Ant guards influence the mating system of their plant hosts by altering pollinator behaviour

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30

31 **Abstract**

32 Ant guards can increase plant fitness by deterring herbivores but may also
 33 reduce it by interfering with pollination, hence ant-plant interactions are ideal
 34 systems in which to study costs and benefits of mutualisms. While ant impacts
 35 on herbivory are well-studied, much less is known about impacts on
 36 pollinators and associated consequences for plant mating systems and fitness.
 37 We used field experiments to quantify the effect of ant guards on pollinator
 38 community composition, frequency and duration of flower visits, and
 39 cascading effects on plant mating system and plant fitness in *Turnera velutina*
 40 (Passifloraceae). Although ant patrolling did not affect pollinator community
 41 composition or visitation frequency, it decreased pollinator foraging time and
 42 flower visit duration. Such behavioural changes resulted in reduced pollen
 43 deposition on stigmas, decreasing male fitness whilst increasing outcrossing
 44 rates. This study contributes to understanding how non-pollinators, such as
 45 these defensive mutualists, can shape plant mating systems.

46

47

48 **Introduction**

49 Ant-plants are excellent systems in which to explore the costs and benefits of
50 multispecies mutualisms. While aggressive ants increase plant fitness by
51 defending their host plants from herbivores (Bentley 1977; Beattie 1985; Martin
52 & Doyle 2003), they can also decrease plant fitness disrupting plant–pollinator
53 mutualisms and repelling other plant-beneficial predatory arthropods (Koptur
54 *et al.* 2015). Ants can disrupt pollination by consuming floral structures,
55 damaging pollen (Stanton *et al.* 1999; Frederickson 2009; Stanton & Palmer
56 2011; Dutton & Frederickson 2012; Malé *et al.* 2015), or deterring flower
57 visitation by pollinators (Assunção *et al.* 2014; Villamil *et al.* 2018). Yet,
58 information on their impacts on pollinators, pollen transfer and seed set is still
59 limited, and only few studies have addressed the ecological costs of ants via
60 pollinator deterrence (Romero & Koricheva 2011).

61 Ant aggressivity may be a double-edged sword underlying the core
62 ecological costs and benefits of myrmecophily. More aggressive ants may be
63 better defenders against herbivores, but may also pose a higher predation risk
64 to other mutualistic guilds such non-ant predators of herbivores or pollinators
65 (Ness 2006; Ohm & Miller 2014; Jones & Koptur 2015; Villamil *et al.* 2018).
66 Furthermore, the metrics commonly used in quantifying the effectiveness of
67 indirect defences against herbivores – reduction in damage by herbivores – are
68 unlikely to reveal pollination-associated impacts on plant fitness (Dukas 2001;

69 Gaume *et al.* 2005; Ness 2006; Goncalves-Souza *et al.* 2008; Frederickson 2009;
70 Romero & Koricheva 2011; Stanton & Palmer 2011; Dutton & Frederickson 2012;
71 Ohm & Miller 2014; Jones & Koptur 2015; Malé *et al.* 2015) and a multispecies
72 approach is needed to improve our estimates of the net outcomes of
73 mutualistic interactions.

74 Ant impacts on pollinators may be consumptive (through predation) or
75 non-consumptive, defined as changes in prey traits or behaviours in response
76 to perceived predation risk (Preisser *et al.* 2005; Sheriff & Thaler 2014). The
77 magnitude of non-consumptive effects on pollinator and plant fitness can be
78 similar to, or higher than, that of direct consumptive effects (Preisser *et al.*
79 2005; Romero *et al.* 2011; Clinchy *et al.* 2013; Sheriff & Thaler 2014). However,
80 the mechanism(s) by which predators influence pollinator behaviour and
81 impact on plant fitness remain entirely unknown for the majority of ant-plants
82 (Romero & Koricheva 2011).

83 The few studies on the effects of ant patrolling on pollinator behaviour
84 suggest that ants can have positive or negative consequences for plant fitness.
85 For example, lower seed set in *Ferocactus wislizenii* plants tended by
86 aggressive ants was attributed to a three-fold reduction in pollinator visitation
87 frequency (Ness 2006). However, this hypothesis was not experimentally
88 tested. Alternatively, an increase in fruit set in ant-patrolled plants of
89 *Psycotria limonensis* was attributed to pollinator relocation, where ant
90 threats might have caused pollinators to spend less time per flower and visit

91 more flowers, promoting pollen transfer (Altshuler 1999). Again, this
 92 mechanism was inferred, but not experimentally tested. Previous experiments
 93 on *Turnera velutina* showed that ant corpses placed inside flowers reduce
 94 pollinator visit duration (Villamil *et al.* 2018). However, such an experimental
 95 setup may differ from natural circumstances as flower occupation by ants is a
 96 rare event, and live ants (in contrast to dead ones) do not remain immobile in
 97 the flowers for long periods. Overall, the presence of ants can promote
 98 changes in pollinator community composition, visit frequency and duration.
 99 This, in turn, could drive positive or negative impacts of ant-pollinator
 100 interactions on plant reproduction (Altshuler 1999; Ness 2006). To date, no
 101 study has quantified the impacts of ant patrolling on pollinator visitation
 102 behaviour, plant mating systems, and fitness under natural conditions.

103 We estimated the ecological and potential evolutionary consequences
 104 of myrmecophily on the pollination biology, mating system, and fitness of
 105 *Turnera velutina* (Passifloraceae), a self-compatible ant-plant using an ant
 106 exclusion field experiment. We addressed the following questions: (i) What is
 107 the effect of ant patrolling on pollinator visitation? (pollinator community
 108 composition, visitation frequency, duration, and behaviour) (ii) Does ant
 109 patrolling affect the host plant mating system? (iii) Does ant patrolling affect
 110 pollen transfer dynamics? (iv) Does ant patrolling affect plant male fitness?
 111 First, because smaller or solitary pollinator taxa are expected to be more
 112 vulnerable to predation risk than larger or social species (Dukas & Morse 2003)
 113 (Clark & Dukas 1994; Abbott & Dukas 2009) we predicted that the pollinator

community composition on ant-excluded plants should be biased, relative to plants with ants, towards smaller and solitary taxa. Second, due to ant-associated predation risk, we hypothesised that flowers of ant-occupied plants would receive fewer and shorter pollinator visits, with higher rates of flower avoidance (pollinator failure to land). Last, we had three different predictions for the effects of ants on pollinator visitation and plant mating system, depending on the magnitude of ant-related impacts on pollinator behaviour: (a) Ants strongly deter pollinators, leading to reduced visitation frequency, shorter visits, pollinator limitation, and reduced seed set in ant-occupied plants. (b) Ants partially deter pollinators forcing them to relocate to other flowers within the same plant, leading to higher visitation frequency but reduced visit duration and higher rates of geitonogamy (intra-plant pollination) in ant-occupied plants. (c) Ants partially deter pollinators, forcing them to relocate to flowers of different plants, leading to higher visitation frequency but reduced visit duration, and higher outcrossing rates (inter-plant pollination) in ant-occupied plants, increasing seed genetic diversity.

130

131 **Materials and methods**

132 *Study site and system*

Field experiments were conducted in coastal sand scrub at Troncones, Guerrero, on the southern Pacific coastline of Mexico (17°47' N, 101° 44' W, elevation < 50 m). *Turnera velutina* (Passifloraceae) is a Mexican endemic

136 shrub (Cuautle & Rico-Gray 2003; Arbo 2005) that establishes a facultative
 137 mutualism with 10 ants species in Troncones (Zedillo-Avelleyra 2017)
 138 rewarding them with extrafloral nectar (Villamil *et al.* 2013). *Turnera velutina* is
 139 a self-compatible, herkogamous species that requires pollinators for seed
 140 production (Sosenski *et al.* 2016). Although it flowers year-round, flowering
 141 peaks during summer (Cuautle *et al.* 2005) and the entomophilous flowers last
 142 one day (Sosenski *et al.* 2016). Pollinator rewards are pollen and floral nectar
 143 (Sosenski *et al.* 2016; Villamil *et al.* 2018). At Troncones, native butterflies are
 144 the dominant flower visitors of *T. velutina*, followed by the introduced
 145 honeybee (*Apis mellifera*); native bees, wasps, and occasionally flies also visit
 146 the flowers.

147

148 *Ant exclusion and experimental setup*

149 We identified six replicate arrays of plants, each of which was at least 10 m
 150 from any other array, and comprised two large focal plants producing > 6
 151 flowers per day, and separated by >2 m. One focal plant was randomly
 152 designated as control, with natural levels of ant-guards. The second was
 153 designated ant-excluded, excluding ants from all stems using Tanglefoot™
 154 (Fig 1a). Both focal plants in each array were isolated from other plants by
 155 trimming or tying back any surrounding vegetation. Exclusion treatments
 156 were checked daily and Tanglefoot™ was replenished if required. Each focal
 157 plant pair was surrounded by 6-10 neighbouring adult plants of *T. velutina* >2
 158 m away (Fig. 1a).

159 To assess the effect of ant patrolling on pollen transfer and its
160 consequences on the rate of selfing, geitonogamy, and outcrossing, we dyed
161 the anthers and pollen of control and ant-excluded plants, using four
162 contrasting dyes (red, blue, green or purple) (Fig. 1b). Within each focal plant,
163 one flower was designated as a focal flower, whilst the other five flowers were
164 designated satellite flowers (Fig. 1a). The anthers of the focal flower were dyed
165 using one colour, whilst the anthers of all satellite flowers were dyed in a
166 second colour. The remaining two colours were used on the other focal plant
167 within the array, differentially dyeing the anthers of focal and satellite flowers
168 (Fig. 1a). Pollen from the neighbouring non-focal *T. velutina* plants within the
169 array was left undyed (naturally yellow-orange). The dyeing treatment was
170 repeated in each of the six plant arrays.

171 Every morning before anthesis, six flower buds per plant (1 focal + 5
172 satellite buds) were bagged to exclude visitors. All additional pre-anthesis buds
173 were removed to standardise floral display across focal plants. Once the
174 corollas were fully open, anthers were dyed and flowers were re-bagged until
175 the dye dried and anthers dehisced, exposing the dyed pollen (Fig. 1b). To
176 ensure a minimum common supply of allogamous pollen across all flower
177 pairs, 10-12 flowers from the neighbouring plants within the array were also
178 bagged before anthesis and remained bagged until the visitation observations
179 started. Stigmas from focal flowers were collected at the end of the anthesis
180 period to count pollen grains received, as detailed below (Fig. 1c).

181 *Pollen dyes*

182 Anthers of focal and satellite flowers were dyed once the corolla opened
 183 completely (~0800-0815), but before anther dehiscence. Anthers were
 184 individually embedded in a droplet of dye until soaked, and flowers were
 185 bagged again until anthers dehisced and the released pollen was dry. The dyes
 186 used were methyl violet (purple), Green S (green), safranin (red), and
 187 methylene blue (blue) (for further details see Supplementary material).
 188 Previous studies showed that dyeing *Turnera velutina* anthers in these colours
 189 effectively dyed pollen grains had no effect on pollinator visitation (Ochoa
 190 Sánchez 2016). Towards the end of anthesis (11:30), pistils from focal flowers
 191 were collected in Eppendorf tubes and slide mounted as a glycerine squash
 192 (Kearns & Inouye 1993; Ochoa Sánchez 2016).

193 *Pollinator visitation*

194 We recorded pollinator visitation to all six flowers on control and ant-
 195 excluded focal plants. Every focal plant was observed for two 20-minute
 196 periods – one immediately after bag removal when flowers had a full pollen
 197 and nectar load and the second 90 minutes later. Flowers remained bagged
 198 until their first observation round started to ensure all flowers had a full pollen
 199 and nectar load. We recorded the identity, frequency, duration, and behaviour
 200 of floral visitors and visits, as detailed below. We conducted a total of 40 h of
 201 observations of 360 flowers on 12 plants over five days. Statistical analyses were
 202 conducted in R version 3.5 (R Core Team 2016). All mixed effects models were

203 fitted using ‘lme4’ R package (Bates *et al.* 2016) and *post-hoc* Tukey
 204 comparisons were tested using the ‘multcomp’ R package (Hothorn *et al.*
 205 2008). All model specifications are reported in detail in Table 1.

206

207 a) Pollinator community composition

208 Flower visitors regarded as potential pollinators (hereafter pollinators) were
 209 identified to one of five taxonomic categories: *Apis mellifera*, native bees,
 210 butterflies, flies, and wasps. To estimate the overall abundance of pollinators
 211 from each taxonomic group, we pooled together observations from control and
 212 ant-excluded plants and calculated the percentage of visitors from each group.
 213 Within each of these taxonomic groups, differences in the total number of
 214 visitors between control and ant excluded plants were assessed using a
 215 Pearson Chi-squared test. Because *Apis mellifera* and butterflies jointly
 216 accounted for 94% of all visitors (Table S1), only these taxonomic groups were
 217 included in all further analyses.

218 b) Pollinator visitation frequency and duration

219 Flower visits were scored each time a pollinator hovered over, landed and
 220 contacted the reproductive organs of a flower, and visit duration was recorded
 221 until the pollinator departed. We recorded visitor identity and considered re-
 222 visitation events. Visitor abundance was estimated as the number of individual
 223 visitors per taxa landing on flowers of a particular plant. For instance, a visitor

224 that landed, hovered, and landed again in another flower was registered as two
 225 visits from one visitor. Ant patrolling effects on visitation frequency were
 226 tested using a Poisson mixed model. The effect of ant patrolling on visit
 227 duration was tested using a Poisson mixed model.

228 c) Pollinator behavior

229 All pollinator visits were allocated to one of two behavioural categories
 230 following Villamil *et al.* (2018): inspection (defined as a pollinator approaching
 231 a flower without landing) or contact (landing on the flower). The effect of ant
 232 patrolling on the likelihood of pollinators displaying inspection behaviours
 233 was tested with a binomial mixed model, considering the presence or absence
 234 of inspection behaviours as the response variable. The effect of ant patrolling
 235 on pollinator deterrence was tested using a binomial mixed model. Pollinator
 236 deterrence is here defined as the absence of contact behaviours following an
 237 inspection behaviour. For every pollinator that displayed an inspection
 238 behaviour, we recorded the presence or absence of contact behaviours and
 239 fitted this as a binomial response variable. For instance, if a pollinator hovered
 240 over a flower, without landing inside it, we would record a zero as the
 241 response variable. The effect of ant patrolling on the duration of each type of
 242 behaviour was tested using a Poisson mixed model, splitting observations into
 243 inspection or contact behaviours. The total duration of each behaviour
 244 (inspection or contact) per visitor was fitted as the response variable.

245

246 *Plant mating system and pollen transfer dynamics*

247 The effect of ant patrolling on pollen transfer and its consequences on plant
248 mating system was assessed by counting differentially dyed pollen grains on
249 focal flower stigma squash slides under a light microscope. The effect of ant
250 patrolling on stigma pollen load, defined as the total number of pollen grains
251 received per stigma, was tested using a Poisson mixed model (Table 1).

252 Pollen colour allowed us to identify pollen grains received from either
253 the same flower (selfing), another flower within the same plant (geitonogamy),
254 the other focal plant in the same array (outcrossing), or another un-dyed plant
255 (outcrossing). The number of pollen grains from each origin (selfing,
256 geitonogamy or outcrossing) was divided by the total number of pollen grains
257 stigma (pollen load) to determine the proportion of pollen from each mating
258 system source. Proportional data were transformed to normality using the
259 logit transformation, with infinite numbers resulting from impossible
260 quotients replaced by zeros. The effect of ant patrolling on the mating system
261 was tested using a linear mixed model, fitting the proportion of pollen from
262 each mating system as the response variable (Table 1).

263 Pollen transfer dynamics were analysed using five categories to describe
264 the mating system and the pollen origin (hereafter referred to as MSPO; Fig.
265 1a). These categories summarise pollen grains received from and donated to
266 every possible pollen source identifiable in this experiment as follows: (i)
267 received/donated to the same flower (selfing), (ii) received from another

268 flower from the same plant (geitonogamy received), (iii) received from the
269 reciprocal focal plant (outcrossing pair received), (iv) donated to the reciprocal
270 focal plant (outcrossing pair donated), (v) or received from another plant from
271 the same species (outcrossing unknown received). The effect of ant patrolling
272 on pollen flow dynamics was tested using a Poisson mixed model (Table 1),
273 fitting as the response variable the number of pollen grains in each of the five
274 MSPO categories.

275 *Male plant fitness*

276 The number of pollen grains donated per flower was an estimate for male
277 plant fitness and quantified as number of pollen grains from each flower
278 donated to focal stigmas. The total number of pollen grains from satellite
279 flowers on the same plant was divided by five, to obtain the mean number of
280 pollen grains donated per flower. The total number of pollen grains from the
281 other focal plant in the same array was divided by six (1 focal + 5 satellite
282 flowers). The effect of ants on male fitness was estimated using a Poisson
283 mixed model fitting as the response variable the number of pollen grains
284 donated per flower in control or ant-excluded plants.

285 The effect of ant patrolling on the destination of the pollen grains
286 donated per flower was tested using a Poisson mixed model, fitting number of
287 pollen grains as the response variable. We only contrasted the number of
288 pollen grains donated by focal or satellite flowers, as every plant had exactly
289 six flowers because floral display was controlled for in our experimental

290 design. Pollen donated by unknown plants was excluded as the number of
291 donor flowers was unknown, and hence pollen grains donated per flower
292 cannot be estimated.

293

294 **Results (720 words)**

295 *Pollinator visitation: composition, frequency and duration*

296 We recorded 967 floral visitors, of which 853 belonged to taxa we regarded as
297 potential pollen vectors (hereafter pollinators) because they were observed
298 contacting male and female plant sexual organs (Table 1), although
299 experimental analyses of their efficiencies as pollen vectors are required.
300 Butterflies and honeybees accounted for more than 80% of all floral visitors
301 and > 94 % of potential pollinators (Table S1). Ant exclusion did not
302 significantly influence the community composition of pollinators visiting *T.*
303 *velutina* flowers ($X^2 = 1.42$, $df = 4$, $P = 0.84$; Fig. 2).

304 Visitation frequency did not differ significantly between pollinator
305 types, or between control and ant-excluded plants (Fig 3a, Table 1). The effect
306 of ant exclusion on visit duration varied across pollinator taxa, as indicated by
307 the significant interaction term (Fig. 4b, Table 1). While flower visits by
308 honeybees were twice as long in ant-excluded plants ($Z = 2.45$, $P = 0.05$; Table
309 1; Fig. 4b), there was no significant effect of ant patrolling on butterflies ($Z =$
310 1.07 , $P = 0.70$; Table 1; Fig. 4b).

311

312 *Pollinator behaviour*

313 Inspection behaviours differed significantly between pollinator taxa (Table 1,
314 Fig. 3c), with butterflies being on average 15% more likely to display inspection
315 behaviours than *Apis mellifera* (Fig. 3c). However, ant exclusion did not affect
316 this behaviour in either pollinator group (Table 1, Fig. 3c). Avoidance differed
317 significantly between pollinator taxa, and the two taxa differed in their
318 responses to ant guards (significant ant exclusion $\times$ pollinator taxon
319 interaction; Table 1). Ant exclusion increased avoidance behaviour in
320 butterflies, but decreased it for *Apis mellifera* (Fig. 3d), resulting in butterflies
321 being deterred from landing on flowers following inspection three times more
322 frequently than *Apis mellifera* (butterflies: 27%, *Apis mellifera*: 8.5%; Fig. 3d,
323 Table 1).

324 When visit duration was split between inspection and contact
325 behaviours, the effect of ant exclusion on visit duration differed between
326 pollinator taxa, and behaviours (Fig. 3e, Table 1). Ant exclusion significantly
327 increased the duration of *Apis mellifera* contact visits ($Z = 2.96$, $P = 0.05$),
328 increasing the time bees spent inside flowers, but did not affect the time
329 butterflies spent inside flowers (contact behaviours: $Z = -2.34$, $P = 0.25$), or the
330 duration of inspection behaviours by either pollinator (*Apis mellifera*: $Z = -$
331 0.48 , $P = 0.99$; butterflies: $Z = -1.73$, $P = 0.65$). Both pollinator groups spent

332 longer periods displaying contact behaviours than inspection behaviours,
333 regardless of the ant exclusion treatment (Table 1, Fig. 3e).

334

335 *Plant mating system and pollen transfer dynamics*

336 Pollen load per stigma was significantly higher in ant-excluded plants (Fig. 4a,
337 Table 1), with focal stigmas on ant-excluded plants receiving on average 155
338 more pollen grains than stigmas on control flowers (control: 85 ± 12 ; ant
339 exclusion: 240 ± 32 (mean $\pm$ se); $LRT = 9.19$, $P = 0.002$; Table 2, Fig. 4a). The
340 proportion of pollen grains from each mating system category differed
341 significantly within and between plant treatments (Fig. 4b, Table 1). In
342 particular, ant exclusion halved outcrossing rates, reduced geitonogamy 33-
343 fold, and tripled selfing rates (Table 1, Fig. 4b).

344 Ant exclusion increased the number of selfing and allogamous pollen
345 grains from non-focal plants received by stigmas, but reduced the number of
346 geitonogamous pollen grains (Table 1, Fig. 4c). But overall, ant exclusion had
347 no effect on the number of pollen grains received and donated by flowers
348 between reciprocal pair plants (OUT_PAIR_REC: control vs. exclusion: $Z =$
349 0.31 , $P = 1.00$; Fig. 4c).

350

351 *Plant fitness*

352 Ant exclusion increased male fitness, assessed as the number of pollen grains
353 donated per flower, from 27.2 ± 6.54 pollen grains in control plants to $163 \pm$
354 23.9 in ant-excluded plants (mean $\pm$ se) (Fig 5a). Ant exclusion, mating system,
355 and their interaction all had significant effects on the number of pollen grains
356 donated per flower to different destinations (Table 1). Most of the pollen
357 donated per flower was received on the same flower's stigma as selfing pollen,
358 regardless of the exclusion treatment (Fig. 5b). Furthermore, ant patrolling
359 had no significant effect on the number of pollen grains donated to the
360 reciprocal pair plant.

361

362 **Discussion**

363 This study provides a comprehensive picture of the interaction between
364 myrmecophily and pollination by showing the ecological and behavioural
365 effects of ant patrolling on pollinators and their cascading effects on plant
366 mating system and fitness. Despite previous experimental evidence in this
367 system suggesting direct ant-pollinator conflicts (Villamil *et al.* 2018), and
368 contrary to our expectations, excluding ants from plants did not affect
369 pollinator community composition (Fig. 2), visitation frequency, pollinator
370 avoidance or inspection behaviours (Fig. 3). However, ant exclusion increased
371 pollinator visit duration (Fig 3), pollen load, male fitness, and selfing rates (Fig.

372 4). To our knowledge, this is the first evidence that ant patrolling can affect
373 the host plant mating system and male plant fitness.

374

375 *How do ants affect the plant mating system and fitness?*

376 Ant exclusion doubled the time *Apis mellifera* spent inside flowers and
377 increased pollen load on stigmas by 150%, but did not affect visitation
378 frequency. These findings are consistent with the pollinator relocation
379 hypothesis, which suggests ants can mildly deter pollinators leading to equally
380 frequent but shorter visits that enhance pollen transfer. Furthermore, ant
381 exclusion promoted a switch in the mating system from outcrossing to selfing
382 (Fig. 4b). The increase in the time *Apis mellifera* spent inside flowers may
383 underlie the increased selfing rates observed in ant-excluded plants: by
384 foraging longer on pollen and nectar bees are likely to transfer more pollen
385 from the anthers to the stigmas within a flower. Longer contact visits by *Apis*
386 *mellifera* in the absence of ants may also be responsible for the increased male
387 fitness, if longer visits allow *Apis mellifera* to collect and transport more pollen
388 grains. Hence, ants cause behavioural changes in pollinator visitation
389 dynamics that have cascading effects on the host plant mating system, and
390 ultimately influence male and female fitness.

391

392 *Effects of ant patrolling on anti-predatory responses and efficiency*

393 Anti-predatory responses in pollinators vary depending on the pollinator and
 394 predator taxa involved (Romero *et al.* 2011), but few studies have documented
 395 how different floral visitors respond to ant patrolling (Ness 2006; Ohm &
 396 Miller 2014; Carper *et al.* 2016) and how different ant partners affect pollinators
 397 (Ness 2006; Miller 2007; Ohm & Miller 2014; Villamil *et al.* 2018). Several
 398 hypotheses have been made regarding how ants may differentially affect each
 399 pollinator depending on body size and lifestyle (social or solitary) (Clark &
 400 Dukas 1994; Abbott & Dukas 2009) (Romero *et al.* 2011). Overall, predation risk
 401 by ant patrolling in *T. velutina* was not strong enough to affect pollinator
 402 composition or increase the natural avoidance and inspection rates of
 403 pollinators. Our behavioural results are consistent with the pattern revealed in
 404 a meta-analysis by Romero *et al.* (2011) showing that pollinator lifestyle (social
 405 vs. solitary) is not a good predictor of anti-predatory sensitivity. In our study,
 406 ant patrolling reduced visit duration in *Apis mellifera*, but not in butterflies
 407 (Fig. 3b, Table 1). Our results show avoidance behaviours differ between
 408 pollinator taxa, a pattern consistent with previous findings suggesting different
 409 pollinators differ in their anti-predatory response behaviours (Romero *et al.*
 410 2011).

411 The net effect of defensive mutualists on the host plant pollination and
 412 fitness can vary depending on whether predators deter efficient pollinators or
 413 inefficient visitors (Romero & Koricheva 2011). For example, guarding ants
 414 decreased plant fitness when they attacked efficient pollinators in *Ficus*

415 *pertusa* (Moraceae: Bronstein 1991) and *Opuntia imbricata* (Cactaceae: Ohm &
416 Miller 2014), but had positive effects in *Banistriopsis malifolia*
417 (Malpighiaceae: Alves-Silva *et al.* 2013) where wasps protected flowers from
418 predation without deterring efficient pollinators. In *T. velutina* butterflies were
419 more abundant than bees, but changes in the behavioural patterns of bees, and
420 not of butterflies, (Fig. 3) seem to be driving changes in plant mating systems
421 (Fig. 4) and fitness (Fig. 5).

422 *Effects of ant patrolling on plant mating system and pollen transfer*

423 Although the deleterious effects of selfing and geitonogamy have been well
424 described for many species (Waser & Price 1991; de Jong *et al.* 1992; Lloyd
425 1992), the decomposition of pollen on stigmas into donor components
426 (intraflower or selfing, intraplant or geitonogamy, interplant or outcrossing)
427 has rarely been performed, and its importance remains underappreciated (de
428 Jong *et al.* 1993; Wu *et al.* 2018). We assessed the effects of ant patrolling on
429 plant mating system decomposing pollen transfer based on its origin and fate
430 finding that in this self-compatible species, ant exclusion shifted the plant
431 mating system from predominantly outcrossing to predominantly selfing,
432 reducing geitonogamy (Fig. 4b). In bisexual flowers self-pollination can be
433 mediated by pollinators (Wu *et al.* 2018) and we suggest that this is the case for
434 *T. velutina* where ant patrolling reduced selfing rates by affecting pollinator
435 visitation behaviour, reducing the time bees spent inside flowers.

437 When contrasting our results from the mating system analyses (which
438 show the proportion of pollen from different origins received by a stigma) with
439 data on pollen transfer (showing counts of pollen grains from different donors)
440 it becomes evident that the increase in pollen load in the absence of ants is
441 driven by selfing and allogamous pollen from other undyed, surrounding, *T.*
442 *velutina* plants. The number of selfing pollen grains on ant-excluded stigmas
443 was much higher, likely driving the change in the mating system from
444 outcrossing to selfing. Yet, ant exclusion also increased the number of
445 allogamous pollen grains received by the stigmas. The significant increase in
446 the number of geitonogamous pollen grains received by ant-patrolled stigmas
447 (Fig. 4c) is consistent with the pollinator relocation hypothesis, which
448 proposes that ant patrolling mildly deters pollinators, causing them to move to
449 a nearby flower and hence increasing the rate of geitonogamy in the plant
450 (Altshuler 1999; Romero & Koricheva 2011). Our results suggest that ants
451 contribute to maintaining outcrossing in the self-compatible *T. velutina*.

452

453 *Effects of ant patrolling on plant fitness*

454 A complete understanding of the effects mutualists and antagonists have on
455 plant fitness requires the assessment of both female and male fitness
456 components, because the magnitude and direction of the effects may differ
457 between plant sexual functions (Schaeffer *et al.* 2013; Carper *et al.* 2016). For
458 instance, male fitness is more constrained by the number of mates reached
459 than female fitness; and pollinator behaviour affects pollen transfer, with

460 longer visits increasing pollen export and pollen deposition on stigmas (Carper
461 *et al.* 2016). Consequently, we expect male fitness to be more susceptible to
462 changes in pollinator behaviour (Krupnick & Weis 1999; Schaeffer *et al.* 2013;
463 Carper *et al.* 2016). To date, very little has been done to assess the effect of ant
464 patrolling on female fitness, and we are not aware of any study assessing the
465 effects of ants on male plant fitness. Our experimental design allowed us to
466 quantify the consequences of ant patrolling on male reproductive fitness
467 (pollen grains donated per flower) and infer potential effects on female fitness
468 (progeny quality). Ant exclusion resulted in a six-fold increase in the number
469 of pollen grains donated per flower (Fig. 5a), suggesting that guarding ants
470 may hinder male fitness.

471 Ant exclusion changed the pollen destination, given that most pollen
472 was donated towards selfing, which contrasts with flowers from ant-patrolled
473 flowers which donated a quarter as much pollen to themselves (Fig. 5b).
474 Hence, ant patrolling in *T. velutina*'s may increase female fitness by promoting
475 outbred seeds and so increasing the offspring quality. Our findings contrast
476 with previous studies showing negative effects of ant patrolling on female
477 plant fitness. In *Opuntia imbricata* ant patrolling decreased seed count by 30%
478 and seed mass by 16% (Ohm & Miller 2014); and in *Heteropterys physophora*
479 ants consumed floral buds, deterred pollinators, reduced pollen transfer and
480 fruit set in buds that escaped ant predation (Malé *et al.* 2012). Furthermore,
481 our findings exemplify how non-pollinators insects interacting with plants and
482 their pollinators may have contrasting effects on female and male fitness

483 components, highlighting the importance of considering both sexual
484 functions.

485 This study provides a comprehensive picture of the interaction between
486 myrmecophily and pollination by showing the ecological and behavioural
487 effects of ant patrolling on pollinators and its cascading effects on plant
488 mating systems that lead to fitness consequences. Contrary to our initial
489 prediction, ant patrolling benefited plant fitness by reducing pollinator visit
490 duration, which promoted pollinator relocation, and led to a reduction in
491 selfing and an increase in outcrossing rates. Although ant patrolling reduced
492 pollen load and male fitness, far from having an ecological cost on the host,
493 ant patrolling seems to be another mechanism – along with herkogamy and
494 pollinator attracting features (Sosenski *et al.* 2016)– to promote outcrossing in
495 this self-compatible species with hermaphroditic flowers. This study
496 contributes towards our understanding on how non-pollinators can shape
497 plant mating systems. We provide the first evidence of the role of patrolling
498 ants on determining plant mating systems and male plant fitness by taking a
499 multispecies approach on plant-animal interactions.

500

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701 **Figure 1.** Experimental design and methods. (a) Diagram of the experimental setup
702 showing a plant pair with or without ant patrolling. Within each experimental plant,
703 the focal flower is represented by a uniquely coloured circle, and satellite flowers are
704 the five circles coloured differently. The surrounding bushes with yellow circles
705 represent undyed (naturally yellow) flowers from neighbouring plants bagged to
706 secure allogamous pollen. (b) Photograph of dyed pollen on the anthers (dp) and
707 dyed pollen grain on the flower stigmas (ps). (c) Photograph of stigma squash slides
708 with dyed pollen grains.

709 **Figure 2.** Composition of floral visitors visiting *Turnera velutina* plants with and
710 without ant patrolling.

711 **Figure 3.** Effects of ant patrolling on pollinator visitation by *Apis mellifera* and native
712 butterflies on *Turnera velutina* flowers on control plants with ant patrolling (black),
713 and ant excluded plants (white) showing mean $\pm$ se for: (a) pollinator visitation
714 frequency, (b) visit duration, and (c-e) pollinator behaviours affecting (c) the time
715 spent displaying alert (circles) or contact/presence behaviours (triangles), (d) the
716 display of alert behaviours, (e) the likelihood of deterrence.

717 **Figure 4.** Effects of ant patrolling on the (a) pollen load, (b) mating system rates and
718 (c) pollen flow (origin and destination) in *Turnera velutina* showing mean $\pm$ se for
719 control (black) and ant-excluded (white) plants.

720 **Figure 5.** Effects of ant patrolling on male fitness, showing (a) average number pollen
721 grains fathered per flower, and (b) the destination of the pollen grains donated per
722 flower (mean $\pm$ se) for control (black) and ant excluded (white) plants.

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724 **Table 1.**

725 Model statistics testing the costs of ant patrolling on *Turnera velutina*'s pollination

726 biology, including ecological, behavioural, mating system, and fitness consequences.

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730 b)

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735 c)

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744 secure allogamous pollen. (b) Photograph of dyed pollen on the anthers (dp) and

745 dyed pollen grain on the flower stigmas (ps). (c) Photograph of stigma squash slides
746 with dyed pollen grains.

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Figure 2. Composition of floral visitors in *Turnera velutina* plants with and without ant patrolling.

Figure 3. Effects of ant patrolling on pollinator visitation by *Apis mellifera* and native butterflies on *Turnera velutina* flowers on control plants with ant patrolling (black), and ant excluded plants (white). (a) Pollinator visitation frequency, (b) visit duration, and (c-e) pollinator behaviours affecting (c) the time spent displaying alert (circles) or contact/presence behaviours (triangles), (d) the display of alert behaviours, (e) the likelihood of deterrence.

Figure 4. Effects of ant patrolling on the (a) pollen load, (b) mating system rates and (c) pollen flow (origin and destination) in *Turnera velutina* showing mean $\pm$ se for control (black) and ant-excluded (white) plants.

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12 **Figure 5.** Effects of ant patrolling on male fitness, showing (a) average number pollen grains fathered per flower, and (b) the
 13 destination of the pollen grains donated per flower (mean $\pm$ se) for control (black) and ant excluded (white) plants.

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Table 1. Model statistics testing the costs of ant patrolling on *Turnera velutina*'s pollination biology, including ecological, behavioural, mating system, and fitness consequences.

	Response	Fixed effects	N	LRT	P-value	Random effects	Variance	SD	Distribution
Ecological consequences	Visitation frequency	Ant exclusion	90	0.64	0.42	Plant	0.00	0.00	Poisson
		Pollinator taxon (Am & butterflies)		3.26	0.07	Day	0.113	0.336	
		Ant exclusion x Taxon		0.75	0.78	OLRE	0.108	0.328	
	Duration	Ant exclusion	90	1.60	0.20	Plant	5.48 ⁻¹⁰	2.34 ⁻⁰⁵	Poisson
		Visitor taxon (Am & butterflies)		8.36	0.0038	Day	9.97 ⁻¹⁰	3.15 ⁻⁰⁵	
		Ant exclusion x Taxon		7.76	0.0053	OLRE	0.681	0.825	
Behavioural consequences	Likelihood of inspection	Ant exclusion	326	1.64	0.19	Plant	0	0	Binomial Bernoulli
		Visitor taxon (Am & butterflies)		10.01	0.001	Day	0	0	
		Ant exclusion x Taxon		1.03	0.30	Pair	0	0	
	Likelihood of avoidance	Ant exclusion	326	1.56	0.211	Plant	0	0	Binomial Bernoulli
		Visitor taxon (Am & butterflies)		14.71	0.0001	Day	0.118	0.343	
		Ant exclusion x Taxon		5.05	0.024	Pair	0.051	0.226	

	Response	Fixed effects	N	LRT	P-value	Random effects	Variance	SD	Distribution
Behavioural consequences	Duration (per behaviour)	Ant exclusion	652	0.84	0.35				
		Visitor taxon (Am & butterflies)		11.31	0.0007	***	Plant	1.09-06	0.0010
							Pair	2.58-07	0.00050
		Behaviour		331.44	2.2-16	***	Day	1.77-07	0.00042
							OLRE	1.70	1.30
		Ant exclusion x Taxon		5.40	0.02	*			
		Ant exclusion x Behaviour		0.90	0.34				
		Taxon x Behaviour		29.90	4.54-08	***			
Mating system consequences	Pollen load	Ant exclusion	72	9.19	0.002	**	Plant	0.12	0.34
							Pair	0.03	0.18
							Day	0.16	0.41
							OLRE	0.52	0.72
	Mating system	Ant exclusion	336	0.08	0.77		Plant	3.92-06	0.001
		Mating system		24.67	4.37-06	***	Pair	0.17	0.41
		Ant exclusion x Mating system		75.92	2.2-16	***	Day	0.53	0.73
							OLRE	6.46	2.54
	Pollen flow (mating system, received and donated)	Ant exclusion	336	0.58	0.44		Plant	0.0006	0.077
		Pollen origin		108.95	2-16	***	Pair	0.22	0.83
		Ant exclusion x Pollen origin		92.71	2.2-16	***	Day	0.69	0.47
							OLRE	4.25	2.06

	Response	Fixed effects	N	LRT	P-value		Random effects	Variance	SD	Distribution
Fitness consequences	Male fitness	Ant exclusion	72	9.56	0.001	**	Plant	0.12	0.34	Poisson
							Pair	0.03	0.18	
							Day	0.16	0.41	
							OLRE	0.52	0.72	
	Destination of donated pollen	Ant exclusion	200	3.93	0.04	*	Plant	0.16	0.40	Poisson
							Pair	0.004	0.06	
							Day	0.44	0.67	
							OLRE	2.99	1.72	