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

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Abstract

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

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;

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

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

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

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

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

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

130

131 **Materials and methods**

132 *Study site and system*

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

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

Ant exclusion and experimental setup

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

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

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

Pollen dyes

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

Pollinator visitation

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

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

a) Pollinator community composition

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

b) Pollinator visitation frequency and duration

Flower visits were scored each time a pollinator hovered over, landed and contacted the reproductive organs of a flower, and visit duration was recorded until the pollinator departed. We recorded visitor identity and considered re-visitation events. Visitor abundance was estimated as the number of individual 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

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

Male plant fitness

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

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

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

Results (720 words)

Pollinator visitation: composition, frequency and duration

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

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

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

Plant mating system and pollen transfer dynamics

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

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

Plant fitness

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

Discussion

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

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

Effects of ant patrolling on plant mating system and pollen transfer

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

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

Effects of ant patrolling on plant fitness

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

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

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

components, highlighting the importance of considering both sexual functions.

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

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

Figure 2. Composition of floral visitors visiting 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) showing mean ± se for: (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 ± se for control (black) and ant-excluded (white) plants.

Figure 5. Effects of ant patrolling on male fitness, showing (a) average number pollen grains fathered per flower, and (b) the destination of the pollen grains donated per flower (mean ± 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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728 a)

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730 b)

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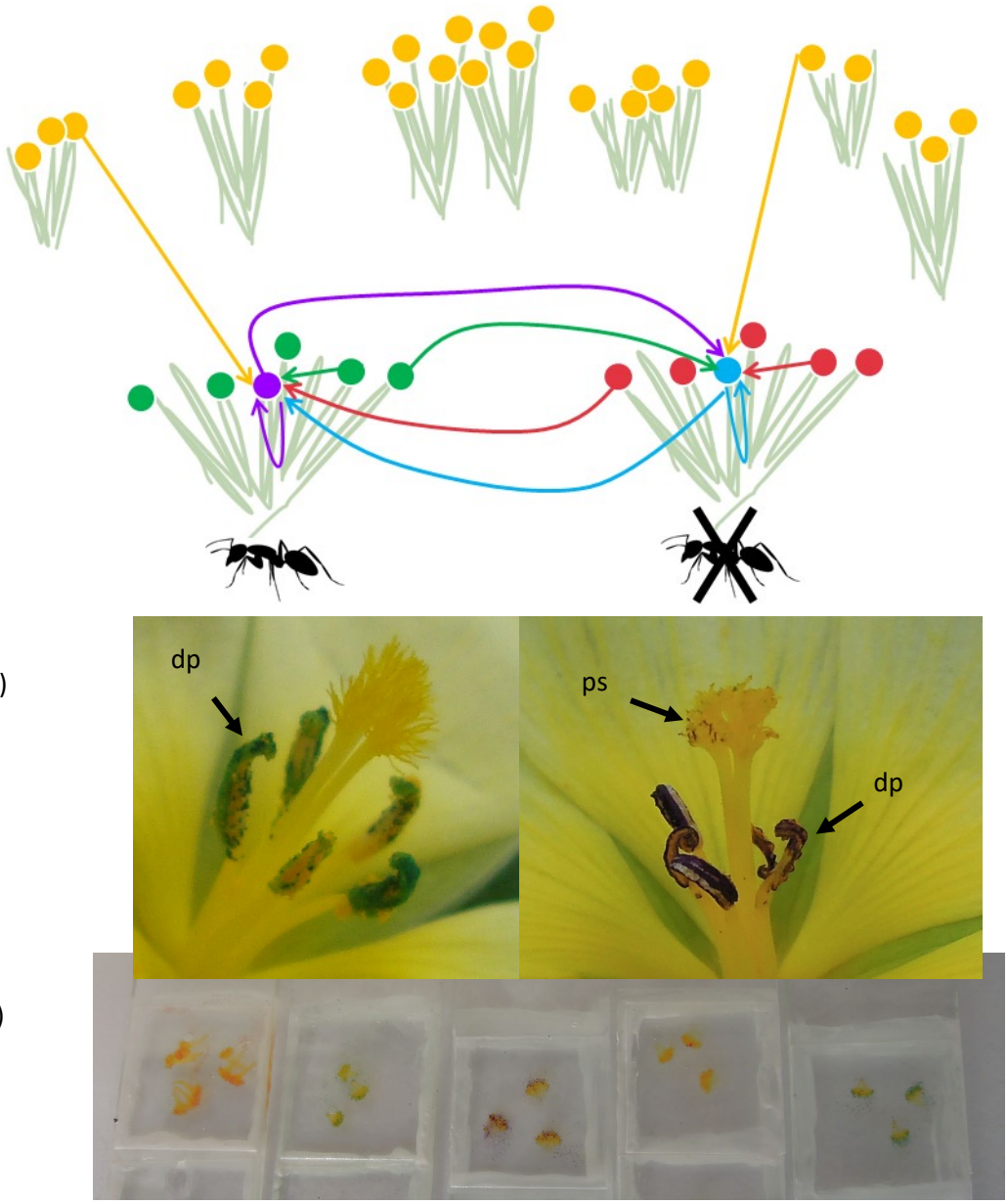

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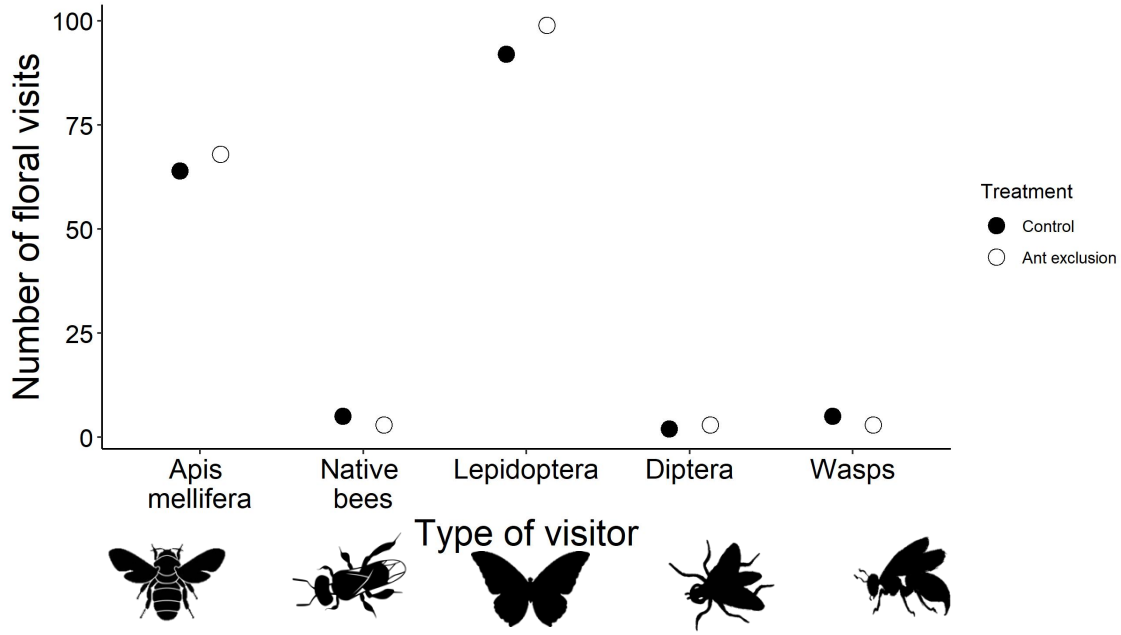

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751 **Figure 2.** Composition of floral visitors in *Turnera velutina* plants with and without
752 ant patrolling.

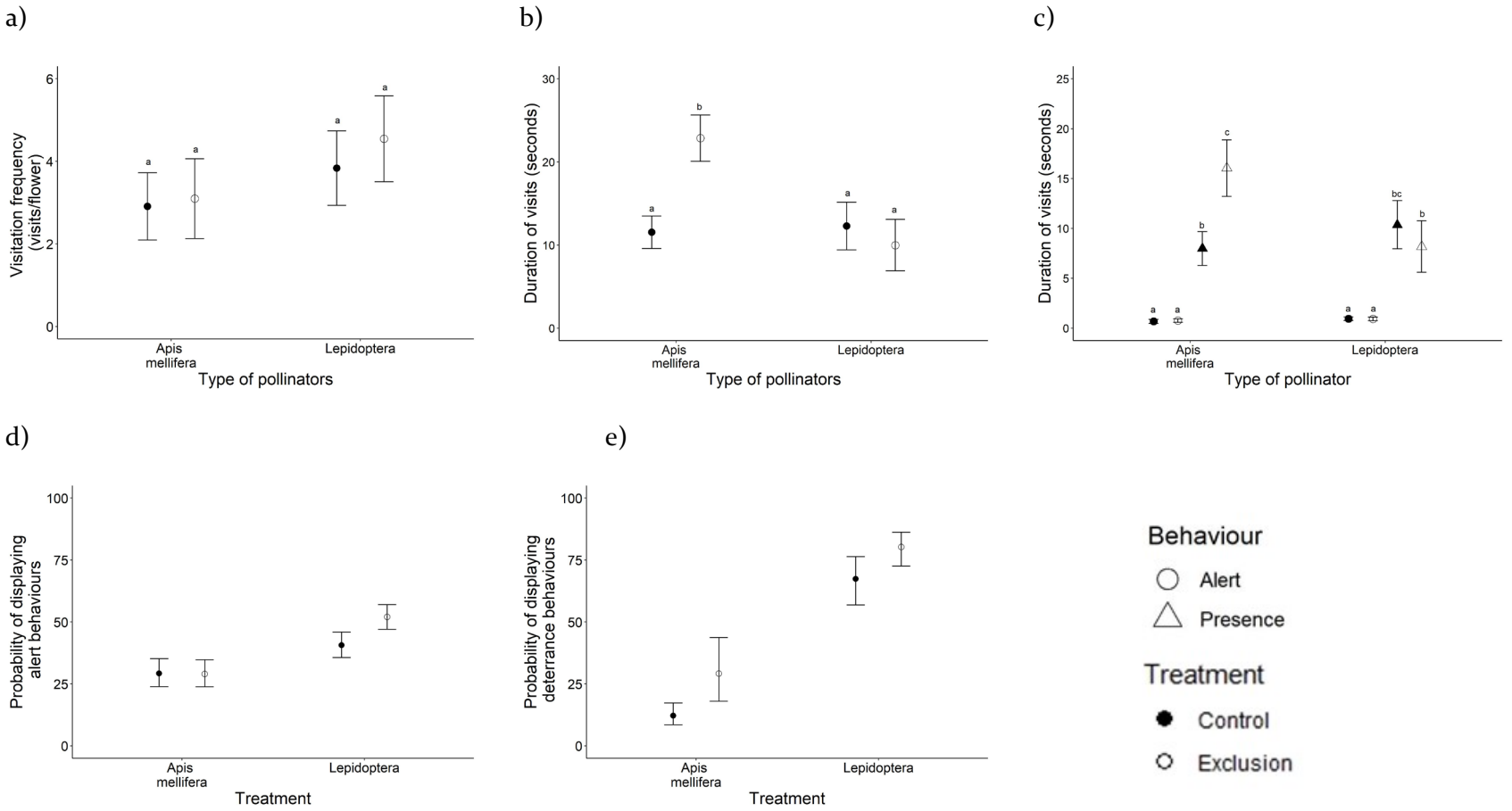
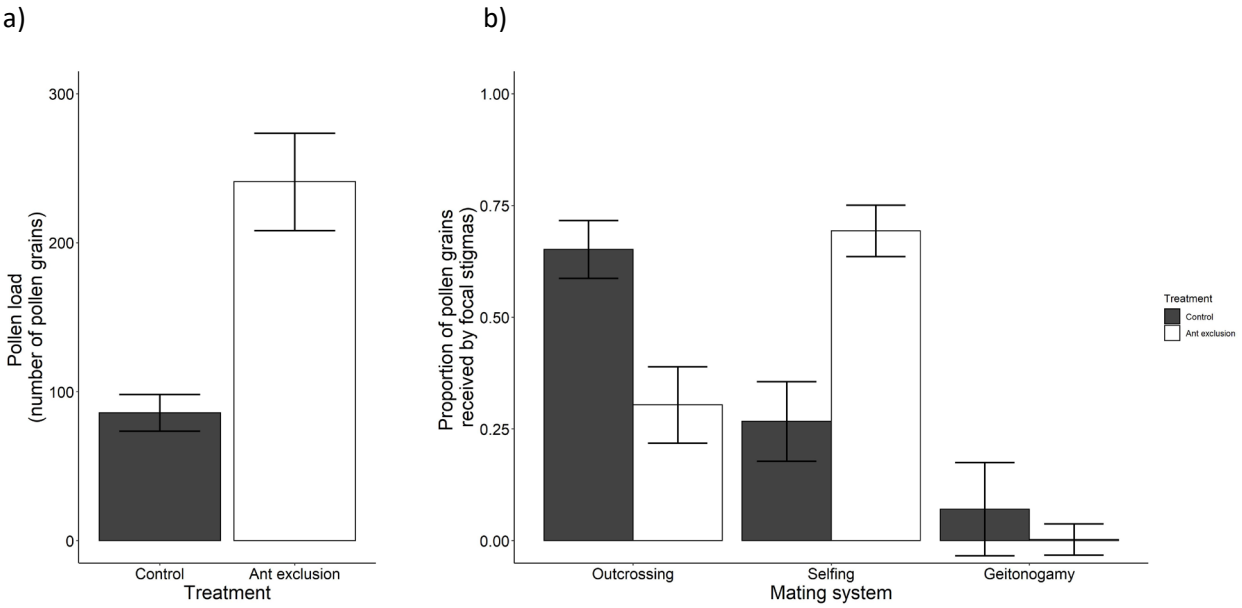


Figure 3. Effects of ant patrolling on pollinator visitation by *Apis mellifera* and native butterflies on *Turnera velutina* flowers on control plants with ant patrolling (black), and ant excluded plants (white). (a) Pollinator visitation frequency, (b) visit duration, and (c-e) pollinator behaviours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



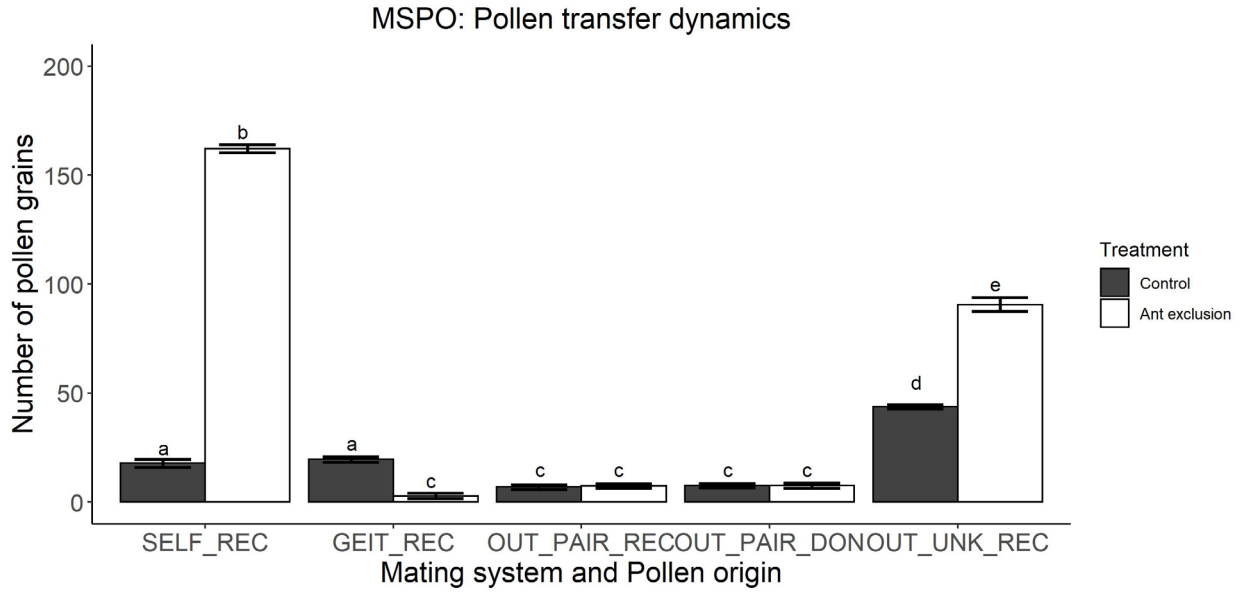


Figure 4. Effects of ant patrolling on the (a) pollen load, (b) mating system rates and (c) pollen flow (origin and destination) in *Turnera velutina* showing mean ± se for control (black) and ant-excluded (white) plants.

a) b)

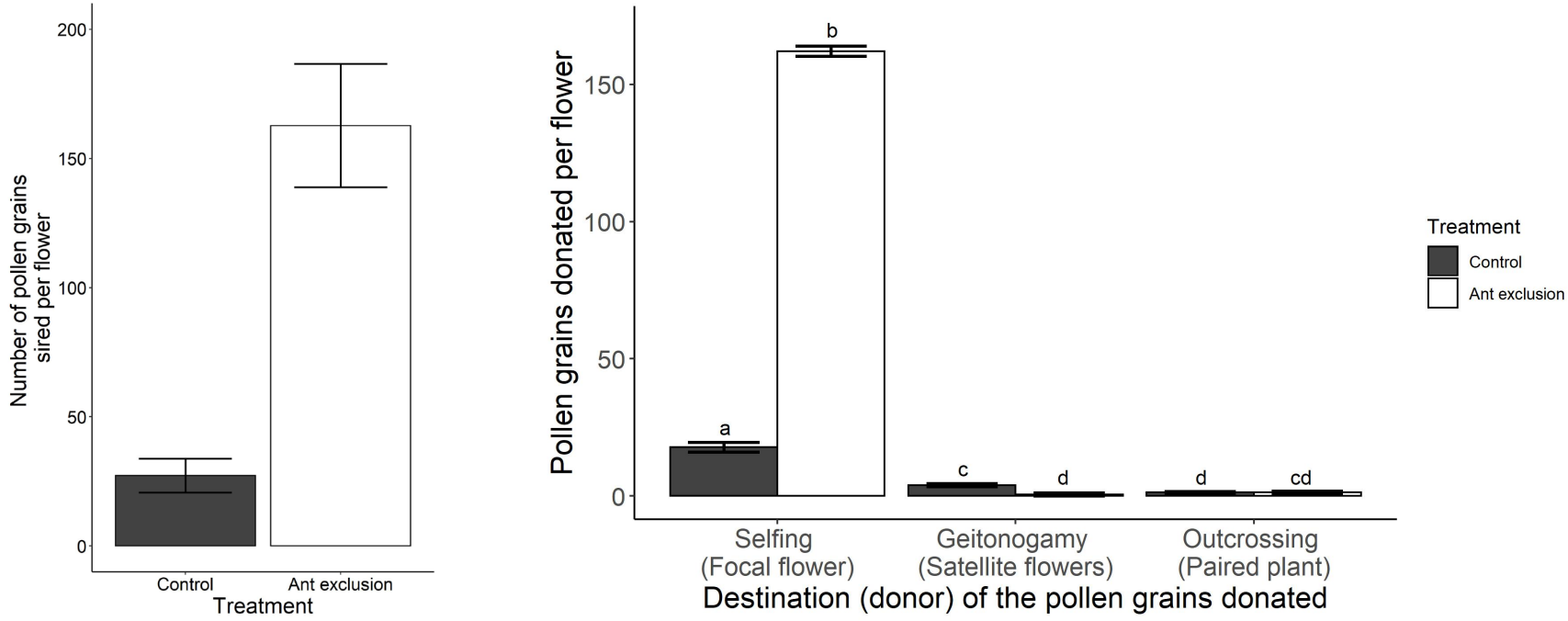


Figure 5. Effects of ant patrolling on male fitness, showing (a) average number pollen grains fathered per flower, and (b) the destination of the pollen grains donated per flower (mean ± se) for control (black) and ant excluded (white) plants.

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					Poisson
		Visitor taxon (Am & butterflies)		**8.36**	**0.0038**	**	Plant	5.48-10	2.34-05	
							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	
							Pair	0	0	
		Ant exclusion x Taxon		1.03	0.30					
	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	
							Pair	0.051	0.226	
		Ant exclusion x Taxon		**5.05**	**0.024**	*				

	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	Poisson
							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	Poisson
							Pair	0.03	0.18	
							Day	0.16	0.41	
							OLRE	0.52	0.72	
	Mating system	Ant exclusion	336	0.08	0.77					
		Mating system		**24.67**	**4.37-06**	***	Plant	3.92-06	0.001	Gaussian
							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					
		Pollen origin		**108.95**	**2-16**	***	Plant	0.0006	0.077	Poisson
							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	