousselin and Kjellberg 2001, Zhang et al., 2019). Further, data on *F.*
148 *fistulosa* and *F. fulva* show that in male figs only pistillate flowers that receive an egg are
149 pollinated (Jousselin and Kjellberg, 2001). These results suggest 1) that female wasp have been
150 selected to only pollinate flower into which they oviposit and 2) that wasp larvae benefit from
151 the induction of endosperm development through the double fertilization. In contrast, in female
152 figs, where no egg is laid, there is no correlation within a fig between pollination of a pistillate
153 flower and oviposition attempt, i.e. introduction of the ovipositor into the style of the flower
154 (Jousselin and Kjellberg, 2001).

155 This difference in pollen deposition pattern suggests that female wasps behave differently
156 in male and female figs. Nevertheless, Galil (1973) did not observe any difference in wasp
157 pollination behaviour between the two types of figs in *F. fistulosa*. To resolve this discrepancy,
158 we investigate here more closely oviposition and pollination by *Ceratosolen solmsi marchali*
159 Mayr in male and female figs of *F. hispida* at the Xishuangbanna Tropical Botanical Garden in
160 southern Yunnan, China

161

162 **2. Materials and methods**

163 *2.1. Wasp behaviour*

164 Wasp behaviour was carefully observed, recorded on video and described. Pollinator-free

165 receptive figs were obtained by bagging figs before their pistillate flowers become receptive to
166 pollen. At fig receptivity, one female wasp freshly emerged from a ripe fig was manually
167 deposited on each receptive fig. When the wasp had entered the fig, the fig was cut in half, and
168 the fig cavity of the half containing the wasp was sealed with a glass slide attached with
169 adhesive tape (Fig. S1). The fig was kept in the dark for 5 min, and then the wasp's behaviour
170 was recorded under a stereoscopic microscope, under LED illumination.

171 Wasp behaviour was then quantified. For this purpose, we defined three successive
172 behaviours. Oviposition represents the behaviour beginning with the insertion of the ovipositor
173 into a style until the onset of pollination behaviour, or when pollination behaviour was missing,
174 until the removal of the ovipositor from the style. Pollination was defined as lasting from the
175 first leg movement to the pollen pockets until the removal of the ovipositor from the style.
176 Searching was the interval separating the removal of the ovipositor from the style until the next
177 insertion of the ovipositor. The behaviour of 11 wasps in figs of each sex was observed. Each
178 wasp was observed for 30 oviposition bouts in the first two hours after entering the fig. All
179 observations were performed between 8:30 and 10:30 in the morning during June and July 2017.

180

181 *2.2. Oviposition rate and pollen deposition rate*

182 In order to estimate oviposition rate and pollen deposition rate, either one or two female
183 wasps were introduced per receptive fig using the same technique as above. Wasps were
184 dissected prior to entry into a fig and at intervals of 2h, 6h, 12h and 24h after fig entry,. The

185 number of unlaidd eggs was counted following Dunn et al. (2011). Pollen was extracted from
186 the pollen pockets by sonication for 20s in an ultrasonic-wave cleaning machine (SB5200) in
187 0.5 ml of 75% EtOH. Samples were subsequently diluted into 20 ml of 2% NaCl, homogenised
188 by sonication, and pollen grains were counted using a particle counter (Elzone II 5390, Norcross,
189 Georgia, USA) (Ashman and Hitchens 2000).

190 *2.3. Presence of eggs in flowers and pollen tube germination on the stigmas*

191 Wasps caught flying around receptive trees were introduced into receptive figs as
192 described above. When all wasps had entered, the figs were re-bagged. Twenty-four hours later,
193 the figs were collected and preserved in FAA (5 ml formalin, 5 ml glacial acetic acid, 90 ml
194 50 % ethanol) for later dissection. The figs were cut into 8 sections along the ostiole-peduncle
195 axis. All the pistillate flowers of one section were placed on glass slides with a droplet of cold
196 distilled water and then dissected under a 40× magnification microscope to assess the presence
197 and number of eggs in each ovule. The flowers from another section were observed to check
198 for the presence of pollen grains and pollen tubes on the stigmas. These flowers were prepared
199 using different concentrations of NaOH, stained with 0.01% aniline blue in 0.1 M K₃PO₄ for
200 48h, then squashed under a cover glass and observed under an epifluorescence microscope
201 (Axio Imager A2) (Jousselin and Kjellberg, 2001).

202 *2.4. Statistical analyses*

203 Statistical analyses were performed in R (v. 3.3.3), and figures were plotted with the
204 package ggplot2.

205 The durations of the successive behaviours (oviposition, pollination, searching)
206 constituting a behavioural sequence were compared between wasps that had entered male and
207 female figs by fitting linear mixed-effect models. The duration of behaviour was set as the
208 response variable, and ‘fig sex’ (two levels, female and male) and ‘behaviour type’ (three levels,
209 searching, oviposition, pollination) were set as fixed effects, whereas wasp individuals were
210 coded as random effects, using the package of lmerTest, multcomp. For each behaviour type,
211 we used Tukey contrast tests for multiple comparisons among the durations of the different
212 behaviours under the different experimental conditions.

213 To investigate the rhythm of pollen deposition, we fitted a linear model with the number
214 of pollen grains left in the pollen pockets as the response variable, ‘time’ (continuous variable),
215 ‘the number of wasps in the fig’ (two levels, one and two), and ‘fig sex’ (two levels, male or
216 female figs) as fixed effects. We used a backward strategy for the model selection followed by
217 a Tukey multiple comparison. The same strategy was used to analyse the decrease of the number
218 of unlaidd eggs over time.

219 **3. Results**

220 *3.1. Oviposition and pollination behaviour in male and female figs*

221 In male figs, the wasp moved around in the fig cavity, inserted its ovipositor deep into a
222 style so that the hypopygial mucro (the spine-like extension of the hypopygium) was also
223 inserted into the style, and oviposited or attempted to oviposit. Before removing the ovipositor
224 from the style, the wasp lifted the forepart of its body at a 45-degree angle, and made a series

225 of movements with its forelegs, apparently removing some pollen from the pollen-pockets with
 226 the extremity of the tarsi (Figs. 1A, 1B). The wasp then took hold with all six legs on the stigmas
 227 and pushed its body away from the fig stigmatic surface, extracting the ovipositor from the style.
 228 Immediately after, the wasp repeatedly passed its fore tarsi on the hypopygial mucro. The wasp
 229 then moved on in search of the next flower into which it introduced its ovipositor (Video S1).
 230 In male figs, all except one of the 330 observed oviposition sequences included the full set of
 231 behaviours. In female figs, the pollination behaviour was entirely missing in 260 out of 330
 232 sequences (79%)(Video S2).

233 **Table 1.** Time spent by pollinators on each behaviour in seconds.

Behaviour	In female figs		In male figs		Significance
	Range	Mean \pm SD	Range	Mean \pm SD	
Searching	2-152	17.66 \pm 15.14	3-55	14.07 \pm 8.16	P<0.05
Ovipositing	3-78	13.36 \pm 7.37	2-33	8.64 \pm 4.37	P<0.01
Pollinating ¹	0-96	3.60 \pm 11.95	0-32	10.62 \pm 4.80	P<0.001
Pollinating ²	1-96	17.00 \pm 12.51	1-32	10.66 \pm 4.80	P<0.001
Total time	10-164	34.48 \pm 21.52	17-79	33.33 \pm 9.94	NS

234 Note: Pollinating¹: with zeros; Pollinating²: without zeros.

235 In female figs, pollinators spent more time attempting to oviposit than pollinating ($P <$

236 0.001, mean durations given in Table 1). In male figs, they spent more time pollinating than
237 ovipositing ($P < 0.05$): pollination behaviour represented one-third of the total duration of the
238 behavioural sequence, against one-tenth in female figs (Table 1). However, as the duration of
239 the oviposition attempt and duration of the search for a flower were longer in female figs, the
240 total duration of a behavioural sequence was similar in male and female figs. When the
241 pollinators in female figs presented the pollination behaviour, it lasted longer than in male figs:
242 each individual pollination behaviour lasted 60% longer in female figs than in male figs.

243 *3.2. Decrease over time of the number of unlaidd eggs*

244 Pollinators that had not yet started ovipositing presented on average 221.7 ± 34.2 (mean \pm
245 SD, N=98) unlaidd eggs. At the end of the behavioural sequence, the number of unlaidd eggs was
246 much larger for wasps that had entered female figs than for those that had entered male figs,
247 both for single-wasp figs ($P < 0.001$) and for two-wasp figs ($P < 0.001$). Indeed, the number of
248 unlaidd eggs decreased over time in male figs, whereas it remained constant over time in two-
249 wasp female figs and markedly decreased only after 24 hours in one-wasp female figs (Fig. 2).

250 *3.3. Decrease over time in the number of pollen grains remaining in the pollen pockets*

251 Before entering a fig, the wasps carried on average 592.5 ± 221.4 (mean \pm SD, N=82)
252 pollen grains in the pollen pockets. The number of wasps in a fig did not affect the variation
253 over time in the number of pollen grains remaining in the pollen pockets ($P = 0.89$), whereas
254 fig sex ($P < 0.001$), oviposition duration ($P < 0.001$), and their interaction ($P < 0.05$) affected
255 this number. In both female and male figs, the number of pollen grains remaining in the pockets

256 decreased over time (Fig. 3). However, the decrease was steeper in female figs than in male figs
257 ($P < 0.001$ for both one-wasp and two-wasp figs).

258 *3.4. Presence of eggs in flowers and pollen tube germination on stigmas*

259 A total of 2,883 pistillate flowers from 22 figs from two male fig trees were dissected. In
260 every fig over 50% (range 57.1%-95.6%) of the ovules contained an egg (Fig. 4A), and 1-5
261 wasps were found within the fig cavity. Two or more eggs were recorded in 127 ovules (5.6%
262 of the ovules that had received an egg), within 18 figs. In contrast, 2,466 pistillate flowers of
263 16 figs from two female fig trees were dissected, and none of the ovules contained an egg, even
264 though 3-5 wasps were found inside the fig cavities.

265 At all concentrations of NaOH (2, 4, 8 mol/l), the treated styles became dark, so that only
266 pollen tubes growing superficially on the stigmatic surface could be observed. We did not
267 observe any superficial pollen tube on the 666 examined pistillate flowers from male figs, while
268 we observed pollen tubes on 453 out of 600 (68%) flowers from female figs and often several
269 pollen tubes were observed per stigma (Fig. 4B). To confirm that pistillate flowers were
270 fertilised in male figs, we dissected some flowers and observed them with the fluorescence
271 microscope: in many flowers, a pollen tube was observed entering the ovule (Fig. 4C).

272 4. Discussion

273 The study presented here is the first to describe and quantify a succession of behavioural
274 sequences associated with active pollination. There is no published data on the frequency at
275 which oviposition is directly followed by pollination behaviour in any *Ficus* species. It is the
276 first study showing that the frequency of pollination behaviour and the actual pollination
277 behaviour differ between male and female figs.

278 In *F. hispida* male figs, the wasps transfer pollen from the pollen pockets to the fore tarsi
279 and then from the tarsi to the tip (the mucro) of the hypopygium. At the next oviposition attempt,
280 when the hypopygium which stabilises the ovipositor is inserted into the funnel shaped style,
281 pollen is deposited deep inside the styles, on the hidden stigmatic papillae. As a result, in male
282 figs, pollen does not germinate at the apex of the style, but deep within the styles of flowers
283 into which the wasp's ovipositor has been inserted. This is why we could not visualise any
284 pollen tubes on the stigmatic surface of flowers in male figs. This behaviour explains previous
285 data on two other actively pollinated *Ficus* species showing a strong association in male figs
286 between oviposition in the ovule and flower pollination (Jousselin and Kjellberg, 2001).

287 Pollen deposition on the hypopygium has been reported for two species of *Pegoscapus*
288 pollinating monoecious *Ficus* species (Frank, 1984). This observation suggests that similar
289 wasp pollination behaviour may occur in monoecious and dioecious *Ficus* species. However,
290 other descriptions of active pollination in dioecious and monoecious figs did not report pollen
291 deposition on the hypopygium (e.g. Galil, 1973; Galil and Eisikowitch, 1974). Images in a film
292 of a species of *Courtella* pollinating the monoecious *F. ottoniifolia* (Miq.) suggest that the wasp

293 deposits pollen directly on the stigmas (Michaloud, 1982). Comparative studies including video
294 recordings of a succession of behavioural sequences are required to establish how pollination
295 behaviour varies among Agaonid wasp species.

296 For wasps ovipositing in male figs, the decrease in egg number over time was numerically
297 close to the decrease in the number of pollen grains, suggesting that each time a wasp lays an
298 egg, it deposits a single pollen grain. As pollen grains in *Ficus* measure only about 10 μm (Wang
299 et al., 2014), this is a remarkable feat. Further, this suggests that the wasps generally avoid
300 inserting their ovipositor into flowers that have already received an egg. Else, more pollen
301 grains than eggs would be deposited. In support of this inference, we only observed one type of
302 behavioural sequence in male figs, and not two, as would have been expected if some ovipositor
303 insertions did not lead to oviposition. Hence, in male figs, the pollen deposition and oviposition
304 behaviour lead to systematic pollination of oviposited flowers and lack of pollination of flowers
305 that do not contain an egg. As a consequence, no seeds are produced in male figs.

306 Active pollination behaviour is costly in *C. solmsi marchali*. It represents one-third of the
307 insect's time budget within male figs, and the wasps spend more time pollinating than
308 ovipositing. Comparatively, in *Yucca* moths, the time spent depositing pollen is equivalent to
309 only 6.7 % of the time spent ovipositing (Pellmyr, 1997). Such a large time investment is
310 beneficial for the wasp. Indeed, at our study site, lack of ovule fertilisation in *F. hispida* resulted
311 in a reduction of brood size of over 50% resulting from fig abortion and wasp larval mortality
312 (Zhang et al., 2019). Such a large investment is unlikely to be sustainable in situations where
313 several wasps compete within a fig for oviposition sites (as is generally the case in *F. hispida*,

314 Liu et al., 2013), unless individual wasp larvae benefit directly from the fertilisation of the
315 flower in which they develop by their mother. This should be confirmed by direct experiments
316 as in Jandér et al. (2012). Our results further confirm that generally a single egg is laid per
317 flower, even when wasps compete for oviposition sites (Jousselin et al., 2001; Ghana et al.,
318 2012). We also confirm that no egg is laid in pistillate flower ovules in female figs. This is
319 probably because the wasp ovipositor is too short to reach the locus of egg deposition in the
320 long styled pistillate flowers of female figs (Jousselin and Kjellberg, 2001). The decrease in
321 number of unlaied eggs contained in the body of single foundresses after 24 hours may be
322 explained either by egg resorption or by females that have reached the end of their life span
323 after many failed oviposition attempts, losing control and releasing eggs into the fig cavity. The
324 decrease in eggs was not observed in figs entered by two foundresses. Further observations will
325 be necessary to detect whether the onset of the decrease in the number of unlaied eggs is delayed
326 in two foundress figs.

327 The behaviour associated with pollination is less streamlined in female figs than in male
328 figs. First, after an oviposition attempt, the pollination behaviour is not expressed in 80% of
329 cases. This may be due to the wasp not depositing eggs in the flower ovules in female figs. In
330 the behavioural sequence in male figs, the removal of a pollen grain from the pollen pockets
331 and its deposition on the hypopygium immediately follows oviposition. Lack of oviposition
332 likely disrupts the behavioural sequence. However, when pollination behaviour was expressed
333 in female figs, it lasted much longer than in male figs. This suggests that the very high precision
334 of this behaviour expressed in male figs could be affected by failure to oviposit. Indeed, the

335 number of pollen grains remaining in the pollen pockets decreased twice as fast in female figs
336 as in male figs. This suggests that each time pollination behaviour was expressed in female figs,
337 about 10 pollen grains were removed from the pockets and placed on the hypopygial mucro,
338 instead of one pollen grain in male figs. Hence, wasp pollination behaviour is very precise in
339 male figs, where it will strongly contribute to the successful development of its offspring. Wasp
340 behaviour is less precise in female figs where it has no incidence on offspring development as
341 no egg is laid, and where it is hence not submitted to selection.

342 We hypothesise that when the wasp moves around in the cavity of female figs, pollen
343 grains located on the hypopygium contact and adhere to the sticky papillae present on the
344 surface of the stigmas, as proposed for two actively pollinated monoecious *Ficus* species (Frank,
345 1984). This mechanism would explain the low correlation in female figs between introduction
346 of the ovipositor into a style and pollination that was observed in *F. fistulosa*, a close relative
347 of *F. hispida* (Jousselin and Kjellberg, 2001). Pollen deposition in female figs is less precise
348 than in male figs as evidenced by multiple pollen tubes growing on individual stigmas (Fig. 4B;
349 Jousselin and Kjellberg, 2001). However, somewhat clumped pollen deposition may be
350 compensated by secondary dispersal of pollen tubes growing on the stigmatic surface to reach
351 neighbouring flowers. This is enabled by the presence of a synstigma in female figs of actively
352 pollinated species (Jousselin and Kjellberg, 2001; Teixeira et al., 2018). Pollen deposition in
353 female figs results in efficient flower fertilisation. Indeed, we observed pollen tubes on 68% of
354 flower stigmas in female figs and previous observations have documented that female figs of *F.*
355 *hispida* produce about 1900 seeds per fig for about 2500 pistillate flowers (Liu et al., 2013;

356 Peng et al., 2005).

357 The foundresses used in our experiments contained on average 213 eggs and carried 592
358 pollen grains. In another set of experiments, introductions of single foundresses into *F. hispida*
359 figs resulted in the production of circa 800 seeds (Liu et al., 2013). The wasps used in those
360 experiments were larger than the ones in our experiments as they contained on average 306 ± 48
361 (N=100) eggs (Liu C. unpublished data), and therefore they probably also carried more pollen
362 grains. Nevertheless, this data suggests remarkable fertilisation efficiency with close to one
363 seed initiated per pollen grain.

364 Hence, while the pollination behaviour of the wasps appears *a priori* far from optimal for
365 seed set in female figs, the presence of an exposed papillate stigmatic surface and the synstigma
366 result in very efficient pollen use for ovule fertilisation. In contrast, the hidden stigmatic
367 papillae help the plant avoid seed set in male figs, as only flowers into which the ovipositor is
368 inserted are fertilised. Stigma presentation in male and female figs, *i.e.* functional anatomy and
369 spatial structure, result in efficient pollen use in male and female figs.

370 The results expose an important intrinsic constraint associated with active pollination: the
371 wasp controls the number of pollen grains it carries in its pockets. While female figs of *F.*
372 *hispida* contained about 2500 pistillate flowers (Peng et al., 2005), each pollinator only carried
373 about 600 pollen grains. Hence, when a single pollinator enters a female fig, seed set is limited
374 by the number of pollen grains the wasp has loaded into its pockets (Liu et al., 2013). We predict
375 that in *Ficus* species where pollinators are scarce and female figs contain many pistillate flowers,
376 the plant can be selected to force more pollen grains onto the wasps. This is the case for *F.*

377 *tikoua* Bur. Despite the pollinating *Ceratosolen* species presenting active pollination behaviour,
378 male figs produce very abundant pollen in their numerous dehiscent anthers, a set of traits
379 usually found in passively pollinated *Ficus* species (Deng et al., 2016; Kjellberg et al., 2001).
380 As a result, female wasps are abundantly dusted with pollen (Deng et al. 2016). Our
381 interpretation is that in male figs, only pollen actively deposited by the wasp deep inside the
382 style will germinate, while pollen passively loaded on the body of the wasps does not end up
383 deep in the stigmas and therefore does not fertilise ovules. On the other hand, in female figs,
384 any pollen that ends up on the surface of the synstigma will germinate and ensure ovule
385 fertilisation. Ovule fertilisation in female figs would thus result from both active pollination
386 behaviour and passive pollen transport.

387 Active pollination by genus *Ceratosolen* wasps is ancestral and generalised in subgenus
388 *Sycomorus*, to which *F. hispida* belongs (Cruaud et al., 2012, Rasplus et al., 2021). The
389 association between *Ceratosolen* and subgenus *Sycomorus* is 50-60 Ma old (Cruaud et al.,
390 2012). We propose, as a working hypothesis pending investigation of further species, that active
391 pollination has persisted for 50 Ma, and is today present in the over 100 dioecious species of
392 the subgenus, because i) floral morphology in male figs facilitates precise pollen deposition,
393 beneficial for the wasp progeny; and ii) floral morphology in female figs compensates for wasp
394 pollination behaviour that is not adapted to female figs. Dioecious fig trees, like so many other
395 large organisms involved in mutualistic interactions with small organisms, control to their
396 advantage the morphology of the arena in which the interactions with their mutualists are played
397 out, stabilising the interaction as a side effect.

398 **CRedit authorship contribution statement**

399 Bai-Ge Miao: Investigation, Validation & Writing—original draft. Ming-Xin Liu: Investigation
400 & Validation. Bo Wang: Formal analysis. Yan-Qiong Peng: Resources, Supervision, Project
401 administration & Funding acquisition. Annick Lesne: Writing—review & editing, Finn Kjellberg:
402 Conceptualization & Writing—review & editing. K. Charlotte Jandér : Methodology & Writing—
403 review & editing.

404 **Declaration of Competing Interest**

405 The authors declare that they have no known competing financial interests or personal
406 relationships that could have appeared to influence the work reported in this paper.

407 **Supplementary materials**

408 Supplementary material associated with this article can be found in the online version.

409 Figure S1. Fig cut open and mounted for behavioural observation. Note the presence of an
410 ovipositing female within the fig cavity.

411 Video S1. Pollinator behaviour in receptive male figs of *Ficus hispida*

412 Video S2. Pollinator behaviour in receptive female fig of *Ficus hispida*

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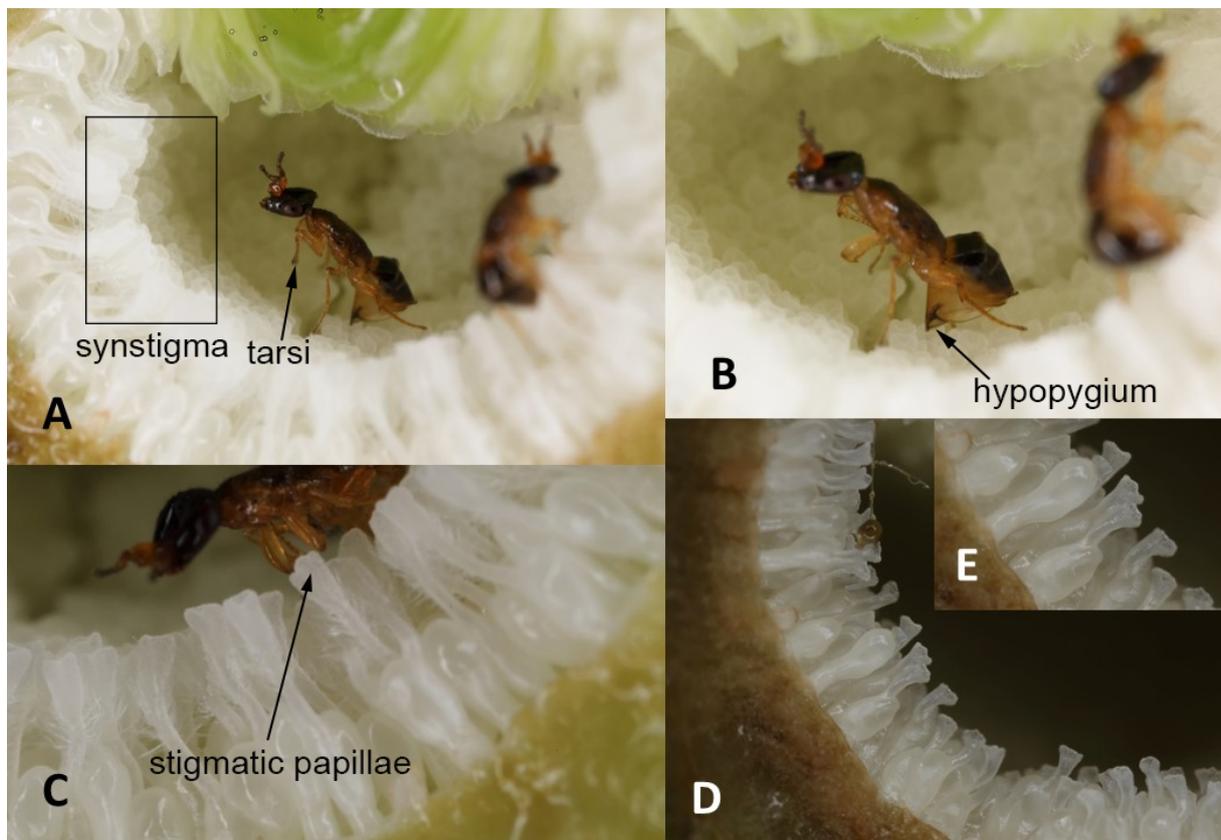
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542 **Figure captions**

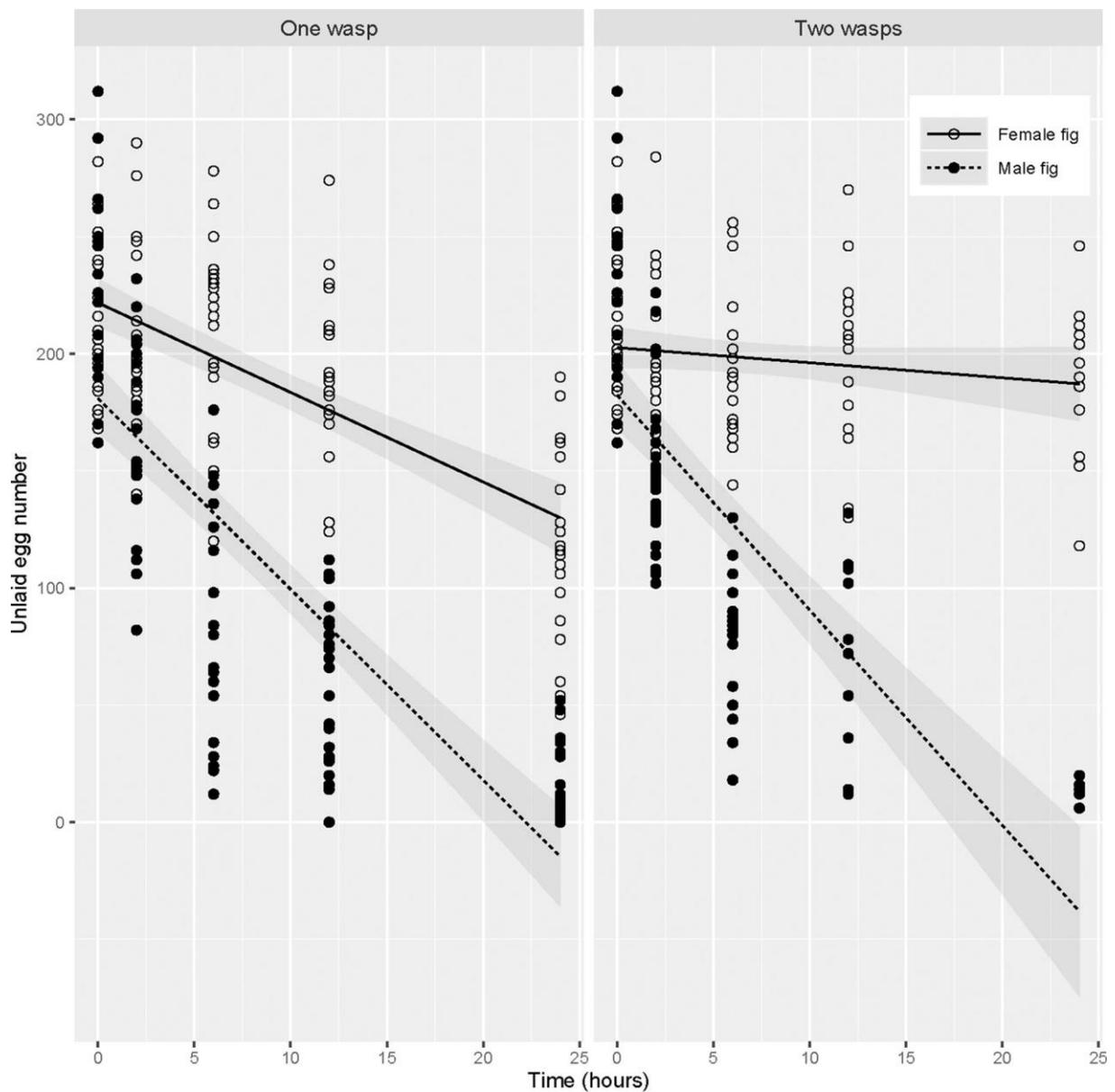
543 **Fig. 1.** Pollination and stigma structure in female (A, B, C) and male (D, E) figs. Stigmas in
544 female figs are tightly packed forming a synstigma (A), the stigmatic papillae are
545 apparent and extend down the external surface of the styles (C). Stigmas in male figs
546 are separate (D) and the stigmatic papillae are located within the stigmatic depression
547 (E). Note the upright position of the wasp collecting pollen from its pollen pockets (A,
548 B). B: the tarsi are inserted into the pockets, collecting pollen. A and B: the hypopygium
549 is the triangular piece of chitin touching the stigma. The hypopygial mucro is inserted
550 into the style.



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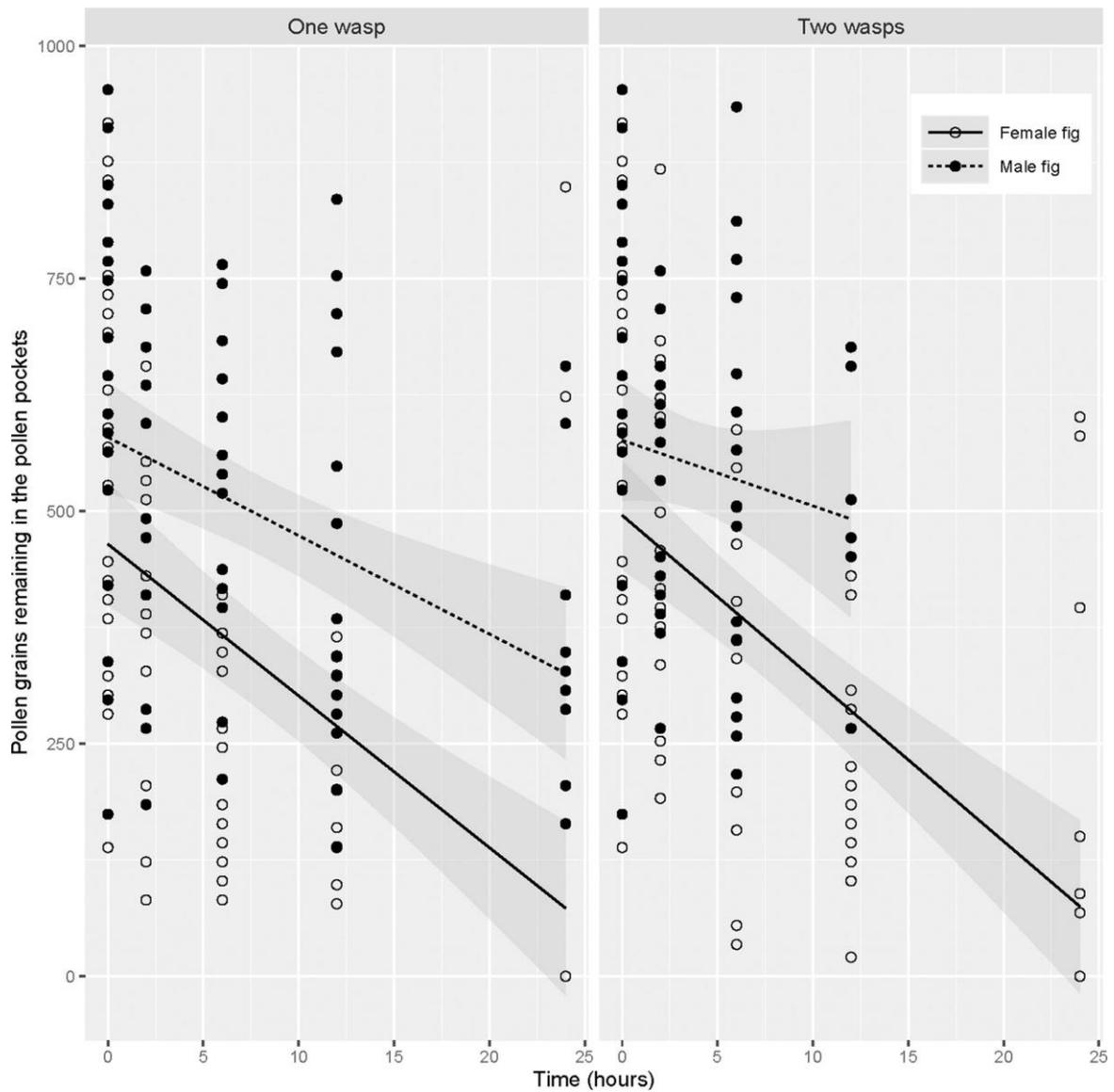
553 **Fig. 2.** Number of unlaidd eggs as a function of time spent in male figs and female figs. Linear
 554 regression lines and 95% confidence intervals are shown. *Left panel:* one-wasp female
 555 figs: $y = 222 - 3.8x$ (x : time in hours, y : egg number), $r^2 = 0.43$, $p < 0.001$; one-wasp male
 556 figs: $y = 181 - 8.15x$, $r^2 = 0.63$, $p < 0.001$. *Right panel:* two-wasp female figs: $y = 203 -$
 557 $0.6x$, $r^2 = 0.02$, $p = 0.13$, non-significant decrease; two-wasp male figs: $y = 182 - 9.2x$, r^2
 558 $= 0.56$, $p < 0.001$.



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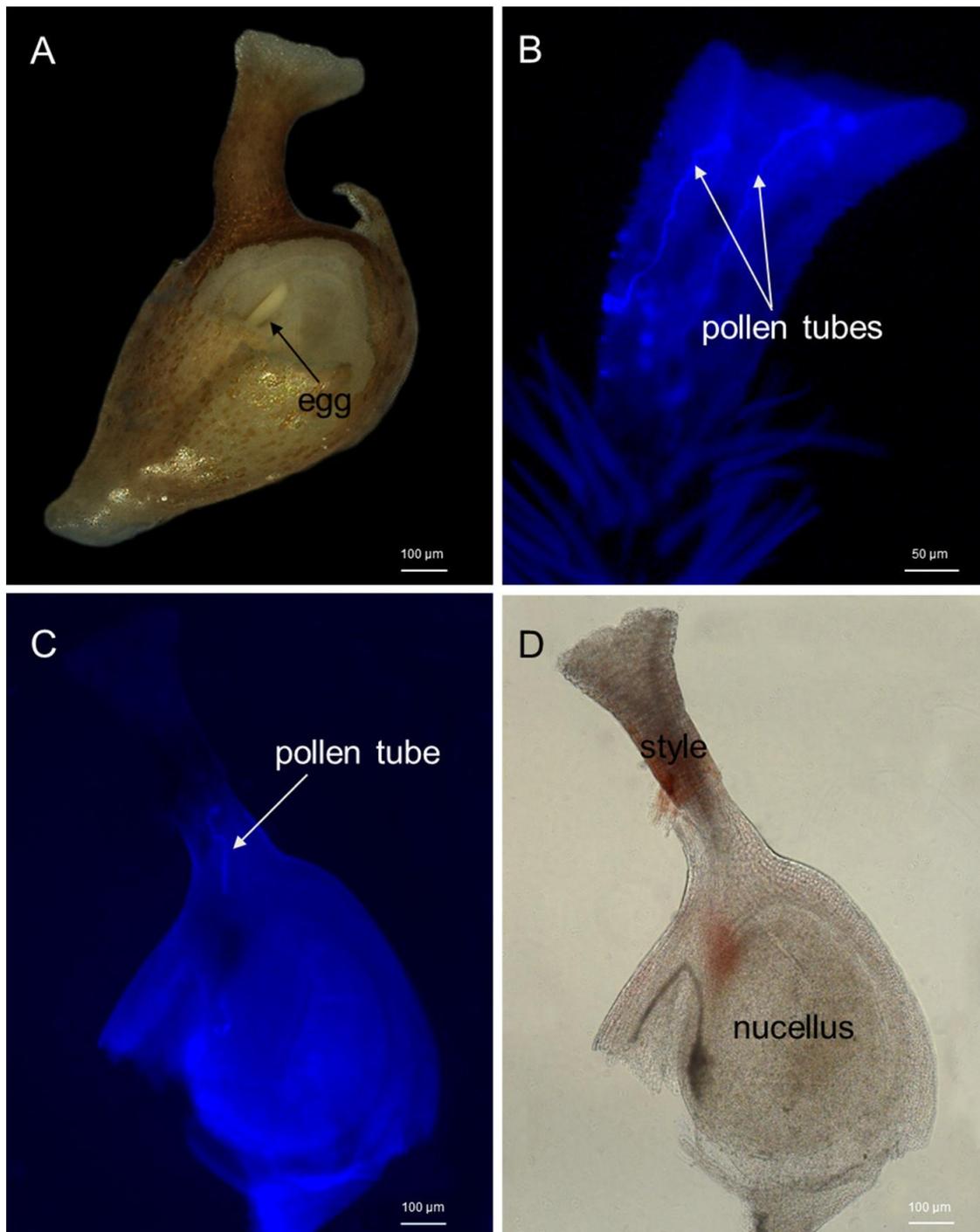
561 **Fig. 3.** Decrease over time of the number of pollen grains remaining in the pollen pockets after
 562 a wasp entered a male fig or a female fig. Linear regression lines and 95% confidence
 563 intervals are shown. *Left panel:* one-wasp female fig: $y = 465 - 16.3x$ (x : time in hours,
 564 y : pollen grain number), $r^2 = 0.32$, $p < 0.001$; one-wasp male fig: $y = 580 - 10.6x$, $r^2 = 0.17$,
 565 $p < 0.001$. *Right panel:* two-wasp female fig: $y = 496 - 17.5x$, $r^2 = 0.36$, $p < 0.001$; two-
 566 wasp male fig: $y = 576 - 7.1x$, $r^2 = 0.02$, $p = 0.24$ slope not significantly different from
 567 zero.



568

569

570 **Fig. 4.** A: a single egg is laid in a pistillate-flower ovary in a male fig. B: pollen tubes
571 growing on the externally apparent papillate zone of the style of a pistillate flower
572 in a female fig. C: pollen tube growing down the style of a pistillate flower in a
573 male fig (epifluorescence imaging); D: daylight view of the same flower as C.



574