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Ant-plants recruit aggressive ants to defend themselves against herbivores. Herbivores, eat plants voraciously. Plants have evolved different mechanisms to defend themselves against herbivores. Some are poisonous: like coffee or tobacco; some are prickly: like cacti or nettles. But others, persuade mercenaries - mostly ant guards - offering them sugary rewards and housing, and hence are called ant-plants. So, in exchange for protection services, these plants provide ants with food and nesting sites. However, most ant-plants are pollinated by animals, different from ants, and they also reward them with food, such as nectar. But guarding ants are usually not great at discriminating between friends and foes that visit the plant. So, pollinators may be weary when visiting the flowers of ant-plants. This plant problem is called ant-pollinator conflict.

My PhD research tries to answer:
Do ants and pollinators fight directly or over nectar? Does having efficient bodyguards scare pollinators away and how does this affect the plants? Can plants bribe ants away with extrafloral sugary rewards?

I found that ants and bees do not fight over nectar, but they may fight directly. Pollinators spend less time visiting the flowers where the most aggressive ants are. Bodyguards scare away pollinators affecting flowers’ sex life. By reducing the time pollinators spend inside flowers, ants help the plants avoid mating with themselves (self-pollinating). Self-pollination can make plant seeds grow poorly, like haemophilia, an illness that made the royals die from bleeding and was caused by them marrying with their relatives. However, to reduce ant-pollinator conflicts, plant bribe ants away from the flowers providing them extrafloral sugary rewards on the outskirts of the flowers. Understanding how guarding ants affect the pollination biology and fruit production of ant-plants is crucial for all of us, since we deeply care about our morning toast, sweet or savoury, and our passionfruit juice to down the toast.
ABSTRACT

Ant-plants recruit ants to defend them against herbivores, but most of them also require pollinator for successful seed set. Interactions between patrolling ants and pollinators on ant-plants have received relatively little attention. Negative ant-pollinator interactions are expected for several reasons. First, ants and pollinators benefit from plant investment in different functions (defence and reproduction, respectively) which can lead to plant trade-offs. Second, although more aggressive ants are likely to be better defenders, they may also deter pollinators, affecting plant fitness. However, ant-plants may have mechanisms to manage ant-pollinator interactions, maximising the benefits from their services whilst minimising the costs.

I used field experiments to investigate the ecological costs and evolutionary consequences of ant patrolling for the pollination biology of a facultative ant-plant, the Mexican endemic *Turnera velutina* (Passifloraceae), addressing the following questions:

a) Which are the most aggressive and best ant defenders of *T. velutina*?
b) Is there direct (pollinator deterrence) or indirect (nectar trade-offs) ant-pollinator conflict?
c) What are the ecological costs and evolutionary consequences of myrmecophily for the host plant pollination and mating system?
d) Do ant-plants have adaptations to cope with both mutualists, avoiding conflict?

Answers:

a) *Cephalotes* sp. ants was detected as parasitic non-defenders, and the remaining ant species were ranked as: *Capmponotus planatus* < *Crematogaster* sp. < *Paratrechina longicornis* < *Brachymyrmex* sp. < *Dorymyrmex bicolor*. 
b) I found evidence for direct but not for indirect conflict.

c) Ant patrolling affected pollinator visit duration, pollen loads, outcrossing rates, and male fitness, leading to negative effects on pollinator foraging efficiency, but such changes had positive effects for plant fitness increasing outcrossing and male fitness.

d) Extrafloral nectar also serves to bribe ants away from reproductive structures during the crucial pollination period, reducing the probability of ant-occupation of flowers, reducing ant-pollinator conflict, and increasing plant reproductive success.
DECLARATION

I declare that this thesis has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree. Except where stated otherwise by reference or acknowledgment, the work presented is entirely my own.

14 January 2019

Nora Villamil Buenrostro
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# TABLE OF CONTENTS

## CHAPTER 1 ..................................................................................................................................... 6

### GENERAL INTRODUCTION .............................................................................................................. 6

**INTRODUCTION** .......................................................................................................................... 7
- Defining ant-plants (myrmecophily) .......................................................................................... 7
- Facultative vs. obligate mutualisms ......................................................................................... 7
- Indirect interactions: a simultaneous approach ................................................................. 8
- Ants as agents for floral traits .............................................................................................. 10

**RESEARCH AIMS AND QUESTIONS** .......................................................................................... 12

**CHAPTERS OVERVIEW** ..................................................................................................................... 12
- Chapter 2: Ant aggressivity ...................................................................................................... 12
- Chapter 3: Ant-Pollinator conflict .......................................................................................... 13
- Chapter 4: Ecological costs .................................................................................................... 13
- Chapter 5: Extrafloral nectaries as ant distractors .............................................................. 14

**STUDY SYSTEM AND SITES** ........................................................................................................... 15
- The study system in brief ...................................................................................................... 15
- The study sites in brief ......................................................................................................... 18
- Conservation of coastal sand dunes in Mexico: an essay ...................................................... 20

## CHAPTER 2 ..................................................................................................................................... 28

### ANT AGGRESSIVITY AND PROTECTION EFFECTIVENESS ................................................................... 28

**INTRODUCTION** .......................................................................................................................... 29
- Quantifying aggressivity: a brief summary ........................................................................... 32

**QUESTIONS & HYPOTHESES** ........................................................................................................ 36

**MATERIALS AND METHODS** ....................................................................................................... 36
- Study site .................................................................................................................................. 36
- Study system .......................................................................................................................... 36
- Anti-herbivore assays ............................................................................................................ 37
- QUANTIFYING AGGRESSIVITY ................................................................................................. 40
- Maximum aggressivity score ................................................................................................. 40
- Likelihood of aggressive behaviours ..................................................................................... 41
- Ant recruitment ..................................................................................................................... 41
- Response time ....................................................................................................................... 42
- Repellence time ..................................................................................................................... 42
- Protection effectiveness ........................................................................................................... 42

**RESULTS** ..................................................................................................................................... 44
- Quantifying aggressivity ......................................................................................................... 45
- Ant recruitment, response and repellence time .................................................................... 49
- Collinearity plots between aggressivity variables .............................................................. 52
- Maximum aggressivity score ................................................................................................. 54
- Protection Effectiveness Index ............................................................................................... 54

**DISCUSSION** .................................................................................................................................. 57
- How to quantify aggressivity? ................................................................................................. 57
THE DISTRACTION HYPOTHESIS OF EXTRAFLORAL NECTAR ................................................................. 140

CHAPTER 5 ......................................................................................................................................... 140

RESULTS ......................................................................................................................................... 116

DISCUSSION ................................................................................................................................... 128

Conclusions ................................................................................................................................... 138

CHAPTER 5......................................................................................................................................... 140

THE DISTRACTION HYPOTHESIS OF EXTRAFLORAL NECTAR ................................................................. 140

INTRODUCTION ............................................................................................................................. 141

QUESTIONS & AIMS ....................................................................................................................... 145

HYPOTHESES AND PREDICTIONS ....................................................................................................... 146

MATERIALS AND METHODS ........................................................................................................... 146

Study site and system ........................................................................................................................ 146

FIELDWORK METHODS ............................................................................................................... 146

Surveys of ants inside flowers ........................................................................................................ 146

Experimental manipulation of EFN secretion ............................................................................. 147

Impacts of EFN secretion on fitness ............................................................................................. 148

STATISTICAL ANALYSES ............................................................................................................ 149

Surveys of ants in flowers .............................................................................................................. 149

Ecological consequences of EFN secretion ................................................................................ 150

Impacts of EFN secretion on plant fitness ................................................................................... 152

Exploring the responses and effects of different ant species ....................................................... 152

Effect sizes ..................................................................................................................................... 153

RESULTS ......................................................................................................................................... 153

Surveys of ants in flowers .............................................................................................................. 153

Floral visitors .................................................................................................................................. 153

Ecological consequences of EFN removal ................................................................................... 157

Impacts of EFN secretion on plant fitness ................................................................................... 158

Comparison of patterns across spatio-temporal scales ............................................................. 162

Ant species-specific responses to clogging ................................................................................... 162

and effects on pollinators .............................................................................................................. 162

DISCUSSION ................................................................................................................................... 164
The Distraction Hypothesis ................................................................. 164
Exploring ant species-specific effects ............................................ 167
The spatio-temporal scale of the Distraction Hypothesis ................. 169
Implications for ant and pollinator foraging strategies ................. 170
Conclusions .................................................................................... 171

SUPPLEMENTARY ............................................................................ 173

MATERIAL 1 .................................................................................. 173
FIELDWORK METHODS ................................................................. 173
STATISTICAL ANALYSES ............................................................. 174
RESULTS AND DISCUSSION ......................................................... 176
  Reduction of EFN secretion ......................................................... 176
  Effect sizes ................................................................................ 178
  Comparing effect sizes and spatio-temporal scales ..................... 179

SUPPLEMENTARY ............................................................................ 185

MATERIAL 2 .................................................................................. 185
STATISTICAL ANALYSES ............................................................. 185
  Effects of clogging on ant species ............................................. 185
  Effects of ant species on pollinators ........................................ 186
RESULTS AND DISCUSSION ......................................................... 187
  Effects of clogging on ant species ............................................. 187
  Effects of ant species on pollinators ........................................ 188

CHAPTER 6 .................................................................................... 192
GENERAL DISCUSSION ................................................................. 192
SUMMARY OF KEY FINDINGS .................................................... 193
SOME LOOSE ENDS ........................................................................ 195
  Natural history ......................................................................... 195
  Is the corolla a natural ant shield for pollinators? ................. 195
  Networks .................................................................................. 196
BROADER IMPLICATIONS ............................................................. 197
  Herbivore vs. pollinator susceptibility to ants ......................... 197
  Are Brachymyrmex ants the best guards by being pollinator friendly? .... 198
  Ant patrolling, outcrossing rates, and plant selective fruit abortion ... 199
  Ant patrolling & dioecy ......................................................... 201
  Herkogamy, outcrossing and ant aggressivity ......................... 202
REFERENCES .............................................................................. 205

NATURAL HISTORY SUPPLEMENTARY NOTES .................................. 222
  Behavioural observations catalogue ...................................... 222

PUBLICATIONS ARISING FROM THIS THESIS ................................ 230
On not writing poetry
By Ellie McDonald

This is yer Muse talkin.
Ye’re on yer final warnin.
Nae mair sclatchin I the kitchen,
nae mair hingin out the washin,
nae mair stour soukin.
This is yer Muse talkin.
Fae the wyste paper basket.
[...]

THE START...
CHAPTER 1

GENERAL INTRODUCTION

Ant-plants

*Turnera velutina*

Coastal sand dunes
INTRODUCTION

Defining ant-plants (myrmecophily)
Mutualisms are defined as interspecific cooperative interactions in which two species are reciprocally exploited, resulting in a net benefit for both partners (Begon et al. 1986, Bronstein 2001). Plants interact with a vast range of animals in mutualistic or antagonistic relationships (Herrera and Pellmyr 2009). Animals that interact with plants can also interact with each other. In fact, to deter herbivores, some plant species recruit ants that predate on herbivores (Belt 1874, Janzen 1966, Bentley 1977). Extrafloral nectaries (EFN), domatia and food bodies have been described as mechanisms by which plants attract ants, by providing nesting sites or nutrients (Rosumek et al. 2009). In return, ants attack herbivores, prune climber vines and prevent fungal and microbial infestation on plant tissues (Bentley 1977, Rosumek et al. 2009). This barter of food and lodging products in exchange for protection services between ants and plants is called myrmecophily, and the plants are often called ant-plants (Rico-Gray and Oliveira 2007a, Herrera and Pellmyr 2009).

Facultative vs. obligate mutualisms
Ant-plant mutualisms can be classified as obligate or facultative depending on the tightness of the interaction, the rewards provided, the specificity of the mutualisms and the number of ant species involved. Plants in obligate mutualisms (myrmecophytes) offer domatia and food bodies which are specific food rewards and nesting sites. Obligate mutualisms often involve only one specialised ant species that tends to nest and feed exclusively on the host plant (Rico-Gray and Oliveira 2007a, Herrera and Pellmyr 2009, Rosumek et al. 2009, Quintero et al. 2013). In contrast, plants in facultative associations (myrmecophiles) tend to be patrolled by many ant species, from different
subfamilies (Rico-Gray and Oliveira 2007a), that do not nest on the plant and show low fidelity to the food association (Kersch and Fonseca 2005). Domatia offer a more specific reward and ants show stronger fidelity to domatia providing hosts than EFN-hosts (Rosumek et al. 2009). Despite exceptions (see Kautz et al. 2009 for specialisation against cheating ants), extrafloral nectaries are general sugar source available for any animal to harvest. Consequently, EFN-based mutualisms tend to be less specialised and specific than those mediated by domatia or food bodies (Rico-Gray and Oliveira 2007b). In fact, providing only EFN is one of the criteria to define an ant-plant mutualism as facultative and the host plant a myrmecophile (Quintero et al. 2013).

Patrolling ants from obligate systems are expected to be more aggressive and better guards than those in facultative systems, because ants in obligate mutualisms are defending their nesting sites and colony, as well as their food source. In contrast, ants from facultative systems are only defending part of their food sources. Differences in their protection efficiency were confirmed by several meta-analyses (Chamberlain and Holland 2009, Rosumek et al. 2009, Trager et al. 2010) revealing that obligate ant-plants suffered, on average, three times more damage when ants were excluded than their facultative counterparts (Rosumek et al. 2009). So far, no obligate ant-plants associations have been reported in temperate regions; whilst in the tropics 70% of the mutualisms are facultative and 30% are obligate, with EFN being the most frequent ant attractor (Rosumek et al. 2009).

Indirect interactions: a simultaneous approach
Although huge progress has been made in understanding different plant-animal interactions through the last decades, most research on plant-animal interactions has been done by uncoupling the community and considering paired interactions (e.g., plant-herbivore, plant-pollinator, plant-fungus) (Strauss 1997, Herrera 2000). This pairwise approach, although convenient, is
artificial and oversimplifies nature (Herrera 2000) since plants interact with pollinators, herbivores, herbivore predators and pathogens, among others, simultaneously or sequentially throughout their lifetime (Armbruster 1997). All these interactions jointly affect plants at many levels, from anatomy to physiology, development, ecology and evolution. Furthermore, plants interactions with a certain animal group or guild may also affect relationships with other groups or guilds (Armbruster 1997, Strauss and Irwin 2004, Adler 2008). By simultaneously investigating more than one interaction it is possible to assess the relative importance of each interacting organism for plant fitness (Herrera 2000) as well as the benefits plants provide for each of the interactors.

A direct interaction is defined as a pairwise relationship between a plant and another living organism mediated by a plant trait; indirect interactions are defined as those involving specialised plant traits, and mediated by a third species (Adler 2008). There are few publications on the effects of indirect interactions on evolutionary ecology (Herrera et al. 2002, Irwin et al. 2004, Strauss and Irwin 2004, Takayuki 2005), and indirect interactions have received little attention in herbivore-plant-pollinator systems and have been almost unexplored regarding ant-plant-pollinator interactions (but see: Assunção et al. 2014, Alves-Silva et al. 2015, Dâtillo et al. 2016, Del-Claro et al. 2016, Del-Claro et al. 2018).

In order to successfully grow and reproduce most plants must attract mutualists and deter antagonists. Beneficial and prejudicial interactions are not a cut-and-dry determination. The involvement of a third species is likely to influence pairwise plant-animal interactions. A growing amount of evidence now suggests that pollinator attracting traits and herbivore resistance traits are not independent (Strauss 1997, Strauss et al. 1999, Herrera 2000, Mothershead and Marquis 2000, Strauss and Irwin 2004, Takayuki 2005, Buchanan and Underwood 2013). The negative effects of herbivores on plant fitness can be
direct (by reducing available resource due to lost photosynthetic area) or indirect (by deterring pollinators or affecting floral or mating system traits). Nonetheless, many studies have implicitly assumed that the effects of herbivores on plant fitness are due to direct effects (Rodríguez-Robles et al. 1992, Johnson et al. 1995, Conner and Rush 1996, Strauss et al. 1996), and little attention has been paid to indirect effects of herbivory on plant fitness. Some of the few studies on indirect effects of herbivores on pollination highlight the importance that induced defences have on pollination biology (Lohman et al. 1996, Kessler and Halitschke 2009, Kessler et al. 2011, Campbell and Kessler 2013), emphasising how pollination services and effectiveness can be influenced by herbivory (Takayuki 2005).

Features that originally evolved to serve one function, can later co-opt to serve the other one, such as anti-herbivore resins that develop into pollinator rewards (Armbruster 1997). The evolution of plant-pollinator interactions is a great example of this: Flowers – a key trait in angiosperms massive adaptive radiation – are nowadays a means to lure pollinators but might have evolved as a defensive mechanism to protect pollen and ovules from herbivores (Mulcahy 1979). Enclosing the reproductive structures in modified leaves (carpels) that later evolved into ovaries, petals, and sepals provided the basis for natural selection to act upon (Willmer 2011). Since some visitors were less detrimental than others, and some carpels were more protective than other, coevolution shaped plant-herbivores interactions into plant-pollinator relationships (Rico-Gray and Oliveira 2007b).

Ants as agents for floral traits
In many species plant reproductive traits seem to be the result of conflicting selective pressures exerted by mutualists and antagonists (Herrera 2000). Pollinators are not always the selective agents for floral traits and the selective pressures exerted by pollinators cannot account for all the variation observed in
floral traits (Galen 1999, Parachnowitsch and Caruso 2008). Several studies have shown that herbivores indirectly modulate the nature, strength and consequences of plant-pollinator interactions (Lohman et al. 1996, Strauss 1997, Strauss et al. 1999, among others) and modify plant mating systems (Leege and Wolfe 2002, Adler 2008, Kessler and Heil 2011). Defensive traits might directly deter pollinators and therefore select for less defended phenotypes; pollinators could also be selecting for highly defended plants when preferring undamaged individuals (Adler 2008). It has been extensively shown that leaf herbivory reduces plant fitness by deterring pollinators (Strauss et al. 1996, Strauss 1997, Mothershead and Marquis 2000, Poveda et al. 2003). Actively patrolled plants are better defended and therefore less damaged, which might attract more pollinators, but, the presence of ants could also imply pollinator deterrence, and this has been poorly studied.

Although the overall effect of ants on plant fitness has been demonstrated as positive (Romero and Koricheva 2011), ant patrolling can affect pollinator dynamics and plant mating systems in a variety of ways revealing interesting community ecological dynamics. Defensive traits, such as aggressive ant patrollers, might directly deter pollinators and therefore select for less defended phenotypes; but pollinators could also be selecting for highly defended plants when preferring undamaged individuals (Adler 2008).

Ant-pollinator interactions are likely to impact pollen transfer, influencing pollination success. Ants might deter pollinators, causing pollen limitation in animal pollinated ant-plants. If this is the case, it would be expected that selfing mechanisms could be selected in plants as a reproductive insurance to compromised animal vectors. Although selfing reduces genetic variability in populations, increasing the risk of inbreeding depression, it has been suggested that a switch from an allogamous to an autogamous mating system might be beneficial in marginal environments, since it provides a back-
up, reproductive insurance when mates or pollen vectors are scarce (Campbell and Kessler 2013). The same logic can be applied when pollinators are in short supply because of ants: those ant-plants capable of selfing are expected to have a better outcome. On the other hand, ant-pollinator interaction may be beneficial for plant fitness if ants increase pollen transfer efficiency by pollinator relocation. In this case, mechanisms that promote outcrossing would be benefitted. More studies are required to gain a better understanding on the outcome of ant-pollinator interactions, their effects on plants and the adaptations plants might have developed to promote or ease these relationships.

Yet, these are only hypotheses. The relative importance of mutualists and antagonists in shaping plant traits is mostly unknown given that studies rarely identify the agents of selection on plant traits (Parachnowitsch and Caruso 2008). Ants have been reported as agents selecting for floral traits in Polemonium viscosum (Galen 1999, Galen and Cuba 2001) and Erica halicacaba (Turner et al. 2012). However, neither of these species are ant-plants and so ants in these cases ants are herbivorous, prejudicial insects, rather than beneficial herbivore predators. To my knowledge, the potential of mutualistic ants as selective agents upon floral traits has not been explored.

**RESEARCH AIMS AND QUESTIONS**

The main aim of this thesis is to investigate the ant-pollinator interactions on a facultative ant-plant, and assess the ecological and evolutionary consequences of ant patrolling on the host plant pollination biology.

**CHAPTERS OVERVIEW**

**Chapter 2: Ant aggressivity**

The net outcome in facultative mutualisms depends heavily on the identity of the partner species involved, the quality of the rewards provided, and the
aggressivity and protection effectiveness of the patrolling ant species (Raine et al. 2004, Ness et al. 2006, Del-Claro and Marquis 2015, Fagundes et al. 2017). In this chapter I quantified the aggressivity of the most abundant ant species patrolling on *T. velutina*.

**Chapter 3: Ant-Pollinator conflict**

During anthesis, conflict between ants and pollinators is expected because aggressive ants inside the flowers might directly attack pollinators or make the flowers unattractive due to high predation risk and low rewards. Plant trade-offs in resource allocation to reproduction or defence is another reason why we expect conflicting interactions between pollinators and ants. Plants have a limited amount of resources they must distribute amongst different physiological needs, such as growth, defence, reproduction, etc. Resources allocated to one of these functions are no longer available for the others, and so trade-offs can occur. Pollinators would benefit from resources invested in reproduction, whilst ants benefit from plant investment in defence.

This chapter explores whether ants and pollinators have a conflicting relationship expressed as direct conflict in which the presence of ants inside flowers hinder pollination, and as indirect conflict over plant reward allocation to each mutualistic guild. Comparing how plants invest in each mutualist's reward and whether reallocation is possible are two key steps towards understanding ant-pollinator interactions.

**Chapter 4: Ecological costs**

Negative interactions between predator ants and pollinators are theoretically expected, and have been confirmed in several species (Rico-Gray and Oliveira 2007, Frederickson 2009, Palmer et al. 2010). Those studies that have addressed ant-pollinator conflict show contrasting evidence: ants can either increase
fitness (Altshuler 1999) or reduce it (Ness 2006). Changes in pollinator composition, frequency and duration of the visits have been hypothesized as potential factors driving the positive or negative outcomes of ant-pollinator interactions for plant reproduction (Altshuler 1999, Ness et al. 2009). Pollinators’ responses to ant threats, may have different consequences for the host reproduction and mating systems. Yet, none of these have been expressly tested or observed in an adequate experiment where pollinator visitation, frequency, and duration of the visits is recorded along with plant fitness associated to plants with and without defensive ants patrolling. In this chapter we used an experimental approach to test the effects of patrolling ants on pollinator visitation frequency, duration, behavior, and their cascading effects on the host plant mating system and fitness.

Chapter 5: Extrafloral nectaries as ant distractors
Previous findings show that EFN secretion volume and sugar content peak when leaves are associated with flowers, compared to EFN secretion in leaves associated buds and fruits (Villamil 2017). I tested if this increased secretion during anthesis could be part of a mutualist management strategy by which the plant lures ants outside the flowers, avoiding the costs of ant-pollinator interference without giving up the protection. This suggested function of EFN is known as the Distraction Hypothesis, and despite being proposed in 1878 (Kerner 1878), it had not been adequately tested (but see: Wagner and Kay 2002, Galen 2005, Chamberlain and Holland 2008 and further discussion in Chapter 5). In this chapter I tested whether EFN secretion was distracting ants from going inside the flowers, whilst luring them to patrol the vicinity of flowers defending them against florivores using an experimental design in which we prevented EFN secretion.
STUDY SYSTEM AND SITES

In this thesis, all data chapters (Chapters 2-5) contain research conducted on the same study plant (*Turnera velutina*) investigated at two different locations in Mexico (La Mancha, Veracruz and Troncones, Guerrero). Since all these chapters are self-contained research pieces, each will have a paragraph describing essential information about the study system and site, and although tailored to the specific needs and context of each chapter, these sections may be slightly repetitive. Apologies to the reader. However, here I contrasted the main differences between the study sites, providing a justification as to why I explore both sites.

The study system in brief

*Turnera velutina* C. Presl (Passifloraceae: Turneroideae) is a Mexican endemic perennial shrub that grows in coastal sand dune scrubs and in tropical dry forests, from sea level to 1300 m of altitude (Arbo 2005). This species has alternate, petiolated, lanceolate-shaped, and pubescent leaves bearing paired cup-shaped extrafloral nectaries on the abaxial blade surface (Arbo 2005, Villamil et al. 2013). Flowers are axillary, yellow, pentamerous, bisexual, actinomorphic, self-compatible, and last only one day opening from three to five hours. Five floral nectaries are located at the base of the receptacle, between each pair of petals (Torres-Hernández and Rico-Gray 2000, Benitez-Vieyra et al. 2010). Although plants flower year round, reproductive output peaks during the rainy, summer season (Arbo 2005). Fruits mature in 10-20 days (Ochoa-López 2013) and reproductive structures from several ontogenetic stages (buds, flowers, and fruits) may be found in adjacent leaves within the same branch.

*T. velutina* is an entomophilous species, whose pollinators include lepidopterans (*Aphrissa statira, Phoebis phillea, Anteos maerula, and*
unidentified members of the Hesperiidae family), hymenopterans (*Apis mellifera*, *Agapostemon* sp., *Auglochlora* sp., *Ceratina* sp., *Auglochloropsis* sp., *Thygater* sp., and *Lassioglossum* sp.), dipterans (Bombyliidae flies) have also been reported as pollinators (Fig. 2.2; Benitez-Vieyra et al. 2010, Ramos-Castro 2013, Sosenski et al. 2016). Besides displaying physical and chemical defenses and tolerance to cope with herbivore damage, this species also relies on myrmecophily to defend against herbivores (Villamil et al. 2013, Ochoa-López et al. 2015). *Euptoteia hegesia* Cramer (Lepidoptera: Nymphalidae) is the main foliar herbivore, and its activity peaks from June to August (Fig. 1.1; Cuautle and Rico-Gray 2003), although it can be found all year round. The foliar extrafloral nectar is made of sucrose, glucose and fructose in similar percentages (Elias et al. 1975) and is consumed by at 7-13 ant species (Fig. 2.1; Cuautle et al. 2005, Zedillo-Avelleyra 2017), and two wasp species (Torres-Hernández and Rico-Gray 2000). Besides defending the leaves, some of these ants also disperse the seeds (Cuautle and Rico-Gray 2003).

This species was initially described as *Turnera ulmifolia* var. *velutina* (C. Presl) Urb. and recorded as a neotropical species distributed from Florida to Argentina, including several Caribbean islands (Schappert and Shore 1995, Arbo 2005). However, the taxonomy was later amended by Arbo, who identified the Mexican specimens as a separate species and renamed it as *Turnera velutina* C. Presl (Arbo 2005). *Turnera velutina* has been recorded only in Mexico in the provinces of Chiapas, Guerrero, Jalisco, Estado de México, Michoacán, Oaxaca, Tabasco, Tamaulipas and Veracruz. Because of this taxonomic amendment many studies on this species have been published under the name of *Turnera ulmifolia* L. (C. Presl) Urb.
**Figure 1.1.** Some of the ant species patrolling *Turnera velutina* plants. a) Two *Crematogaster* ants (ca) harvesting seeds (se) from a *T. velutina* fruit (fe); b) *Brachymyrmex* sp.; c) *Camponotus mucronatus*; d) *Camponotus bicolor*; e) *Dorymyrmex bicolor*; f) *Pseudomyrmex gracilis*; g) *Cephalotes*.

**Figure 1.2.** Main herbivores of *Turnera velutina*. Left: *Euptoieta claudia*; right *Euptoieta hegesia*. 
Figure 1.3. Main pollinators of *T. velutina*. Top row: Flower and *Apis mellifera*. Middle row: lepidopterans. Bottom row: Native bees, Diptera (Bombyliidae); and wasp.

The study sites in brief
Two different populations of *T. velutina* were studied because the array of pollinators differs greatly between both populations (Fig. 1.4). The European honeybee *Apis mellifera* was introduced in Mexico in the 1800’s (Guzmán-Novoa et al. 2011) and is the dominant pollinator at La Mancha, Veracruz (Ramos-Castro 2013, Sosenski et al. 2016) where most of my PhD research has taken place. The population in Troncones, Guerrero, differs from La Mancha, in that plants are visited by a more diverse array of native insects, mostly butterflies. Furthermore, the aggressivity of patrolling ants differ between locations, with ants from Troncones reported as more aggressive than those at La Mancha (Zedillo and Boege pers. comm.). Increased ant aggressivity creates a larger
potential for direct ant-pollinator conflict. Such features made Troncones an interesting place to study ant-pollinator interactions and investigate how different pollinators (social and solitary) interact with the ant assemblage.

**Figure 1.4.** Map of Mexico indicating with stars the study sites: Troncones, Guerrero and La Mancha, Veracruz.
Chapter 1: General introduction

Conservation of coastal sand dunes in Mexico: an essay

On doing research on a sandy patch...

During my PhD, the most biologically interesting field site I visited was located in Troncones, a seaside village in the state of Guerrero, on the southern coast of Mexico: A vibrant and effervescent area in all respects. Just a few hundred kilometres west along the coast from the legendary beach of Acapulco, a prime holiday destination for 1950’s cinema divas and jet-setters, Troncones lies 380 km south-west of the historic city of Iguala, where (at least) 43 Ayotzinapa College students – training to be rural teachers - were killed on 26th September 2014. Although officially these students were mistakenly killed by the organised crime, it is common knowledge that Mexican army and police forces were involved. Furthermore, recent revelations, sparsely-covered in the mainstream media, suggest that the slaughter was requested by a drug dealer held for trial in the US, whose mercenaries coordinated with the Mexican military and police forces to kill and disappear students heading towards a political rally (although this remains as unofficial news; withheld information by the USA DEA agency). Regardless of Marylin Monroe and Elizabeth Taylor or the 43 missing students (and the DEA’s secrets), the sandy patches of Troncones were of great interest to me. These patches host a large array of the

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1 VICE News Documentary. The Missing 43: Mexico’s Disappeared Students. Link: https://www.youtube.com/watch?v=0jt-urgNN3A
native insects that pollinate the plant I study, *Turnera velutina*: a relative of the passion-fruit. However, my study site stretched between two signposts reading “Se vende”; hence, my field site was on sale.

This sandy patch hosts an ecosystem called coastal sand dune or coastal scrub, which is located at sea-level, just a skip and a jump away from the sea! With the soil being mostly sand, the rainfall quickly runs through, making for a rather dry and water-restricted soil and habitat, even within the humid tropics. You can tell where this ecosystem starts and where the sandy beach ends because the sand in these scrubs is held in place by the roots of vegetation. Precisely because of this, these habitats are often called stabilised dunes, as opposed to the nearby sunbathing-type of dunes, which are mobile and shaped by the wind.

The first settlers of these sandy cords are prostrate plants like *Ipomoea*, the genera of sweet potato. Little by little, these shy sweet potato strings recruit other non-woody species, and then shrubs, until mixed grasslands cover all the sand dunes. The shrubs gather in thickets that recruit more species and form richer communities that spread inland, away from the sea and towards the tropical forest.

Until recently, the 1st of March 2018\(^7\) to be precise, there were no laws or regulations that protected coastal ecosystems in the infamous Mexican LGEEPA: the General Law for Ecological Equilibrium and Environmental Protection. Despite being a country with over 800,000 ha of coastal sand dunes, all we had were six incongruent and mutually cancelling criteria regarding the

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\(^7\) Federal Official Gazette (DOF:23/04/2018). Decree by which several dispositions within the General Law for Ecological Equilibrium and Environmental Protection are amended or added. Diario Oficial de la Federación (DOF:23/04/2018) Decreto por el que se reforman y adicionan diversas disposiciones de la Ley General del equilibrio Ecológico y la Protección al Ambiente.
development requirements on Mexican beaches in a lower ranked law, called the Official Mexican Norm (NOM) (NMX-AA-120-SCFI-2006).

_Duna_ is the Spanish word for dune, and a Norm is a lowly ranked law within the legal hierarchy of Mexican Law. Searching for _duna_ within the NMX-AA-120-SCFI-2006 norm which regulates the quality of Mexican beaches provides just eleven hits. Seven of those hits comprise the paltry and incongruent criteria that do not protect sand dunes, but “establish the requirements and specifications on the quality and sustainability of the beaches”:

1. Native coastal sand dune vegetation shall not be removed, and the beaches must display adequate information regarding the efforts towards sand dune protection. (section 5.4.g)
2. There shall not be any infrastructure upon the coastal sand dunes. (section 5.3.d)

[So far, so good, but...]
3. Any infrastructure that is to be built upon sand dunes, shall be built 5 metres behind the second cord of dunes. In those places where the presence of dunes is not easily identifiable due to size or because these have been removed, such infrastructure should be built behind the stripe of existing vegetation. If such stripe exists. (section 5.9.b)

[Further down the norm states that...]
4. It is forbidden to remove any vegetation from the sand dunes. (section 5.9.d)
5. Beach accesses through the dunes should be wooden skywalks built using appropriate techniques to prevent erosion, allowing constant influx of users into the dune without deteriorating the sand dunes. (section 5.9.e)
6. No vehicle shall be allowed to circulate or park upon the beach or the sand dunes. Except for those vehicles involved in public services such as cleaning, bin collection, security, or towing vehicles. (section 5.5.i)

In March 2018 the Congress finally approved the petition to include the concept “coastal ecosystems” in the LGEEPA. Finally, ecosystems such as mangroves, wetlands, marshes, intertidal zones, coastal sand dunes, coastal lagoons, macroalgae, coral reefs, marine prairies, cenotes, oases, floodable rainforests, and rocky cliffs, were included in the Environmental Protection Legislation! Given the richness of these habitats, and that they are amongst the most lucrative spaces for touristic development, it is hard to believe that the long-standing lack of regulation for coastal environments was a coincidence, or a by-product of the overwhelmingly busy and understaffed Mexican Environment Council.

And so, back from fieldwork at Troncones. Counting is done, fieldwork is over, and I return to the lab desk in Auld Reekie. As I am writing the findings from Troncones I am left with a list of interesting wee puzzles to solve. Ecological nooks and crannies I am desperate to pry into. Ant gossip I am desperate to get! But… all of a sudden I am assaulted by a humongous concern:

What if this patch finally gets sold?

What if someone builds there another holiday house? One more addition to that long tail of holiday rentals which are not inhabited, but rented or occupied for only a few weeks per year.

What if when I go to do the follow-up experiments I dream of, instead of this unregulated patch of trees, hives, plants, beetles, ants and butterflies, I find - a fence. A fence lined-up beautifully with cacti and Aloe vera plants, a couple of magueyes sprinkled here and there. A fence of breath-taking plants, especially
Chapter 1: General introduction

when they decide to flower, which they will certainly do thanks to the caring hand of the local gardener. Him who gets paid per month what the owner probably charges the tourists for a night stay. And when not in flower, these majestic plants are really green, and really thorny, providing a natural discouragement from getting too close to the wooden planks lining the fence from inside. Behind the fence lays an enormous pool, surrounded by an orange clay-tiled patio and white, comfy sofas on which to sunbathe. In the corner, over a bar table, Margarita glasses hang upside down from a black, wrought-iron rail. The thick, blue, blown-glass rims of these harebell cocktail glasses stare into the infertile, orange clay tiles. Upstairs, a balcony under a thatch-roofed _palapa_ will make for the perfect holiday let picture and title.

One more holiday let to go up on Airbnb under the sexy name of: “Casa Prana Yama: Oceanfront Balcony”, or “Casa Joya del Mar: Luxurious beachfront villa”, or perhaps as “La mariposa: Luxury beachfront villa”. All of these names aiming to be the most appealing punchline by grasping different avenues of uniqueness. But all these ads are in fact strikingly similar, sharing a key feature: the beachfront or oceanfront offer. A dangerous punchline, or shall I say a destructive one. The beachfront or oceanfront is, in ecological terms, the stabilised sand dunes ecosystem, an overlooked but heavily endangered habitat worldwide and particularly in Mexico.

Marketing alongside the capitalistic tourist culture has made many of us dream and drool of a beach holiday at a tropical, sandy beach with powdery white sands and warm seas. Many dream of an all-inclusive resort, with a choice of infinity pools, each with a bar, looking out over the ocean. Others dream of a modest hotel, but with the essential air-con, or maybe a very low-budget, low-impact ‘beachfront’ thatched-roof hut. Yet, all of these facilities are built upon coastal habitats. All of them. Mostly on stabilised sand dunes. No matter how
modest, luxurious, environmentally-friendly or wasteful, posh or tacky, or hipster- all of these facilities displace the coastal ecosystems.

On top of these threats comes the icing of the cake, with plenty of room left for a cherry. Lack of protection regulations; developer's interests: these threats have just budded, like bacteria, doubling themselves overnight, with the cheeky approval of the so-called Biodiversity Law. Approved in early April 2018 by a minimal quorum in an under-attended Congress; against the wishes and loud voices of hundreds of scientists, artists, NGO's, and citizens⁸. Nonetheless, the 'biodiversity' law was supported by the Mexican Green party, which is in bed with the ruling party (the PRI). The only 'Green' party in the world that promotes mining and industrial activities inside nature reserves. With this law the Mexican Green party is pushing to deregulate and legalise the exploitation of Nature Reserves in a megadiverse country, such as Mexico.

In Troncones, Guerrero, with the “Se vende” posts marking its east and west limits, facing the sea to the south, and with its northern back against a Tarmacked road, lies one of the last strongholds of coastal sand dunes in Troncones that has not been built upon. Yet. But given the immense pressure for holiday houses in this area, the clock is ticking louder and louder every second. Meanwhile, while I spend days and weeks sorting out the databases, finding the right model, getting plots just right, and writing up my findings, I suddenly realise the hourglass has been dripping dry on the corner of my desk.

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Chapter 1: General introduction

On doing research in a sandy patch, watching it slip away, grain by grain out of your hands as you carry on counting leaves and critters. Keep calm and count critters? Impossible to keep calm!

I am the sole author of all the text used here, with comments on earlier drafts from Jorge Comensal and Thomas Godfrey.

This essay has been published in Spanish as:

*Dunas costeras. Un ecosistema codiciado, soslayado y amenazado.* Revista de la Universidad de México. Daños
‘Scotland small?’
By Hugh MacDiarmid

Scotland small? Our multiform, our infinite Scotland small?
Only as a patch of hillside may be a cliché corner
To a fool who cries ‘Nothing but heather!’ where in September another
Sitting there and resting and gazing around
Sees not only the heather but blaeberry
With bright green leaves and leaves already turned scarlet.
Hiding ripe blue berries; and amongst the sage-green leaves
Of the bog-myrtle the golden flowers of the tormentil shining;
And on the small bare places, where the little Blackface sheep
Found grazing, milkworts blue as summer skies;
And down in neglected peat-hags, not worked
Within living memory, sphagnum moss in pastel shades
Of yellow, green, and pink; sundew and butterwort
Waiting with wide-open sticky leaves for their tiny winged prey;
And nodding harebells vying in their colour
With the blue butterflies that poise themselves delicately upon them;
And stunted rowans with harsh dry leaves of glorious colour.
‘Nothing but heather!’ — How marvelously descriptive!
And incomplete!
CHAPTER 2

ANT AGGRESSIVITY AND PROTECTION EFFECTIVENESS

Which are the most aggressive ant species?
INTRODUCTION

Mutualisms, defined as reciprocal exploitation that results in a net benefit for both partners (Begon et al. 1986), are ubiquitous in nature and involve organisms from every kingdom (Bronstein 2001). Given their frequency there are many opportunities for exploitation of mutualisms (Bronstein 2001, Raine et al. 2004), in which one partner (now defined as a parasite) exploits resources offered by a second partner but, but without being mutually exploited by their partner, e.g., without also providing a net benefit for its partner (Bronstein 2001). And so, the mutual exploitation with a net benefit for both parts is broken.

Ant-plant mutualisms are also widespread, with over 4000 angiosperm species bearing EFN to attract ants, plus more species that attract ants via other rewards such as food bodies or nesting sites (Keeler 2014). In tropical rainforests approximately one third of woody dicots, herbs and vines produce ant-luring structures (Davidson and Cook 2008), and ants represent 50% of arthropods (Davidson and Cook 2008).

EFN is a general resource, easily accessible to all agents encountering it (Carroll and Janzen 1973, but for exceptions see: Kautz et al. 2009). EFN-bearng plants are thus usually associated with multiple ant species, leading to facultative and diffuse interactions, in contrast with obligate mutualisms which are biunivocal interactions, usually involving specialised partners (Rico-Gray and Oliveira 2007, Herrera and Pellmyr 2009, Melián et al. 2009, Dáttilo et al. 2016). On average, facultative ant-plants are visited by 6-9 ant species, whilst obligate ant-plants are visited by one or occasionally two ant species (Rico-Gray and Oliveira 2007, Rosumek et al. 2009). The diffuse nature of many ant-plant associations limits the potential for tight coevolutionary interactions between partners, and these mutualisms are often associated with great variation in the net benefits received.
Several factors can influence the net outcome of facultative ant-plant interactions and these have been classified by Del-Claro and Marquis (2015) as internal (the identity of the particular species involved in the interaction) or external (abiotic factors that affect at least one partner). Internal variation is driven by differences among species in the quality (effectiveness) and quantity (frequency and abundance) of their interactions (Ness et al. 2006, Alves-Silva et al. 2015, Fagundes et al. 2017) and can often be stronger than external, abiotic factors such as fire (Del-Claro and Marquis 2015).

Although, on average, ant species within facultative mutualisms are less aggressive than those in obligate mutualisms (Rosumek et al. 2009), interacting with multiple ant partners may be advantageous for plant for several reasons (Fagundes et al. 2017). First, if found simultaneously, ant species may differ in their anti-herbivore strategy, and so defensive success may increase with the number of partners (for exception on host sharing: Raine et al. 2004, Heil et al. 2009, Palmer et al. 2010). Redundant species, can have a synergic effect and enhance overall protection levels at a lower colony cost. Second, redundant species can replace absent protector species under different conditions, as these diffuse mutualists are variable in time and space.

Furthermore, despite the abundance of facultative mutualisms (Keeler 2014), the diversity of their associated species (Rosumek et al. 2009, Fagundes et al. 2017), the accessibility of EFN (Carroll and Janzen 1973, but for exceptions see: Kautz et al. 2009) and the consequent ease to cheat in such mutualisms (Bronstein 2001), very few examples of cheating species have been documented. According to
Raine et al. (2004), until 2004 only four ant species had been detected as parasites (Janzen 1975, Young et al. 1996, Yu and Pierce 1998, Gaume and McKey 1999, Stanton et al. 1999), although now at least fifteen cases have been added to the list (Raine et al. 2004, Kautz et al. 2009, Del-Claro and Marquis 2015, Fagundes et al. 2017). This low frequency of cheaters amongst facultative ant mutualists may reflect a biological truth, or represent a research underestimation bias deriving from the fact that few studies have actually estimated the protecting benefits of individual ant species.

Functional variation among ant species in apparent mutualisms must be kept in mind when using the abundance of ants to gauge the level of protection experienced by a plant. Although the number of ants recruited may be an estimate of plant attractiveness, ant identity is crucial for estimation of defensive benefits (Box 2.1). Many studies assume that all ant species collecting extrafloral nectar defend plants against herbivores, although very few studies have tested the protection services (Box 2.1) provided by each ant species. When aggressivity is evaluated, ant guards are often ranked based on a qualitative approach, using observations of clearly aggressive behaviours, such as biting and attacking, or ambiguous behaviours, such as recruitment as indicators of aggressivity (Ness et al. 2006). However, a quantitative approach is a more accurate estimate that reduces subjectivity and misinterpretation of aggressive behaviours (Ness et al. 2006, Fagundes et al. 2017), as for instance, it is easier to detect a biting attack than other attacking modes such as acid spraying. Hence, a currency to objectively measure the protection effectiveness (Box 2.1) of ants – such as herbivore damage prevented – in terms directly linked to plant benefits is crucial. But even fewer have quantified these parameters (but see: Apple and Feener Jr 2001, Raine et al. 2004, Ness et al. 2006, Del-Claro and Marquis 2015, Fagundes et al. 2017).
Chapter 2: Ant aggressivity

The protection effectiveness of an ant species is influenced by its taxonomic identity, which influences multiple morphological and behavioural traits including aggressivity (Del-Claro and Marquis 2015, Fagundes et al. 2017), frequency of interaction with the host plant (Apple and Feener Jr 2001, Cuautle and Rico-Gray 2003, Ness et al. 2006), strength of recruitment, effectiveness in capturing or repelling a herbivore (Itioka et al. 2000, Raine et al. 2004, Ness et al. 2006, Fagundes et al. 2017), the plant ontogenetic stage and nectar availability (Villamil et al. 2013, Lange et al. 2017), and environmental conditions (Chamberlain and Holland 2008, Del-Claro and Marquis 2015). The most effective ant protectors would be those ants that patrol often, and are likely to find, capture or deter herbivores from the host plant.

Box 2.1 Three key definitions

AGRESSIVITY
Refers to the antagonistic behaviours displayed by ants. Particularly the probability of attacking, harassing, or displaying other behaviours to deter a visitor and the severity of such response. This is an estimate of ant behaviour and may play a role in other interactions such as pollinator deterrence.

PROTECTION EFFECTIVENESS
Refers to the anti-herbivore capacity of ants and their ability to prevent or reduce plant damage by herbivores. This is an estimate of their benefits to the plant (although it inherently involves ant behavioural traits) and a proxy of their quality as plant defenders.

DEFENSIVE QUALITY
The defensive quality is a qualitative, categorical and descriptive assessment of the overall ability of ants to prevent or reduce plant damage (e.g. good, bad, effective, non-effective, non-defenders).
Chapter 2: Ant aggressivity

Quantifying aggressivity: a brief summary

Few studies have assessed the defensive quality of different ant species associated to ant-plants and even less have experimentally quantified the aggressivity of different ant species (Apple and Feener Jr 2001, Raine et al. 2004, Ness et al. 2006, Del-Claro and Marquis 2015, Fagundes et al. 2017). Some studies have assessed ant aggressivity and responses when their host plant is artificially damaged (Christianini and Machado 2004, Romero and Izzo 2004, Zedillo-Avelleyra 2017). However, experimental quantification of ant aggressivity via *in situ* assays involving real herbivores on a host plant is, in my opinion, the best and most realistic method to standardise and quantify aggressive behaviours by defensive mutualists. Some studies using herbivores use surrogates that may not usually damage the plants but are experimentally convenient. Examples include termite workers (Apple and Feener Jr 2001, Fagundes et al. 2017), diverse local caterpillars and orthopterans (Fiala et al. 1989), or caterpillars of the generalist herbivore *Manduca sexta* (Ness et al. 2006). However, the degree to which ant aggressivity to surrogate herbivores mimics natural conditions remains unknown and is a cause for concern. My experiment is one of the few, perhaps the first one, to use natural herbivores of a host myrmecophile. The metrics and calculations of papers that, in my opinion, have best quantified ant aggressivity in an ant-plant context are summarized in Box 2.2.
Box 2.2 Aggressivity quantification methods

**FIALA**

Fiala et al. (1989) evaluated protection effectiveness by placing a diverse array of local herbivore surrogates (several lepidopteran caterpillar species, orthopterans, beetles and bugs) and eggs on *Macaranga* leaves. They assessed the protection effectiveness of the ant species *Crematogaster bornbeensis* in four congeneric host plants (*M. triloba, M. hosei, M. hypoleuca, M. hulletti*). They evaluated ant protection using only a **qualitative** aggressivity metric: attack and repellence rates (Fiala et al. 1989).

**NESS**

Ness et al. (2006) estimated the differences in the quality and quantity of defensive services provided by the four most common ant visitors to *Ferocactus wislizeni* when larvae of the generalist lepidopteran herbivore *Manduca sexta* were placed on the host plant.

They multiplied the quantity of the service by its quality to quantify the non-linear relationship between both components. They used Michaelis-Menton equations to describe this relationship, arguing that defensive behaviours behave in a similar fashion to enzyme saturation dynamics.

They used ant per capita effectiveness as the **quality** metric, defined as the number of workers required to remove an experimentally placed *Manduca sexta* caterpillar on the host plant. The **quantity** metric was the number of ants that visited the plant and the frequency of visitation (Ness et al. 2006).

They suggest that the quantitative and qualitative components of an interaction are often not independent because any bodyguards beyond those needed to remove all herbivores may be redundant (Ness et al. 2006). Based on this assumption they predict that as the quantity of an interaction increases, the quality will often decline. Consequently, the quantity and quality components are inversely proportional and linked via a non-linear relationship (Ness et al. 2006).
Box 2.1 continued. Aggressivity quantification methods

FAGUNDES
Fagundes et al. (2017) quantified ant aggressivity in the most comprehensive study so far, by placing termites (Sintermes sp.) as surrogate herbivores on 20 plants for each of 10 host plant species.

They quantified the protection effectiveness of the ant community within the whole plant community in a riparian grassland, producing detailed quantitative outputs with a network approach that included every ant species associated with every plant species.

Aggressivity was quantified as the product of a qualitative and a quantitative component. The qualitative metric was based on ant behaviour and effects on the termites. They recorded defence as successful in their assay when ants found, captured or repelled the termite, and a failure when the ants found but ignored the termite. The quantitative component was the inverse of the ant response time, multiplied by the repellence efficiency within that essay. Since they placed multiple termites per assay, they could produce a repellence efficiency estimate per assay.

\[ PE = QNC \times QLC \]

- \( PE \) = protection effectiveness
- \( QNC \) = number of ants recruited
- \( QLC = \frac{1}{\text{time}} \times \text{repellence efficiency} \)

Based on the QNC and QLC components, ant species were classified into four functional groups:

a) **highly effective protectors**: high recruitment and quick removal of herbivores on most plant species

b) **low effective protectors**: high recruitment and quick removal of herbivores on a few plant species

c) **ineffective protectors**: low recruitment, low detection and removal of herbivores

d) **Non-protectors**: no recruitment, no detection or removal of herbivores.
QUESTIONS & HYPOTHESES

Here I tested and quantified the aggressivity of six species within the ant community associated with *T. velutina* at La Mancha, Veracruz using larvae of the natural herbivore *Euptoieta hegesia* (Lepidoptera), to answer the following questions:

(i) Do ants vary in their aggressivity displays and in their ability to repel herbivores (protection effectiveness)? If so, which are the most aggressive ant species?

(ii) Does any observed variation in aggressivity correlate with previous observations of ant-herbivore interactions for the same or related ant taxa?

My null hypothesis was that ant species do not differ in their aggressivity display or protection effectiveness.

MATERIALS AND METHODS

Study site

All experiments and observations were conducted at La Mancha, Veracruz in coastal sand dunes within the CICOLMA Field Station in the Gulf of Mexico (19°36’N, 96°22’W, elevation <100 masl).

Study system

*Turnera velutina* (Passifloraceae) is a Mexican endemic perennial shrub (Arbo 2005) that is also a myrmecophile (Cuautle and Rico-Gray 2003) that grows in lowland coastal environments. It establishes a facultative mutualism with up to 13 ant
species (Cuautle et al. 2005, Zedillo-Avelleyra 2017). Extrafloral nectar is provided in paired cup-shaped glands located at the bottom of the leaf blade or on the leaf petiole, on the underside of the leaves. *T. velutina* is a self-compatible, monomorphic herkogamous species that requires pollinators for adequate seed production (Sosenski et al. 2016). Although it flowers year-round, flowering peaks in summer (Cuautle et al. 2005). Flowers are bisexual, last one day, and are insect-pollinated (Sosenski et al. 2016). As rewards for pollinators, flowers offer pollen and floral nectar that is easily accessible at the base of the yellow, pentameric, bell-shaped corolla. The main pollinators at La Mancha are honeybees, and the main herbivores are the caterpillars of the butterfly *Euptoieta hegesia* (Nymphalidae), although caterpillars of the congeneric butterfly *Euptoieta claudia*, grasshoppers (Acrididae) and hemipterans also feed on the plants (Cuautle and Rico-Gray 2003, Villamil-Buenrostro 2012, Ochoa-López et al. 2018). The fruits are dry septicidal capsules that dehisce to expose the seeds, which are dispersed by ants. The seeds bear an elaiosome rich in fatty acids and lipids, which needs to be removed to allow imbibition and germination (Ochoa-López 2013). Myrmechochorus ants swarm onto newly open fruits, carry the seeds towards their nests and detach the elaiosome for consumption (Cuautle et al. 2005, Salazar-Rojas et al. 2012).

**Anti-herbivore assays**

Larvae of *Euptoieta hegesia* were reared and maintained in greenhouse conditions under mesh exclusions feeding on *Turnera velutina* plants from multiple ontogenetic stages (Rebollo Hernández 2018). For the aggressivity assays, larvae were kept in 0.5 L plastic containers with a tulle mesh window lid for ventilation. Containers were lined with wet filter paper and fresh *T. velutina* foliage was provided for food and shelter. The frontal width of the head capsule of larvae was measured and larvae were classified in three size groups: small (1.58±0.054 mm), medium (1.77±0.027 mm), and large (2.77±0.019 mm). Only large larvae were used in aggressivity assays.
In the field, adult plants patrolled by a given ant species were selected haphazardly and the numbers of leaves, buds, flowers, and fruits of a haphazardly selected branch were counted. The species identity and number of patrolling ants on that branch was also recorded. One larva was placed on a vegetative leaf (i.e. not associated with any reproductive structure) of the selected branch and the behavioural response of ants and the larva was observed for 10 minutes. As ant behaviours I recorded evasion, attending, recruiting, attacking and repelling behaviours, and the time to these events. As larval behaviours I recorded relocation (moving to another part of the plant) and drop-off (falling / launching themselves from the plant), and the time to these events, as described below.

Ant behaviours:

- Ignore / Not find / Avoid: Did the ants ever reach the leaf with the larva? If found, did ants ignore the larva? In ignore / fail to find behaviours ants neither respond to the larva nor do they remain on the leaf with the larva. Avoidance behaviours are those in which ants find the larva but then move away from the leaf or branch with the larvae and do not return during the assay, and neither patrolled nor recruited more ants to the same leaf or branch. (Categorical (yes/no) variable)
- Response time: How long after it was placed was the larva found by ants? (Continuous variable)
- Patrol: Once the larva has been found, do ants patrol around it or on the leaf bearing it? (Categorical (yes/no) variable)
- Recruitment: Did the first ant to find a larva recruit more ants? If so, then the number of ants recruited. (Continuous variable)
Chapter 2: Ant aggressivity

- **Attack**: Did the ants attack the larva? Attack can be either by bites, direct harassment (such as antennation or stinging), or by spraying the larvae through the acidopore (see further details in Results). (Categorical (yes/no) variable)
- **Time to first attack**: How long after the larva was placed on the plant was it attacked? (Continuous variable)
- **Repelling**: Did the ants cause the larvae to abandon the leaf or the plant? (Categorical (yes/no) variable)

Larval behaviours:

- **Relocation**: Did the larva abandon the leaf where it was initially placed and moved to another leaf within the same plant?
- **Time to relocation**: How long after placement did the larva relocate?
- **Drop-off**: Did the larvae drop itself off the plant as a consequence of ant interference?
- **Time to drop-off**: How long after placement did the larva drop-off?
- **Foraging**: Was the larvae observed feeding on any plant structure?

If a larva was attacked on one assay, they were not used again in another assay that same day. If they were not attacked after an assay, larvae were used again for a maximum of four assays in a single day. After a trial, larvae were placed back into their individual containers at the greenhouse. Larval containers were washed and the wet lining was changed every night, foliage was replenished as required. These assays were conducted between 22 October – 4 November, 2016.

All statistical analyses were done in R software. Mixed models were fitted using the ‘lme4’ (Bates et al. 2016) and MCMCglmm (Hadfield 2010), and *post-hoc* model comparisons were fitted using the ‘multcomp’ package (Hothorn et al. 2008), unless otherwise specified.
I quantified ant aggressivity and protection effectiveness by assessing ant responses to larvae placed on their host plant by evaluating several metrics in each assay. These metrics were the maximum aggressivity score displayed per assay, the likelihood of each species to display each of the different behaviours defined in response to a larva on their host plant, the number of ants recruited, the time to first response and the time to herbivore repellence. Furthermore, I combined several of these metrics into a Protection Effectiveness Index, allowing us to evaluate and compare the aggressivity display and anti-herbivore efficiency of the different ant species tested.

**Maximum aggressivity score**

I assigned each behaviour a number between 0-5, in rank of increasing apparent aggressivity:

0 – Ignore / Not find  
1 – Find  
2 – Patrol  
3 – Recruit  
4 – Attack  
5 - Repel

Based on this score, I assigned every assay a score reflecting the maximum level of aggressivity displayed by the patrolling ants. The effect of ant species identity on the maximum aggressivity score was analysed using a Poisson mixed model, with maximum aggressivity score as the response variable and ant species identity fitted as a fixed effect. Plant identity and assay identity were fitted as random effects, along with an observation-level random effect (OLRE) to account for overdispersion (Hinde 1982). Post hoc Tukey model comparisons were used to test for significant differences between all species.
Chapter 2: Ant aggressivity

**Likelihood of aggressive behaviours**
The effect of ant species identity on the likelihood with which ants displayed each of the aggressive behaviours recorded when encountering a larva on their host plant, was evaluated using binomial mixed models. These likelihoods are hereafter referred to as efficiencies for each behaviour recorded, such as attacking or patrolling efficiency.

Species varied greatly in their likelihood of behaviours, with some always displaying a given behaviour whilst others never displayed it, causing an extreme category problem (Hauck-Donner effect) in this data for recruiting, attacking, and repelling behaviours. Because of this, I analysed these models using the ‘MCMCglmm’ R package (Hadfield 2010), fitting binomial Bernoulli models with a ‘probit’ link function. I fitted one model for each behaviour independently (ignore/not find, patrol, recruit, attack, repel). I fitted the presence or absence of each behaviour as the response variable and ant species as a fixed effect. Plant identity and assay identity were fitted as random effects. Wald tests were used as post hoc contrasts to detect significant differences between species. However, I decided to plot only the mean from the raw data, as standard errors and confidence intervals from the model outputs are extremely wide due to the Hauck-Donner effect.

**Ant recruitment**
The effect of ant species on the number of ants recruited per assay was analysed using a Poisson mixed model. The number of ants was fitted as the response variable, and ant species as a fixed effect. Plant identity, assay identity and OLRE were fitted as random effects. Post hoc Tukey comparisons were used to detect significant differences between species in the number of ants recruited.
Chapter 2: Ant aggressivity

**Response time**
The effect of ant species on the ants’ response time per assay was analysed using a Poisson mixed model. For those assays in which ants did not ignore the larva, the number of seconds after which ants found the larva was fitted as the response variable, and ant species as a fixed effect. Plant identity, assay identity and OLRE were fitted as random effects. *Post hoc* Tukey comparisons were used to detect significant differences between species in the response time.

**Repellence time**
The effect of ant species on the time it took for a larva to move away from the leaf where it was originally placed was analysed using a Poisson mixed model. Repellence included larvae relocating themselves to another leaf, or larvae dropping-off entirely from the plant. For those assays in which ant interference lead larvae repellence, the number of seconds after which the larvae was repelled was fitted as the response variable, and ant species as a fixed effect. Plant identity, assay identity and OLRE were fitted as random effects. *Post hoc* Tukey comparisons were used to detect significant differences between species in the time to repellence.

**Protection effectiveness**
I evaluated the Protection Effectiveness (PE) of different ant species by creating an index combining several of the previously described defensive metrics as follows:

\[
PE = QNC \times QLC
\]

\[
QNC = \text{Number of ants recruited}
\]

\[
QLC = \frac{1}{\text{Response time}} \times (4 \times \text{Attack} + 5 \times \text{Repel});
\]

where PE is the protection effectiveness index; QNC is the quantitative component of the index and is the number of ants recruited; QLC is the qualitative part of the index which includes the *Response time* (the time between larval placement and ant detection), and the attack and repel score combined. *Attack* and *Repel* are the
Bernoulli variables for presence (1) or absence (0) of either behaviour, each multiplied by their aggressive score, respectively. I decided to include the score for attack and repellence in order to account for differences in attack and repellence efficiency between species, as some species may be aggressive attackers but not effective at repelling larvae from this particular species and instar.

Differences between ant species in their PE score were initially analysed using a Poisson mixed model. The PE index values resulted in a continuous variable between 0-4 that did not follow any usual distribution. Because of this, I recalculated the PE by removing the response time component \((PE = Number\ of\ ants\ recruited \times (4\ Attack + 5\ Repel))\) in order to obtain an integer number that was fitted as the response variable, and ant species identity was fitted as a fixed effect. The reciprocal of response time \((Rt^{-1})\) was fitted in the model as a log-transformed offset to account for this variable whilst maintaining Poisson data in the response variable. However, this statistical approach was abandoned as the model could not run, producing unconstrained predicted values that lead to the arising of infinite numbers, despite having a log link function that is intended to constrain predicted values. Probably these problems are inherent to the unusual distribution of the PE data. Hence, I finally analysed the data using non-parametric statistics. I used a Kruskal-Wallis test to determine whether Protection Effectiveness scores differed among ant species. I used Dunn tests as post hoc model comparisons to find significant differences between species pairs.

Ant species were quantitatively ranked according to the values of PE. Furthermore, I quantitatively classified them into four categories depending on their high, low or null values of QNC and QLC (Fagundes et al. 2017). Those ant species with high values of QNC and QLC were considered highly effective defenders and highly aggressive species. Species with low QNC and high QLC were
considered as low effective defenders and aggressive species. Species with low QNC and low QLC were considered as ineffective defenders and non-aggressive species. Finally, species with a $PE$ value of zero were considered as parasitic species.

**RESULTS**

At La Mancha I observed ten ant species interacting with *T. velutina* from four families and quantified and ranked the aggressivity of the six most frequent species (Table 1).

**Table 1.** Ants species reported in *Turnera velutina* at CICOLMA, Veracruz (Zedillo-Avelleyra, 2015) and observed in these studies. Species whose aggressivity was tested are marked with an asterisk (*).

<table>
<thead>
<tr>
<th>Family</th>
<th>Sting</th>
<th>Acidopore</th>
<th>Species</th>
<th>Patrolling habit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dolichode rinae</td>
<td>Present (slit)</td>
<td>Dolichoderinae</td>
<td>Dorymyrmex bicolor</td>
<td>Gregarious *</td>
</tr>
<tr>
<td>Formicinae</td>
<td>Present (circular)</td>
<td>Formicinae</td>
<td>Camponotus planatus</td>
<td>Loner *</td>
</tr>
<tr>
<td></td>
<td>Absent</td>
<td></td>
<td>Camponotus claviscapus</td>
<td>Not observed</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Camponotus mucronatus</td>
<td>Loner</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Camponotus novogranadensis</td>
<td>Loner</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Brachymyrmex sp.</td>
<td>Gregarious *</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Paratrechina longicornis</td>
<td>Gregarious *</td>
</tr>
<tr>
<td>Myrmicinae</td>
<td>Present</td>
<td>Present retracted</td>
<td>Myrmicocrypta sp.</td>
<td>Not observed</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Monomorium ebenium</td>
<td>Gregarious</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cephalotes</td>
<td>Loner/few *</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Crematogaster</td>
<td>Gregarious *</td>
</tr>
<tr>
<td>Pseudomyrmecinae</td>
<td>Present well</td>
<td>Present</td>
<td>Pseudomyrmex gracilis</td>
<td>Loner</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Absent</td>
<td>Pseudomyrmex sp.1</td>
<td>Not observed</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pseudomyrmex sp.2</td>
<td>Not observed</td>
</tr>
</tbody>
</table>
Quantifying aggressivity

Ant species differed significantly in their recruiting, attacking and repelling efficiencies, but not in their herbivore finding efficiency or patrolling likelihoods (Table 2). Behavioural differences were very consistent between some species, with certain species always displaying a given behaviour, whilst others never displayed it. For instance, Cephalotes ants never attacked the larvae, whilst Dorymyrmex bicolor always did (Figs. 1,2).

Figure 1. Frequency of assays with each of the maximum aggressive behaviour scores per ant species, when encountering a larva on their host plant.

Cephalotes sp. ants were the least aggressive ants, never observed attacking or repelling any of the larvae, and ignoring larvae in 25% of the essays (Table 2; Figs, 1-2). Even when they found the larvae, they only patrolled around the leaf and were observed recruiting in only one assay (Fig. 1).
Chapter 2: Ant aggressivity

Except for *Cephalotes*, all ant species recruited fellow ants in all assays where they found the larva (Fig. 1,2, Table 2). Highly aggressive species prone to attacking the larvae found were not always the most efficient larvae repellers (Fig. 2, Table 2). Further details regarding behavioural observations of ants are available in the Supplementary Natural History Notes: Ants.

Although *C. planatus* were efficient at larvae repellence, repelling 56% of larvae they found (Fig. 1) their repellence efficiency predicted by the model was only 32% because they seem to be very inefficient at detecting the larvae on the plant they are patrolling, ignoring or failing to find 23% of the larvae. Hence, they ranked lower on the maximum aggressivity score (Table 2).

*Paratrechina longicornis* had an average finding efficiency of 87%, and often engage in aggressive behaviours with the larvae found. Yet, with a repellence efficiency of only 15% this species is the least efficient at repelling larvae among ant species found to have this effect (excluding *Cephalotes*) (Table 2, Fig. 1).

*Crematogaster* sp. had an average finding efficiency of 82%, and always engaged in aggressive behaviours once they found the larvae. Despite attacking the larvae in 75% of the assays, they had a low repellence efficiency of 54% (Table 2).

*Brachymyrmex* sp. had an average finding efficiency of 87%, and almost always engage in aggressive behaviours with the found larvae (88%), and attacking the larvae in 87% of the assays, resulting in a very high repelling efficiency of 81% of the larvae found.

*Dorymyrmex bicolor* ants were the most efficient species at larvae detection, finding 100% of the larvae placed on their plant, and always engaging in some sort of aggressive defensive behaviour, and attacking the larvae in 98% of the assays. They were also the most efficient at repellence, repelling 95% of the caterpillars found.
Figure 2. Per species probability of ants displaying different behaviours when encountering a larva on their host plant. Dots represent the proportion of the data (positive/total events), the $P$ value displayed indicates the significance of the effect of species on each behaviour, and different letters indicate significant differences between species ($P < 0.05$).
Table 2. Model estimates for the effect of ant species on the likelihood of ants displaying different defensive behaviours.

<table>
<thead>
<tr>
<th>Response</th>
<th>Fixed effects</th>
<th>N</th>
<th>DIC</th>
<th>$X^2$</th>
<th>P-value</th>
<th>Random effects</th>
<th>Posterior mean</th>
<th>Lower 95% CI</th>
<th>Upper 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ignore/Not find</td>
<td>Ant species</td>
<td>93</td>
<td>70.10</td>
<td>3.21</td>
<td>0.66</td>
<td>Assay</td>
<td>8.1</td>
<td>8.37$^{-0.05}$</td>
<td>27.6</td>
</tr>
<tr>
<td>Patrol</td>
<td>Ant species</td>
<td>93</td>
<td>101.89</td>
<td>8.20</td>
<td>0.14</td>
<td>Assay</td>
<td>5.37</td>
<td>4.14$^{-0.06}$</td>
<td>33.81</td>
</tr>
<tr>
<td>Recruit</td>
<td>Ant species</td>
<td>93</td>
<td>90.11</td>
<td>21.71</td>
<td>0.0005</td>
<td>Assay</td>
<td>0.98</td>
<td>4.26$^{-0.08}$</td>
<td>4.46</td>
</tr>
<tr>
<td>Attack</td>
<td>Ant species</td>
<td>93</td>
<td>86.78</td>
<td>19.36</td>
<td>0.001</td>
<td>Assay</td>
<td>2.41</td>
<td>2.04$^{-0.07}$</td>
<td>8.93</td>
</tr>
<tr>
<td>Repel</td>
<td>Ant species</td>
<td>93</td>
<td>79.58</td>
<td>13.74</td>
<td>0.01</td>
<td>Assay</td>
<td>2.79</td>
<td>0.0001</td>
<td>11.38</td>
</tr>
</tbody>
</table>
Chapter 2: Ant aggressivity

**Ant recruitment, response and repellence time**

I found significant differences between ant species in the number of ants recruited after finding a *Euptoieta hegesia* caterpillar on their host plant (Fig. 3a, Tables 3 & 5). *Cephalotes* had the smallest ant recruitment, recruiting on average less than an ant per assay, contrasting with *Brachymyrmex* ants which recruited on average 24 ants (Table 3 & 5, Fig. 3). Based on the post hoc Tukey contrasts I can split ant species into three groups with significant differences in the number of ants recruited towards herbivores: high recruiters (*Brachymyrmex* sp., *Dorymyrmex* sp., *Crematogaster* sp., and *Paratrechina longicornis*: >5 ants), low recruiters (*Camponotus planatus*: <5 ants), and non-recruiting species (*Cephalotes* sp.: < 1 ant) (Tables 3, Fig. 3a). This and the following two analyses on response and repellence time only included ants which did engage in any response; those that ignored or did not find the larvae were excluded from these analyses, but accounted for in the analyses regarding likelihood of behaviours and protection effectiveness estimates.

Ant species differed significantly in their response time (Tables 3 & 5), with the ant community being split into two groups: slow responders, finding larvae in >20 sec. (*Cephalotes*, *Camponotus planatus*, *Crematogaster* sp., and *Paratrechina longicornis*), and fast responders finding larvae in <20 sec. (*Brachymyrmex* sp. and *Dorymyrmex* sp.) (Fig. 3b). The time it took for larvae to be repelled also differed significantly between ant species (Table 3 & 5), with the ant community being split into three significantly different groups: *Cephalotes* ants who were never seen repelling a larva; slow repelling species managed to repel a larva in >120 sec. (*Camponotus planatus*, *Crematogaster* sp., and *Paratrechina longicornis*), and fast repelling species that achieved this in <120 sec (*Brachymyrmex* sp. and *Dorymyrmex* sp.) (Fig. 3c).
Figure 3. a) Ant recruitment, b) response time and c) time to larval repellence times for different ant species after encountering a larva placed on their host plant. Different letters indicate significant differences between species ($P < 0.05$).
**Table 3.** Model predictions for the number of ants recruited, ants’ time to first response and larval time to repellence after ant interference in assays for different ant species interacting with *Turnera velutina*.

<table>
<thead>
<tr>
<th>Ant species</th>
<th>Ant recruitment</th>
<th>Response time</th>
<th>Repellence time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimate</td>
<td>SE</td>
<td>Prediction (ants)</td>
</tr>
<tr>
<td><em>Cephalotes</em> sp.</td>
<td>-0.29</td>
<td>0.44</td>
<td>0.74 ± 1.5</td>
</tr>
<tr>
<td><em>Camponotus planatus</em></td>
<td>1.44</td>
<td>0.33</td>
<td>4.22 ± 1.4</td>
</tr>
<tr>
<td><em>Paratrechina longicornis</em></td>
<td>2.26</td>
<td>0.39</td>
<td>9.58 ± 1.47</td>
</tr>
<tr>
<td><em>Crematogaster</em> sp.</td>
<td>1.67</td>
<td>0.47</td>
<td>5.31 ± 1.61</td>
</tr>
<tr>
<td><em>Brachymyrmex</em> sp.</td>
<td>3.18</td>
<td>0.40</td>
<td>24 ± 1.5</td>
</tr>
<tr>
<td><em>Dorymyrmex bicolor</em></td>
<td>2.43</td>
<td>0.39</td>
<td>11.35 ± 1.48</td>
</tr>
</tbody>
</table>
Collinearity plots between aggressivity variables

I explored the correlation between different metrics used to assess the aggressivity displays and protection effectiveness of the ant species associated with *T. velutina* using Pearson correlations. The number of ants recruited was negatively and exponentially correlated with response time (Fig. 4a). Assays with the greatest number of ants recruited also occurred in a relatively shorter period of time, suggesting that the high recruiters are also fast recruiters (Fig. 4a).

Ignoring efficiency (the likelihood to ignore or not find a larva) was positively and significantly correlated with response time \( r = 0.33, t(5,77) = 3.07, P = 0.002 \), so ants more likely to ignore or fail to find the larva also had longer response times (Fig. 4b). Repellence efficiency was significantly and negatively correlated with response time \( r = -0.35, t(5,77) = 3.35, P < 0.001 \) and with repellence time \( r = -0.32, t(5,36) = 2.07, P = 0.008 \), implying that more efficient repelling ants were faster at finding the larva and removing it in a reduced period of time (Fig. 4c,f). Repellence efficiency was significantly and positively correlated with the number of ants recruited \( r = 0.23, t(5,90) = 2.33, P = 0.02 \), implying that more ants are more efficient at repelling a given herbivore (Fig. 4d). Repellence efficiency was also positively and significantly correlated with attacking efficiency \( r = 0.89, t(5,91) = 19.08, P < 0.0001 \), implying that attacking efficiency is in fact an excellent predictor of ant taxon defensive quality and efficiency at repelling herbivores. However, I also found that some species may be very prone to attack but have lower repelling efficiency than less aggressive species, as is the case for *Paratrechina longicornis* and *Camponotus planatus* ants, and hence I decided to include both attack and repelling behaviours in the aggressivity and protection effectiveness index, aiming to obtain a more comprehensive metric of both aspects.
Figure 4. Correlations between the different aggressivity and defensive variables assessed for the ant community interacting with *T. velutina*. For significant correlations, the Pearson correlation coefficient (r) and the associated *P*-value are indicated. a) Response time vs. number of ants recruited; b) Probability of ignoring a larva vs. response time; c) Repellence efficiency vs. response time; d) Repellence efficiency vs. number of ants recruited; e) Repellence efficiency vs. attacking efficiency; f) Repellence efficiency vs. time to repellence.
Chapter 2: Ant aggressivity

**Maximum aggressivity score**
Ant species differed significantly in their maximum aggressivity score (Table 5) allowing me to split the ant community into two groups: defensive and non-defensive ants, with only *Cephalotes* in the latter category (Fig. 5f, Table 4-5).

**Protection Effectiveness Index**
The quantitative (QNC) and qualitative (QLC) components used to calculate PE were not significantly correlated \((r = 0.19, t_{(5,77)} = 1.77, P = 0.08;\) Fig. 5a). The protection effectiveness index differed significantly between ant species \((X^2_{(5, 92)} = 23.68, P = 0.0002)\). This index split the ant community into two groups, here termed defenders and non-defender species, with *Cephalotes* identified as the only non-defender ant species (Fig. 5b). Ant species were classified according to their high or low QNC and QLC values as follows: *Dorymyrmex bicolor* and *Brachymyrmex* ants had high QNC and QLC values, and hence were classified highly effective defenders and highly aggressive species (Fig. 5c). *Paratrechina longicornis* had a high QNC but low QLC value, and hence was classified as a low aggressive and low effective defender, whilst *Camponotus planatus* and *Crematogaster* ants were considered ineffective defenders. Finally, *Cephalotes* ants were identified as parasitic species because of their null QNC and QLC values.
Table 4. Model predictions of maximum aggressivity score for different ant species interacting with *Turnera velutina*.

<table>
<thead>
<tr>
<th>Ant species</th>
<th>Maximum aggressivity</th>
<th>Estimate</th>
<th>SE</th>
<th>Prediction (ants)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cephalotes</em> sp.</td>
<td></td>
<td>0.16</td>
<td>0.23</td>
<td>1.18 ± 1.26</td>
</tr>
<tr>
<td><em>Camponotus planatus</em></td>
<td></td>
<td>1.06</td>
<td>0.13</td>
<td>2.90 ± 1.14</td>
</tr>
<tr>
<td><em>Paratrechina longicornis</em></td>
<td></td>
<td>1.14</td>
<td>0.15</td>
<td>3.14 ± 1.16</td>
</tr>
<tr>
<td><em>Crematogaster</em> sp.</td>
<td></td>
<td>1.29</td>
<td>0.17</td>
<td>3.64 ± 1.18</td>
</tr>
<tr>
<td><em>Brachymyrmex</em> sp.</td>
<td></td>
<td>1.38</td>
<td>0.14</td>
<td>3.99 ± 1.16</td>
</tr>
<tr>
<td><em>Dorymyrmex bicolor</em></td>
<td></td>
<td>1.53</td>
<td>0.13</td>
<td>4.65 ± 1.47</td>
</tr>
</tbody>
</table>

Figure 5. Metrics used to assess the aggressivity levels and protection effectiveness of different species associated with *T. velutina*. a) Correlations between the quantitative and qualitative components per assay; b) Protection effectiveness index; c) Average quantitative and qualitative protection effectiveness components per species; d) Maximum aggressivity score.
Table 5. Model estimates for the effect of ant species on recruitment, repellence, and protection effectiveness metrics.

<table>
<thead>
<tr>
<th>Response</th>
<th>Fixed effects</th>
<th>N</th>
<th>AIC</th>
<th>LRT</th>
<th>P-value</th>
<th>Random effects</th>
<th>Variance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Recruitment and time</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ants recruited</td>
<td>Ant species</td>
<td>92</td>
<td>650.4</td>
<td>37.60</td>
<td>4.53&lt;sup&gt;-07&lt;/sup&gt;</td>
<td>***</td>
<td>Assay</td>
</tr>
<tr>
<td>Response time</td>
<td>Ant species</td>
<td>79</td>
<td>816.3</td>
<td>16.69</td>
<td>0.005</td>
<td>**</td>
<td>Assay</td>
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<tr>
<td>Repellence time</td>
<td>Ant species</td>
<td>38</td>
<td>431.1</td>
<td>18.28</td>
<td>0.001</td>
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<td>Assay</td>
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<td><strong>Aggressivity</strong></td>
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<tr>
<td>Maximum aggressivity</td>
<td>Ant species</td>
<td>93</td>
<td>376.4</td>
<td>34.23</td>
<td>2.13&lt;sup&gt;-06&lt;/sup&gt;</td>
<td>***</td>
<td>Assay</td>
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<td>Protection Effectiveness</td>
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<td>5,92</td>
<td>23.68</td>
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Chapter 2: Ant aggressivity

DISCUSSION

For six ant species associated with *T. velutina* I estimated the likelihood of ants ignoring, patrolling, recruiting, attacking and repelling a caterpillar of *Euptoieta hegesia* placed on their host plant. I used two metrics to assess the aggressivity and defensive level of the ant species: the maximum aggressivity score and the protection effectiveness index. Both metrics converged in the same result, splitting the ant community into defender and non-defender species, with the only non-defender species being *Cephalotes* sp. A more detailed picture of the protection and aggressivity of each species was achieved using the qualitative and quantitative components classifying species into four categories: highly effective defenders and highly aggressive species (*Dorymyrmex bicolor* and *Brachymyrmex* ants), lowly aggressive and lowly effective defender (*Paratrechina longicornis*), ineffective defenders (*Camponotus planatus* and *Crematogaster* ants), and parasitic species (*Cephalotes*).

**How to quantify aggressivity?**

Overall, Fagundes et al. (2017) proposed that the number of plants visited (interaction frequency), ant recruitment (patrolling intensity) and the rate of herbivore removal (aggressivity), but not the time spent by ants foraging on the plant (foraging time), are important components of ant foraging behaviour and regulate protection effectiveness of ant species. I agree with this, except that I consider that time to first response or time to finding is a crucial aspect of protection effectiveness, as voracious herbivores can cause severe damage in a short period of time. Hence, I think this should be part of the protection effectiveness metric.

In contrast to Fagundes et al. (2017), I did not evaluate assays as an overall success or failure, but evaluated the presence or absence of each behaviour to produce a more comprehensive picture of ant aggressivity modes and frequency.
within the species associated with *T. velutina*. Fagundes et al. (2017) had multiple termites per plant, and so could estimate the removal percentage per assay and then average such estimates per species. I did not have enough larvae to do this, and so I produced an estimate per species for each behaviour. This is a limitation of this study; however, an advantage of this study is being the first to use the main natural herbivore of the ant-plant, and that it provides a comprehensive picture of ant defensive and patrolling behaviour.

Ness et al. (2006) suggest that the quantitative and qualitative components of an interaction are often not independent, and hence as the quantity of an interaction increases, the quality will often decline. In particular, they suggest that excessive bodyguards beyond those needed to remove all herbivores may be redundant, and so quantity and quality are inversely proportional (Ness et al. 2006). However, I am unsure about this statement and think it may be related to the way these authors defined it. It is possible to envision very effective mutualists that are also very frequent and abundant, and the opposite scenario, with very infrequent and scarce mutualists that are also very inefficient, and a linked scenario in which per capita effectiveness is low, but mutualists are very abundant and overall, are a very effective species due to large recruitment and frequencies.

When quantifying the aggressivity of defensive mutualists, it is crucial to account for differences in the behavioural ecology of each species, reflected in differences in patrolling habits. Ness et al. (2006) showed that relatively few highly aggressive ants can provide similar levels of protection to milder but more abundant ants, particularly if the milder ants are more likely to discover the herbivore due to a higher patrolling frequency. I agree with Ness et al. (2006) in that the interaction frequency (patrolling frequency) is a crucial factor to be accounted for. Patrolling frequency estimates are likely to change the interpretation of PE values, because an average guard may become highly
efficient if it patrols very frequently, being likely to find herbivores much faster than a highly aggressive species, with infrequent and lone patrolling habits.

Del-Claro and Marquis (2015) showed that ant species that recruited more foragers were also those that visited more shrubs (higher frequency of interaction) and more shrub species. Yet there was no difference in the time to find the herbivore between species that interacted with a high or low number of plants. These findings lead to the following questions:

- Is per capita protection effectiveness a better or worse estimate than the PE metric used here?
- Is the number of ants required to repel a larva an important factor in ant-based defence?

Given that ants are social organisms, if the colony is big and the ants are capable of quickly recruiting more ants, then the number of ants required to repel a larva becomes a less important factor. I think the PE estimate should include time, which may be a more crucial factor than a per capita estimate as voracious herbivores can eat a flower or a leaf in less than 10 minutes. Furthermore, constant patrolling by a mild ant species may also deter adults from ovipositing on the plant. Ant patrolling is a key factor in deterring oviposition by *E. hegesia* on more heavily patrolled plants of *T. velutina* (Ochoa-López et al. 2018). Older *T. velutina* plants rely more heavily on biotic defences, whilst younger stages rely on physical and chemical defences (Villamil et al. 2013, Ochoa-López et al. 2015) and butterflies oviposited more frequently on younger stages, with less nutritional quality but also with lower ant patrolling (Ochoa-López et al. 2018).
Which are the most aggressive ant species?

Behavioural differences were very consistent between some species, with certain species always displaying a given behaviour, whilst others never displayed it (Figs. 1, 2).

Despite finding efficiencies (the inverse of ignoring rates) between ant species ranging from 75-100%, no significant differences were detected between species (Fig. 2, Table 5). The same pattern was true for patrolling efficiency, which ranged from 35-100% but no significant differences were detected between species. This suggests that even very infrequent patrolling levels by poor defenders are sufficient for ants to notice a herbivore or plant disturbance.

Recruitment differed significantly between species and the efficiency range between species was even greater, from 1% in *Cephalotes*, to 96-98% in *D. bicolor* and *Brachymyrmex* ants, respectively. Recruitment efficiencies were significantly different for every single species, suggesting this may be a species-specific trait, although further research investigating whether the recruiting efficiency of a given ant species varies depending on the host plant it occupies would shed light on plant traits driving ant behaviour.

The likelihood of attacking displayed by ant species was not always directly correlated with their repellence efficiency (Fig. 4e). This was an interesting and useful finding that helped discriminate between the aggressive and the defensive efficiency traits of ant species. Incapacitated *Manduca sexta* larvae were either killed or knocked off *F. wislizeni* plants by the guarding ants (Ness et al. 2006). This same behaviour was observed in *T. velutina*, where guarding ants would induce *E. hegesia* larvae to relocate or drop-off the plant, and killed the larvae that did not do so.

*Cephalotes* ants were inefficient finders, the slowest species to find the larvae, with low ant recruitment and no evidence of aggressive behaviours,
leading to attacking and repelling efficiency levels of zero (Figs. 1-2). *Cephalotes* had a null PE index and had the lowest maximum aggressivity score (Fig. 5). All these traits suggest this is a parasitic species that harvests EFN without providing any defensive services. These findings in *T. velutina* are consistent with previous evidence of other *Cephalotes* species being non-defenders on *Ouratea spectabilis* Engl. (Ochnaceae) (Byk and Del-Claro 2010) and *Banisteriopsis malifolia* (Alves-Silva et al. 2015) in the Brazilian cerrado, and across many host plants within a riparian grassland community in Brazil (Fagundes et al. 2017). Del-Claro and Marquis (2015) also suggested that the weak protective levels in plants with a mixed array of ant species was due to the predominance of *Cephalotes pusillus* ants in those ant mixes. *C. pusillus* are ineffective defenders against herbivores consuming pollen and nectar without reciprocating with defensive services for the plant (Byk and Del-Claro 2010). Overall, I suggest that *Cephalotes* may be a parasitic genus that exploits the host plants without providing any guarding services, although additional evidence is required to confirm this hypothesis.

In *T. velutina*, *C. planatus* ants did attack herbivores, biting and spraying the larvae in the assays, suggesting that in this associations they are guarding ants, despite their low protection effectiveness (Figs. 1, 2). However, in *Acacia mayana*, *Camponotus planatus* ants were never seen providing any protective service, leading to the conclusion that they were true exploiters (Raine et al. 2004). Yet, non-aggressive ants can still reduce herbivory rates through non-consumptive effects or cleaning services, such as egg removal, although these roles as plant cleaners and egg removers have yet to be tested.

The maximum aggressivity score and the PE index both allowed us to discriminate between defensive and non-defensive species, and detect *Cephalotes* as a parasitic species (Fig. 4), with both metric showing similar degrees of resolution. However, these metrics did not have enough resolution
to rank defensive species according to their aggressivity and protection effectiveness. Splitting the PE index into its quantitative and qualitative components allowed a higher resolution analysis of ant species (Fig. 5c).

Although PE index and the maximum aggressivity score could only split the ant community into defender mutualists and cheating species, they are useful metrics because they produce a quantitative estimate. Such numerical measurement of the protective benefits provided by each ant species can later be used in other analyses, investigating the drivers and consequences of such ant behavioural responses. However, mapping the quantitative (QNC) and qualitative (QLC) components of ant aggressivity displays produced a more detailed description of individual species protection effectiveness. Despite being qualitative and not producing a concrete numerical output, the cartesian QNC-QLC mapping generated a hierarchy of ant aggressivity and defenders’ effectiveness, discriminating between highly efficient, lowly efficient, ineffective defenders and parasitic species (Fig. 5a,c).

Based on all the metrics and behaviours described here, I conclude that the hierarchy of ant aggressivity and protection effectiveness for *T. velutina* at La Mancha, Veracruz is as follows: *Dorymyrmex bicolor* > *Brachymyrmex* sp. > *Paratrechina longicornis* > *Camponotus planatus* > *Crematogaster* sp. > *Cephalotes* sp..

**Are ant traits good predictors of aggressivity?**

**Attack mode: sting vs. spray**

It has been suggested that ants that sting are less aggressive and inferior defenders than those that deploy chemical defences (Davidson et al. 1988). However, this does not seem to be the case in *Acacia mayana* where spraying *Camponotus planatus* ants aggressively outcompeted *Pseudomyrmex*
Chapter 2: Ant aggressivity

*ferrugineus* stinging ants (Raine et al. 2004). As plant defenders this does not seem to be the case either amongst the guarding partners of *T. velutina*, since biting attacks (by *Dorymyrmex* or *Brachymyrmex*) resulted in higher larvae repellence efficiency levels than spraying attacks (by *Crematogaster* or *Camponotus* ants).

**Patrolling habits**

I suggest that significant differences in the numbers of ants recruited revealed by the assay with *Euptoieta hegesia* larvae may reflect inherent differences in behavioural ecology and patrolling habits among ant taxa. Despite *C. planatus* ants having a higher herbivore repelling efficiency than *P. longicornis* (Fig. 2), the latter ranked higher in the Protection Effectiveness index (Fig. 5b) by having a higher recruitment size (Table 4, Fig. 3). I acknowledge that the PE index calculated here may underestimate lone patrolling species with low recruitment sizes. However, lone patrollers will visit plant sections less frequently because fewer ants will take longer to patrol a whole plant than a numerous group of patrolling ants. If this is true, this PE index may be correctly reflecting the quality of ants as defenders by penalizing infrequent patrollers who may take longer to find a herbivore. In ants associated with *Peixotoa tomentosa* (Malpighiaceae), species with higher recruitment numbers also had higher frequencies of interaction (visited more shrubs) and from a larger number of species. Yet there was no differences in the time to find the herbivore between species that interacted with a high or low number of plants (Del-Claro and Marquis 2015). However, this data do not have enough resolution to show a robust pattern on the correlation between the likelihood of ants finding a herbivore and their response time. Having one herbivore per assay per plant, I can only estimate finding efficiency (1-ignorance) at a species level, and not per assay. Other studies using termites as herbivores instead of the natural herbivores managed larger replicate sizes and efficiency estimates per assay (Fagundes et al. 2017). Although studies with this experimental design sacrifice on natural history realism, they achieve more replication and increased
Chapter 2: Ant aggressivity

statistical resolution and would be an ideal follow-up experiment to answer whether lone patrollers are less efficient at finding herbivores than gregarious ant species.

**Ant size**

Small ants have often been regarded as poor defenders (Fiala et al. 1989, Cuautle and Rico-Gray 2003, Cuautle et al. 2005). Although the defensive effectiveness of *Crematogaster borneensis* was questioned mostly based on their small size and their lack of a functional sting, worker ants were observed biting intruders and displayed very strong attaching abilities with high repellence efficiency (Fiala et al. 1989). In *T. velutina* (Cuautle and Rico-Gray 2003) also suggested that larger ants with larger mandibles would provide greater defensive benefits to the plants, but this hypothesis was not supported by my quantitative results of aggressivity. *Dorymyrmex bicolor* and *Brachymyrmex* sp. ant species had the smallest body sizes but the highest aggressivity displays and repellence efficiency levels (Figs. 2, 4, 5). I advocate avoiding *a priori* judgements on the ant traits that may drive their protection effectiveness, and instead suggest quantitative assessments using surrogate or naturally encountered herbivores.

**Protection effectiveness varies between hosts**

Ant recruitment and herbivore removal efficiency are key aspects in determining the protection effectiveness of ants, and these traits have been thought as intrinsic to each ant species, but recent studies show the opposite. The protection effectiveness of a given ant species is likely to differ depending on the host plant it is occupying (Apple and Feener Jr 2001, Fagundes et al. 2017). Defensive attributes can be up- or downregulated by external factors such as the quality of rewards provided (Grover et al. 2007, Fagundes et al. 2017). In a comprehensive community study on the effect of individual ant species on different host plants, Fagundes et al. (2017) found that ant protection was positively associated with sugar concentration of nectar, which explained the
variation in leaf damage among plant species. This demonstrates that ant protection varies among ant species and is enhanced by the plant investment in nectar reward (Fagundes et al. 2017).

Plants providing better nectar quality are likely to interact with more aggressive and territorial ants, reducing the frequency of commensal and parasitic species (Grover et al. 2007, Koptur et al. 2015, Fagundes et al. 2017) and such investment may result in plant benefits via reduction in damaged leaf area (Fagundes et al. 2017). Since increased ant aggressivity increases the reproductive success of the host plant, it may be advantageous for plants to produce more sugar-rich nectar to attract more and better ant guards. Although this may be limited, as more abundant and aggressive partners may also result in ecological costs such as repellence of other natural predators like wasps (Cuautle and Rico-Gray 2003), coccinellid beetles, or spiders (Koptur et al. 2015), and may also deter pollinators (Ness 2006, Assunção et al. 2014). Despite this, the effects of EFN availability, sugar, prey and other resources on ant foraging patterns have rarely been studied (but see: Apple and Feener Jr 2001, Grover et al. 2007, Fagundes et al. 2017).

The more, the merrier
Herbivore traits, such as behaviour or morphology may affect their susceptibility to attacks. Ants are generally more effective against small, and less sclerotised herbivores, with caterpillars being more susceptible than beetles (Fiala et al. 1989). In Macaranga plants, Crematogaster borneensis was more effective at deterring small, non-sclerotised animals, such as caterpillars, opposed to beetles. In T. velutina the main herbivores are the larvae of Euptoieta butterflies, which are not sclerotised and hence are susceptible to ant attacks. Curculionidae and Staphylinidae beetles have also been recorded as florivores in T. velutina (pers. obs.) and these beetles do not seem to be susceptible to ant
attacks as their abundance did not vary between control and ant excluded plants (See Natural History Notes for further details). Their highly sclerotised bodies make them highly resistant to ant biting or acid spraying attacks. Nonetheless, *Pseudomyrmex gracilis* ants have been seen actively and exclusively hunting Staphylinidae beetles and thrips from the flowers (pers. obs., further details in Natural History Notes). If ant species differ in their effectiveness against different kinds of herbivores, an apparently redundant array of patrolling ant species as those in facultative mutualisms may be beneficial for plants.

ACKNOWLEDGEMENTS

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CHAPTER 3

ANT-POLLINATOR CONFLICT

Nectar trade-offs and pollinator deterrence

The weekend naturalist
By Tom Buchan

My humanoid friend, myself, a limited animal
In love with the planet
Escaping across the dumb topographies of Assynt
With maps and a compass
Taking incorrect fixes on anonymous Bens
Staring into bog-pools

Entertaining myself with half-formulated notions
of a non-utilitarian character
and applying my ragbag of ecological data
to flowers which I recognize
absentmindedly as if they were old friends
whose names I’ve forgotten […]
INTRODUCTION

Extrafloral nectaries, domatia and food bodies are all means by which ant-plants (comprising facultative species termed myrmecophiles and obligate ones termed myrmecophytes) (Rosumek et al. 2009; Del-Claro et al. 2016) attract and support ants by providing nesting sites or nutrients (Rico-Gray & Oliveira 2007; Rosumek et al. 2009). In return, ants attack herbivores, prune climbing vines and prevent fungal and microbial infestation on plant tissues (Bentley 1977; Rosumek et al. 2009). This mutualistic interaction is termed myrmecophily.

Interactions involving two or more types of mutualists of a single host are common in nature, but multispecies interactions are much less studied than pairwise and intraguild mutualisms (Strauss 1997; Tscharntke & Hawkins 2002; Strauss & Irwin 2004; Adler 2008; J. et al. 2009; Melián et al. 2009; Koptur et al. 2015). To date, most research on plant-animal interactions has focused on pairwise relationships (e.g., plant-herbivore, plant-pollinator, plant-fungus) in isolation from the community in which they are embedded (Strauss 1997; Herrera 2000; Dáttilo et al. 2016; Del-Claro et al. 2018). This pairwise approach necessarily oversimplifies reality (Herrera 2000) since plants interact sequentially or simultaneously with each of pollinators, herbivores, herbivore predators and pathogens, (Armbruster 1997). Furthermore, plant interactions with one partner or guild can also affect relationships with other groups or guilds (Armbruster 1997) and alter outcomes from mutualistic to antagonistic (Strauss 1997; Strauss et al. 1999; Herrera 2000; Strauss & Irwin 2004; Del-Claro et al. 2016). As a result, a growing number of studies are focusing on multispecies and multitrophic interactions (Melián et al. 2009; Fontaine et al. 2011; Nahas et al. 2012; Pineda et al. 2013; Dáttilo et al. 2016). It might be expected, for example, that the presence of predatory ants can influence pollinators, with top-down effects on plant fitness. This makes ant-plants, which rely on ants for defence against herbivores and on pollinators for seed set seeds, a model tritrophic
system in which to explore the dynamics of multispecies and multitrophic interactions.

Here, I focus on disentangling ant-pollinator interactions that occur when both mutualists share a host plant. Previous work has revealed evidence of ant-pollinator conflict in such systems (Yu & Pierce 1998; Stanton et al. 1999; Gaume et al. 2005; Ness 2006; Palmer & Brody 2007; Frederickson 2009; Stanton & Palmer 2011; Malé et al. 2012; Assunção et al. 2014; LeVan et al. 2014; Hanna et al. 2015). Ant-pollinator conflict is expected for two main reasons. Firstly, both mutualists share with their host interest in different plant functions. Pollinators benefit from plant resource allocation to reproduction (i.e., flowers, floral nectar (FN) and pollen), whilst ants benefit from allocation to growth and defence (i.e., vegetative structures bearing extrafloral nectar (EFN) or domatia) (Yu & Pierce 1998; Frederickson 2009; Palmer et al. 2010). This could derive in a conflict mediated by plant rewards, known as indirect conflict. Floral and extrafloral nectar share sugar as a common currency, providing potential for a trade-off and also a means of quantifying investment in each. Secondly, because as ant guards actively defend their host plant as a means of protecting food and/or nesting sites, they may also repel or attack pollinators (Ness 2006; Stanton & Palmer 2011; Chamberlain & Rudgers 2012), and this drawback of patrolling is known as direct conflict.

Castration is an extreme example of direct ant-pollinator conflict in which guarding ants destroy or consume the reproductive meristems, floral buds or flowers of their host plant (Yu & Pierce 1998; Stanton et al. 1999; Gaume et al. 2005; Palmer & Brody 2007; Frederickson 2009; Malé et al. 2012). Such castrating behaviour inevitably reduces availability of flowers and hence floral rewards for pollinators. It has been suggested that the ultimate cause of castration by patrolling ants is that it promotes reallocation of plant resources from reproduction to growth (Yu & Pierce 1998; Frederickson 2009; Malé et al.
Chapter 3: Ant-Pollinator conflict

2012), and hence increasing the availability of resources on which ant colonies depend. In ant species that are obligate inhabitants of ant-plants, colony size is limited by the number of domatia (Fonseca 1993; Fonseca 1999; Orivel et al. 2011), which is positively correlated with plant investment in growth. Resource allocation strategies towards these two mutualists should be approached in a linked way, as plants interact with both mutualists simultaneously and the presence of one mutualist may increase or decrease the presence of the other. Also, because investment in one may come at the cost on investment in the other, affecting plants investment in the other mutualists reward, positively via linkage, or negatively via trade-offs.

Even in those species that do not castrate their host, ants’ aggressive behaviours might threaten and deter pollinators, compromising plant reproduction (Ness 2006; Assunção et al. 2014; LeVan et al. 2014). Avoidance of such direct conflict has been suggested to explain plant architecture or behaviours that reduce spatial (Raine et al. 2002; Malé et al. 2015; Martínez-Bauer et al. 2015) or temporal overlap of ant guards and pollinators (Gaume & McKey 1999; Gaume et al. 2005; Nicklen & Wagner 2006; Ohm & Miller 2014; Malé et al. 2015), and repelling compounds which exclude ants from flowers when pollen is released (Junker et al. 2007; Willmer et al. 2009; Ballantyne & Willmer 2012).

Extrafloral nectar is a key resource mediating multispecies interactions in many plant communities, and plants bearing extrafloral nectaries comprise up to a third of species in some biomes (Dyer & Phyllis 2002; Rudgers & Gardener 2004; Davidson & Cook 2008; Dyer 2008), particularly in tropical dry forests, savannas and cerrados (Rico-Gray & Oliveira 2007; Assunção et al. 2014). The importance of ant-plants as food resources for mutualists in a given plant community is enhanced if these plants also secrete FN and pollen for pollinators. Management of ant-pollinator conflict in such a way that the crucial
services provided by both mutualist groups are maintained is thus likely to be part of the adaptive landscape of many plant species. Ant-plants with extrafloral and floral nectaries represent an excellent system to test for trade-offs in resource allocation, competition amongst mutualistic guilds, and assess plant strategies that minimise direct and indirect conflicts between their mutualists. To my knowledge, no study has addressed both direct and indirect ant-pollinator conflict in a single ant-plant system.

QUESTIONS & AIMS

Here I tested for direct and indirect ant-pollinator conflict on a Mexican endemic ant-plant, *Turnera velutina*. In particular, I assessed whether *Turnera velutina* reduces the potential for conflict through the daily timing of FN and EFN release. I also tested for potential indirect (nectar-mediated) and direct (deterrence) conflicts between ants and pollinators. I addressed the following specific questions:

(i) What are the daily timings of nectar reward secretion, ant activity, and floral visitation?

(ii) Does the presence of patrolling ants deter pollinators from the flowers? (Direct conflict)

(iii) Do ant species vary in their impacts on potential deterrence to pollinators? (Direct conflict)

(iv) Are *T. velutina* plants able to re-allocate extrafloral nectar resources into floral nectar resources (increasing reward availability to flower visitors)? (Indirect conflict)
MATERIALS AND METHODS

**Study site**
Field experiments were conducted in coastal sand dunes at the CICOLMA Field Station in La Mancha, Veracruz, in the Gulf of Mexico (19°36’ N, 96°22’W, elevation < 100 m). The climate is warm sub-humid, with a rainy season during the summer (June to September), an annual precipitation of 1100 – 1500 mm, and a mean annual temperature ranging between 24 and 26 °C (Travieso-Bello & Campos 2006). Experiments were carried out in November 2014 at four sites with high densities of *Turnera velutina* (Passifloraceae). Greenhouse experiments were conducted in a shade house at CICOLMA.

**Study system**
*Turnera velutina* is an endemic perennial shrub (Arbo 2005) and myrmecophile (Cuautle & Rico-Gray 2003). At La Mancha, *T. velutina* is patrolled by at least seven ant species (Cuautle *et al.* 2005; Zedillo-Avelleyra 2017) and its main herbivores are the specialist caterpillars of the butterfly *Euptoieta hegesia* (Nymphalidae). Extrafloral nectar is provided in paired cup-shaped glands located at the bottom of the leaf blade at the junction with the leaf petiole, on the underside of the leaves (Fig. 1a, Elias *et al.* 1975; Villamil *et al.* 2013). Although it flowers year-round, flowering peaks during summer (Cuautle *et al.* 2005). Flowers last one day, are animal-pollinated (Sosenski *et al.* 2016) and provide FN at the base of the corolla (Fig. 1). Honeybees (*Apis mellifera*) are the dominant pollinators at La Mancha, accounting for 94% of the visits (Sosenski *et al.* 2016) and collect both pollen and floral nectar, but the role of other floral visitors as effective pollinators is yet to be investigated. There is no spatial segregation of patrolling ants and floral visitors in *Turnera velutina* since flower buds emerge from the axillary meristems of leaves bearing extrafloral nectaries (Villamil 2017). Nor are they segregated in daily time, since, EFN volume and sugar content both peak during anthesis (Villamil 2017).
FIELDWORK METHODS

Mutualist activity curves: patrolling ants and pollinators
I quantified daily activity of patrolling ants and flower visitors on *T. velutina* by surveying flowers (n=120 plants, n=1604 flowers;) and their associated leaves at all four La Mancha sites in November 2014. I observed all open flowers for 2 minutes every hour throughout anthesis (0800–1300 hours), with one observer at each site. Every hour, I counted the total number of floral visitors and ants patrolling extrafloral nectaries across all flowers within a site. I sampled the same sites over multiple days. Since these are one-day flowers, I considered each site-day as a replicate (n = 10 site-days; 43.23 ± 2.89 flowers/site-day), and incorporated site-and-day effects into the statistical modelling (see below).

Nectar secretion and pollen deposition curve
Nectar secretion and pollen deposition data were collected from 4 sites within CICOLMA over 5 consecutive days in September 2015. I visited a single site per day (with one exception which was visited twice) and sampled FN and EFN secretion rate and pollen deposition from 1 flower per plant for 5 plants per site (n=20 plants). Flowers were sampled every hour during the anthesis period.
Chapter 3: Ant-Pollinator conflict

(o800-1300 hours). Flowers were bagged with tulle bags before anthesis and FN and EFN were collected every hour during anthesis. The first collection was taken as soon as the corolla was fully open. Flowers were re-bagged between measurements to avoid nectar consumption and a masking tape band with Tanglefoot was applied on the stem below the flower to exclude ants for the duration of the experiment. FN was extracted using a 1 μl capillary inserted into each of the five nectaries in a single flower (Minicaps Disposable capillaries, Hirschmann Laborgerate, In 20°C, ISO 7550, R<0.5%, CV<1.0%, Germany). EFN was also collected from the glands using 1 μl capillaries. Nectar volume was measured using a digital calliper (Mitutoyo Digimatic) and sugar concentration was determined in °Brix (g sucrose per 100 g solution) using a 0-50°B hand held refractometer (Reichert, Munich, Germany). To obtain all of the sugar from extrafloral nectaries, the glands were washed with 2 μl of deionized water using a 0-5 μl micropipette and the sugar concentration of the wash was quantified using the refractometer. Total sugar content in FN and EFN was calculated from volume and °Brix values according to Comba et al. (1999) using the formula:

\[ s = \frac{dvC}{100} \]

where \( s \) is the sugar content (µg), \( v \) is the nectar or wash volume (µl), and \( d \) is the density of a sucrose solution at a concentration \( C \) (g of sucrose per 100 g solution) as read on the refractometer. The density was obtained according to Comba et al. (1999) using the formula:

\[ d = 0.0000178C^2 + 0.003791C + 0.9988603 \]

A different flower from each of these twenty plants was chosen and marked, but never bagged. I collected one stigma every hour, to sample the pollen deposited one, two and three hours post-anthesis. A small proportion of flowers contained a fourth stigma, contributing to a small dataset for four hours post anthesis. Stigmas were stored individually in labelled Eppendorf tubes. Stigmas were individually mounted on slides for fresh squash glycerin preparations. Slides were sealed using nail polish and kept in a fresh and dry environment at 22°C. Each slide was labeled with the site, day, hour, and plant identity. The number
of pollen grains on each stigma was counted under a microscope, and changes in numbers at each time interval plotted to generate the pollen deposition curve.

**Direct conflict**

To test whether non-ant flower visitors detect and avoid flowers with ants, visitation was observed in flowers with and without dead ants for 4 flowers on each of 40 plants (n=160 flowers). Plants > 80 cm in height and with at least four flowers, were haphazardly selected and flowers bagged at 0700 before anthesis. Once the corollas had opened, three ant corpses from a single ant species (either *Dorymyrmex bicolor*, *Brachymyrmex* sp., or *Paratrechina longicornis*) were placed inside each of three flowers/plant. A fourth flower was left as an ant-free control. Ant corpses were placed on the inner surface of the petals in each flower. These species were chosen because they were amongst the most abundant species (see Results), displayed patrolling behaviours on *T. velutina*, and were detected as potentially differing in their effects on *Apis mellifera* pollinators by a previous study (Villamil *et al.* submitted). Additional flowers were removed from the plant to control for floral display across all individuals. Ants were killed in 50% ethanol, which was allowed to evaporate for 30 minutes at ambient temperature (28-35°C) from all samples to prevent ethanol vapours from influencing pollinator behaviour. Flowers containing dead ants were observed for 20 minutes, recording pollinator identity, visit frequency and duration, and associated pollinator behaviours. Observations were conducted during October-November 2016 with four simultaneous observers working collecting data from different plants.

When assessing the effects of ants inside the flowers on pollinator visitation I only considered visits by *Apis mellifera*, since this is the dominant *T. velutina* pollinator in this population (Sosenski *et al.* 2016) and accounted for 91% of the visits in this experiment. I classified honeybee behaviours as ‘inspection’ or ‘contact’. Inspection behaviours are defined as those displayed by the pollinators at the floral headspace before or without landing on the flower comprised either
Chapter 3: Ant-Pollinator conflict

approaching or hovering over a particular flower. Contact behaviours were those that occurred inside the flower, between landing and take-off, and comprised foraging on pollen or nectar resources or standing on the petals, anthers or stigmas.

**Indirect conflict**

To test possible trade-offs in plant resources between FN and EFN I conducted a greenhouse experiment on 72 plants from 18 different maternal lines (2-4 siblings per maternal family, generated from field individuals). Plants were kept under greenhouse conditions in a shade house located within CICOLMA field station. Plants were grown in 1L plastic pots with a substrate of local soil and vermiculite (50:50) and watered every other day (for rearing details see: Ochoa-López et al. 2015). During the experiment, plants were watered every night, and extrafloral nectaries were sprayed with water to wash away any nectar secretion from previous days and to prevent fungal infections on the glands. Pre-anthesis buds were bagged either the night before or during the morning before anthesis to prevent any nectar theft by unexpected insects that may occasionally enter the nursery.

Pairs of maternal siblings were chosen and randomly assigned to either control or experimental groups. The extrafloral nectaries in all leaves of experimental plants were clogged by applying Mylin transparent textile paint in the nectary cup, as in Villamil et al. (in press). Extrafloral nectaries from control plants were left intact and droplets of the same paint were applied on the leaf blade above the glands to match any unintended effects on plants across treatments. Very young floral buds were marked, and their extrafloral nectaries clogged from their emergence, throughout their development, and until anthesis. The droplets on the extrafloral nectaries or leaf blades were checked daily and replenished when required, especially when a new leaf emerged in order to guarantee uniform and continuous application of the clogging treatment across all leaves.
FN secretion was measured before the clogging treatment was applied, and once again after the young clogged/ marked buds became flowers. The aim was to test whether flowers that were unable to secrete EFN invested more sugar resources in floral nectar. FN was collected from control and treatment flowers between 1300 and 1500 h using a 1µl microcapillary pipette. Nectar volume and total sugar mass was estimated and calculated as described above.

**STATISTICAL METHODS**

All statistical analyses were conducted in R version 3.23 (R Core Team 2016). All mixed effects models were fitted using the ‘lme4’ R package (Bates *et al.* 2016) and *post hoc* Tukey comparisons were fitted using the ‘multcomp’ R package (Hothorn *et al.* 2008), unless stated otherwise.

**Mutualist activity and reward secretion curves**

To test whether ant or pollinator activity changed over daily time, I fitted a Poisson mixed model with either the number of ants patrolling or the number of floral visitors as the response variable. I fitted time of day as a fixed effect, with linear and quadratic terms to detect non-linear activity patterns over time. The number of flowers per site was fitted as a log-transformed offset to control for floral display, since I recorded visitor counts per site rather than counts per individual flower (see fieldwork methods). Flowers of *T. velutina* last for a single day, and because multiple flowers were sampled on a given site on a given day, I fitted site identity as a random effect to account for differences between site and day variation in variables that could influence ant abundance, such as resource availability, ant diversity, or the abundance/proximity of ant nests. I also included an observation-level random effect (OLRE) where each data point receives a unique level of a random effect to control for overdispersion (Hinde 1982).
Chapter 3: Ant-Pollinator conflict

To test if FN and EFN secretion changed over the anthesis period I fitted a Poisson mixed model independently for each nectar type, using the sugar mass (µg) value rounded to the nearest integer as the response variable. Nectar sugar content is usually estimated in µg, and I report raw data in such units. However, to facilitate model convergence I re-scaled the response variable (sugar content) from µg to g, and rounded it to the next integer to better fit a Poisson distribution. I fitted time of day as a fixed effect, with linear and quadratic terms to detect non-linear activity patterns over time. I fitted plant identity as a random effect, and, included an observation-level random effect.

Timing of daily activity and secretion peaks
I computed the time at which mutualist activity and nectar secretion reached their maximum by calculating the time at which the slope (i.e., the differential of the fitted model with respect to time) for each variable is zero and then solving for hour, as follows:

\[
\begin{align*}
    y &= \beta_{\text{hour}} \times \text{hour} + \beta_{\text{hour}^2} \times \text{hour}^2 \\
    \frac{dy}{d\text{hour}} &= \beta_{\text{hour}} + 2\beta_{\text{hour}^2} \times \text{hour} \\
    0 &= \beta_{\text{hour}} + 2\beta_{\text{hour}^2} \times \text{hour} \\
    -\frac{\beta_{\text{hour}}}{2\beta_{\text{hour}^2}} &= \text{hour} \\
    \text{hour} &= -\frac{1}{2} \times \frac{\beta_{\text{hour}}}{\beta_{\text{hour}^2}}
\end{align*}
\]

Direct conflict
The effect of different ant species on the visitation frequency was tested using Poisson mixed effects models. I fitted the number of visitors as the response variable, and ant species inside the flower (Control, Dorymyrmex bicolor, Brachymyrmex sp., or Paratrechina longicornis) was fitted as a fixed effect. Because these are one day flowers, plant-day identity was chosen as a random effect to control for individual variation in floral and extrafloral nectar investment. I also included an observation-level random effect. Post hoc Tukey comparisons were used to test differences in visit duration between the four treatments.
Chapter 3: Ant-Pollinator conflict

I tested whether ant species inside the flower differed in their effect on the likelihood with which a pollinator displayed an inspection behaviour using a binomial mixed model. The presence or absence of inspection behaviours was coded as the response variable and ant species was fitted as a fixed effect. As random effects I fitted the plant-day identity, and the visitor identity. For those visits where the pollinator displayed an inspection behaviour, I fitted the proportion of time per visits spent displaying inspection behaviours using a Gaussian mixed model with Logit transformation for data normality. Ant species was included as a fixed effect, and plant-day identity, and the visitor identity were fitted as random effects.

Finally, differences in the duration of inspection or contact behaviours in flowers containing different ant species were analysed using Gamma mixed models. Mixed effects models were fitted independently for each behaviour, but using the same model structure fitting visit duration per flower as the response variable. Ant species inside the flower was fitted as a fixed effect. Plant-day identity was chosen as a random effect to control for individual variation in floral and extrafloral investment, and daily weather variations. I also included an observation-level random effect. Post hoc Tukey comparisons were used to test differences in visit duration between the four treatments.

**Indirect conflict**

For each plant, I estimated the difference in FN produced before and after the extrafloral nectaries were clogged as follows: \( D_{\text{FN}} = Post_{\text{FN}} - Pre_{\text{FN}} \).

Where \( D_{\text{FN}} \) is the difference, \( Post_{\text{FN}} \) is the FN production after the extrafloral nectaries secretion was prevented and \( Pre_{\text{FN}} \) is the FN production before the extrafloral nectaries were clogged. Differences in volume and sugar content between control and experimental flowers were tested using mixed effects models. Both variables had normal distributions and so Gaussian mixed effects models were fitted using the same model structure: The clogging treatment was
fitted as a fixed effect, and the maternal family was fitted as a random effect to independently explain variation in both nectar volume and sugar content.

RESULTS

Mutualist activity, reward secretion, and pollen deposition curves
Activity curves show that both patrolling ants and floral visitors were most active within the first two hours post-anthesis (Fig. 2), although visitation by potential pollinators peaked on average over an hour before ant activity (9 min post-anthesis for potential pollinators, 90 min post-anthesis for ant patrolling; Table 1).

On average, a flower and its associated leaf secreted a total of 2815 ± 767 µg of sugar via floral and extrafloral nectar throughout the 4.5 hour anthesis period. The total sugar content in FN was 149 ± 19.3 µg of sugar, whilst total extrafloral sugar was 2665 ± 765 µg. Thus, the relative sugar contributions of floral and extrafloral nectar in a leaf-flower module were 5.3% and 94.7%, respectively.

Floral and extrafloral nectaries of T. velutina are both able to quickly replenish nectar after experimental removal by non-destructive sampling of the same flowers over the entire anthesis period (Fig. 2a). FN and EFN are secreted simultaneously during anthesis and with the highest amount of sugar content secreted during the first two hours of anthesis (Fig. 2a), although their secretion peaks predicted from model estimates are slightly mismatched (Table 1). EFN secretion peaked 68 minutes after the first collection, which was taken as soon as flowers were fully open, whilst FN peaked 23 min after the first collection. The timing of peaks in secretion of the two types of nectar matches that for the mutualists that harvest each resource. Peak pollen deposition occurred at the beginning of anthesis and steadily declined over time (Fig. 2c). Hence, pollen
deposition data were analysed using a linear model without fitting hour as a squared term and I did not estimate timing of daily maxima using model derivations (Table 1).

I recorded 1535 ant visitors from nine ant species patrolling extrafloral nectaries of T. velutina at CICOLMA (Table 2). Dorymyrmex bicolor, Paratrechina longicornis, and Brachymyrmex spp. accounted for 68.5% of the total ants observed, and 77.35% of the patrolling ants, after excluding Monomorium spp. that were never observed displaying patrolling behaviours on T. velutina and are mostly parasitic consumers of FN and EFN (lestobiotic) (Ettershank 1966; Bolton 1987).
Figure 2. Timing of a) reward secretion (n = 20 flowers-leaves), b) mutualist activity (n = 1604 flowers; n = 10 sites/day) and c) pollen deposition (n = 20 flowers) in Turnera velutina during the anthesis period (08:30 – 12:30), showing on the right raw data from field observations (mean ± se). Red circles show floral nectar and the activity of pollinators in flowers, whilst green triangles show extrafloral nectar and the activity of ants at extrafloral nectaries.
Table 1. Model statistics for the timing of mutualists activity, reward secretion and pollen deposition in *Turnera velutina*. Estimates at which the maxima for mutualist activities and reward production are reached are shown in the last three columns. Peak hour is the time in hours estimated, mpa shows minutes post-anthesis when the maxima is reached, and time of day indicates an approximation of when that activity is likely to occur, although this varies depending on the season and the time of sunrise.

<table>
<thead>
<tr>
<th>Response</th>
<th>Fixed effects</th>
<th>N</th>
<th>Estimate</th>
<th>LRT</th>
<th>P-value</th>
<th>Random effects</th>
<th>Variance</th>
<th>SD</th>
<th>Maxima estimates</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mutualist activity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patrolling ants (number)</td>
<td>Log(flowers)</td>
<td>33</td>
<td>0.804</td>
<td>7.967</td>
<td>0.004</td>
<td>OLRE</td>
<td>0.221</td>
<td>0.471</td>
<td>1.5 90 09:30</td>
</tr>
<tr>
<td></td>
<td>Hour</td>
<td>33</td>
<td>0.088</td>
<td>0.148</td>
<td>0.699</td>
<td>Site ID</td>
<td>0.462</td>
<td>0.680</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hour²</td>
<td>33</td>
<td>-0.028</td>
<td>0.201</td>
<td>0.653</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Floral visitors (number)</td>
<td>Log(flowers)</td>
<td>42</td>
<td>0.507</td>
<td>2.619</td>
<td>0.105</td>
<td>OLRE</td>
<td>0.097</td>
<td>0.311</td>
<td>0.15 9 08:09</td>
</tr>
<tr>
<td></td>
<td>Hour</td>
<td>42</td>
<td>0.040</td>
<td>0.036</td>
<td>0.849</td>
<td>Site ID</td>
<td>0.991</td>
<td>0.991</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hour²</td>
<td>42</td>
<td>-0.131</td>
<td>3.838</td>
<td>0.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Reward secretion</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Floral sugar (µg)</td>
<td>Hour</td>
<td>78</td>
<td>0.1445</td>
<td>0.1019</td>
<td>0.7495</td>
<td>OLRE</td>
<td>0</td>
<td>0</td>
<td>0.39 23 08:23</td>
</tr>
<tr>
<td></td>
<td>Hour²</td>
<td>78</td>
<td>-0.1818</td>
<td>1.3222</td>
<td>0.2502</td>
<td>Plant ID</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Extrafloral sugar (µg)</td>
<td>Hour</td>
<td>78</td>
<td>0.4647</td>
<td>1.0070</td>
<td>0.0316</td>
<td>OLRE</td>
<td>0.2425</td>
<td>0.4925</td>
<td>1.13 68 09:08</td>
</tr>
<tr>
<td></td>
<td>Hour²</td>
<td>78</td>
<td>-0.2053</td>
<td>1.8949</td>
<td>0.1686</td>
<td>Plant ID</td>
<td>0.4013</td>
<td>0.6335</td>
<td></td>
</tr>
<tr>
<td>Pollen deposition (number of grains)</td>
<td>Hour</td>
<td>44</td>
<td>0.3240</td>
<td>21.38</td>
<td>3.73e-06</td>
<td>OLRE</td>
<td>0.1049</td>
<td>0.3240</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Site ID</td>
<td>0.0957</td>
<td>0.3094</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 3: Ant-Pollinator conflict

Direct conflict

I recorded 991 floral visitors, of which 907 (91.5%) were by the honeybee *Apis mellifera*. Of the remaining 8.5%, 61 visits were by native bees, 11 by flies (Diptera), 10 visits by Lepidoptera, one visit by a beetle (Coleoptera) and one by a wasp (Hymenoptera) (Table 2).

Neither the presence of ants inside flowers nor their identity had any significant effect on the number of honeybees visiting the flowers (Fig. 3a, Table 3). The presence of the most aggressive ant species, *Dorymyrmex bicolor*, increased the likelihood of a pollinator displaying inspection behaviours by 20% (Fig. 3b), and increased by 12% the proportion of time per visit spent displaying inspection behaviours rather than foraging or pollinating the flower (Fig. 3c). Finally, the presence of *Dorymyrmex bicolor* and *Paratrechina longicornis* inside the flowers halved the duration of contact visits compared to control flowers without ants, or to flowers with *Brachymyrmex* sp. ants inside.

Indirect conflict

Clogging extrafloral nectaries on the leaves associated with newly emerged floral buds had no effect on their FN volume (LRT \((r, 49) = 0.21; P = 0.64\), Table 3, Fig. 4b) or sugar content (LRT \((r, 49) = 0.087; P = 0.77\), Table 3, Fig. 4a). Differences in FN volume and sugar content \((F_{\text{post-treatment}} - F_{\text{pre-treatment}})\) were positive in plants under both control and clogged treatments (Fig. 4).
Figure 3. The effect of dead ants inside the flowers of *Turnera velutina* on different aspects of the behaviour of visiting honeybees: A) visitation frequency, B) the probability of displaying inspection behaviours, C) the duration of contact visits (time spent inside the flower), and D) proportion of time spent inspecting the flowers per visit bout (hovering over the floral head space). Ant species are arranged in order of increasing aggressivity and names are abbreviated: C = control with no ants, 1P are *Paratrechina longicornis*, 2M are *Brachymyrmex* sp., and 3D are *Dorymyrmex bicolor* ants. (*n* = 40 plants; *n* = 160 flowers). Different letters indicate significant differences between species (*P*<0.05).
Table 2. Abundance and identity of the ants recorded patrolling extrafloral nectaries during the 2014 census and the floral visitors recorded during the direct conflict experiment in 2016 on *Turnera velutina* plants.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Visitor(s)</th>
<th>Percentage</th>
<th>Subfamily</th>
<th>Patrolling</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Paratrechina longicornis</em></td>
<td>487</td>
<td>31.72</td>
<td>Formicinae</td>
<td>Gregarious</td>
</tr>
<tr>
<td><em>Dorymyrmex bicolor</em></td>
<td>421</td>
<td>27.42</td>
<td>Dolichoderinae</td>
<td>Gregarious</td>
</tr>
<tr>
<td><em>Monomorium ebenium</em></td>
<td>270</td>
<td>17.58</td>
<td>Myrmicinae</td>
<td>Gregarious</td>
</tr>
<tr>
<td><em>Camponotus planatus</em></td>
<td>184</td>
<td>11.98</td>
<td>Formicinae</td>
<td>Loner</td>
</tr>
<tr>
<td><em>Brachymyrmex sp.</em></td>
<td>73</td>
<td>4.75</td>
<td>Formicinae</td>
<td>Gregarious</td>
</tr>
<tr>
<td><em>Camponotus mucronatus</em></td>
<td>60</td>
<td>3.90</td>
<td>Formicinae</td>
<td>Loner</td>
</tr>
<tr>
<td><em>Crematogaster sp.</em></td>
<td>23</td>
<td>1.49</td>
<td>Myrmicinae</td>
<td>Gregarious</td>
</tr>
<tr>
<td><em>Camponotus novogranadensis</em></td>
<td>15</td>
<td>0.97</td>
<td>Formicinae</td>
<td>Loner</td>
</tr>
<tr>
<td><em>Pseudomyrmex gracilis</em></td>
<td>2</td>
<td>0.13</td>
<td>Pseudomyrmicinae</td>
<td>Loner and very rare</td>
</tr>
<tr>
<td><em>Apis mellifera</em></td>
<td>907</td>
<td>91.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native bees</td>
<td>61</td>
<td>6.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diptera</td>
<td>11</td>
<td>1.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lepidoptera</td>
<td>10</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coleoptera</td>
<td>1</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wasps</td>
<td>1</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4. Effect of clogging on nectar secretion and sugar re-allocation in *Turnera velutina* for nectar volume (A) and sugar content (B), between control (C) and treatment (T) plants (means ± se; n = 50 plants). Values shown are a difference in floral nectar, defined as: [FN post-treatment – FN pre-treatment].
### Table 3. Model statistics for the experiments testing indirect (nectar-mediated) and direct (pollinator deterrence) ant-pollinator conflict.

<table>
<thead>
<tr>
<th>Response</th>
<th>Distribution</th>
<th>Fixed effects</th>
<th>N</th>
<th>LRT</th>
<th>P-value</th>
<th>Random effects</th>
<th>Variance</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Indirect conflict</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Floral nectar (µl)</td>
<td>Gaussian</td>
<td>Clogging treatment</td>
<td>50</td>
<td>0.0813</td>
<td>0.7754</td>
<td>Family</td>
<td>0.6987</td>
<td>0.8359</td>
</tr>
<tr>
<td>Floral nectar (µl)</td>
<td>Gaussian</td>
<td>Clogging treatment</td>
<td>50</td>
<td>0.21006</td>
<td>0.6467</td>
<td>Family</td>
<td>138.5</td>
<td>11.77</td>
</tr>
<tr>
<td><strong>Direct conflict</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of visitors</td>
<td>Poisson</td>
<td>Ants in flowers</td>
<td>95</td>
<td>1.1848</td>
<td>0.7567</td>
<td>OLRE</td>
<td>2.77e-08</td>
<td>0.0001</td>
</tr>
<tr>
<td>Likelihood of being alerted</td>
<td>Binomial</td>
<td>Ants in flowers</td>
<td>373</td>
<td>10.715</td>
<td>0.01337</td>
<td>Plant</td>
<td>0.144</td>
<td>0.3803</td>
</tr>
<tr>
<td>Proportion of time per visit spent displaying inspection behaviours</td>
<td>Gaussian (logit)</td>
<td>Ants in flowers</td>
<td>112</td>
<td>7.5728</td>
<td>0.055</td>
<td>Plant</td>
<td>8.279e-10</td>
<td>2.877e-05</td>
</tr>
<tr>
<td>Duration of presence behaviours (sec)</td>
<td>Gamma</td>
<td>Ants in flowers</td>
<td>307</td>
<td>392.37</td>
<td>2.2e-16</td>
<td>OLRE</td>
<td>0.1202</td>
<td>0.3468</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ID visitor</td>
<td>0.0009313</td>
<td>0.03052</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Plant</td>
<td>0.0201</td>
<td>0.14198</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>ID visitor</td>
<td>0.1202</td>
<td>0.3468</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Plant</td>
<td>0.001587</td>
<td>0.03984</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
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<td></td>
<td>OLRE</td>
<td>1.928e-10</td>
<td>0.001389</td>
</tr>
</tbody>
</table>
DISCUSSION

My findings show that both ants and pollinators are active while flowers are open (Fig. 2b), that FN and EFN are simultaneously secreted (Fig. 2a), and that pollen deposition occurs when ants are actively patrolling (Fig. 2c). Consequently, in *T. velutina* guarding ants and pollinators operate in close spatial and temporal proximity, implying that direct and indirect conflict could occur in this system. I found, however, no evidence for indirect, nectar-mediated, conflict between ants and pollinators, since plants did not reallocate resources towards floral nectar, even when 95% of the sugar investment during anthesis, which is in EFN, is prevented (Fig 2). I found evidence for direct conflict, as the presence of dead individuals of patrolling ant species inside flowers was associated with both higher frequency of inspection behaviours in potential pollinators, and reduced visit duration (time spent inside flowers) (Fig. 3). Taken together, these effects increased handling time per flower and reduced pollinator foraging efficiency. Nonetheless, this result was obtained under an experimental setting using dead ants placed inside flowers. Further studies are required to test (i) the effect of living patrolling ants on pollinator visitation, and (ii) the impact of any such effects on plant fitness. The latter are crucial to understanding whether there is ongoing selection on *Turnera velutina* to manage direct ant-pollinator conflict.

**Mutualist activity, reward secretion and pollen deposition**

Floral and extrafloral nectar were secreted simultaneously and rapidly replenished in *T. velutina*, especially during the first two hours post anthesis (Fig. 2). Replenishment is a general feature amongst EFN secretion (Pacini et al. 2003; Pacini & Nepi 2007; Pacini & Nicolson 2007). In fact, I am unaware of any report documenting extrafloral nectaries incapable of replenishing secretion after consumption (Pacini et al. 2003; Pacini & Nepi 2007; Pacini & Nicolson 2007; Escalante-Pérez & Heil 2012; Orona-Tamayo et al. 2013; Heil 2015). In contrast, species vary in whether FN is replenished or not (for details on floral
Chapter 3: Ant-Pollinator conflict

nectar dynamics see: Pacini et al. 2003; Nicolson et al. 2007; Willmer 2011). I suggest that rapid resupply is crucial in short-lived flowers, such as the one-day flowers of *Turnera* species, because it makes the flower attractive again for another visit, potentially increasing pollen transfer, pollen deposition, and seed set. Interestingly, Dutton et al. (2016) reported no FN resupply in flowers from three congeners (*Turnera ulmifolia*, *Turnera subulata*, and *Turnera joelii*), when sampling FN in the morning and afternoon, finding no FN secretion in the afternoon collection. These observations show either variation in nectar resupply within closely related species displaying similar floral biology, or perhaps suggest that shorter term dynamics in the nectar supply of the other three species were not detected by the sampling methods used. The latter highlights the need to test floral resupply within short time scales, because over long sampling intervals, flowers can both secrete and reabsorb nectar (Pacini et al. 2003; Nicolson et al. 2007; Willmer 2011). Finding no standing crop when a flower is resampled in the afternoon does not rule out replenishment after emptying in the morning, and then reabsorption later in the day (Kearns & Inouye 1993).

Model-based estimation of the daily timing of the maxima of nectar secretion suggest that floral nectar secretion peaks a few minutes after the corolla is fully open (Table 3) and 45 min before peak EFN sugar secretion (Fig. 2a). This represents a slight mismatch in the timing of rewards for ants and pollinators, which may underlie the 85 min mismatch in estimated peaks of ants and floral visitor activity (Table 1). To my knowledge, temporal segregation in the activity of ants and pollinators has been reported only for obligate (myrmecophytic) species patrolled or tended by a single ant species at a time (*Humboltia brunonis* (Fabaceae; Gaume et al. 2005), *Hirtella physophora* (Chrysobalanaceae; Malé et al. 2015), and *Opuntia imbricata* (Cactaceae; Ohm & Miller 2014)). In some specialised systems, ant and pollinator activity occurs in close proximity and simultaneously, but conflict is prevented by ant-repellent
Chapter 3: Ant-Pollinator conflict

floral volatiles (*Vachellia zanzibarica* (Fabaceae; Willmer & Stone 1997)) and *Vachellia hindsii* (Fabaceae; Raine *et al.* 2002)). On the other hand, temporal overlap in ant activity at extrafloral nectaries and pollinator visitation to flowers has been reported for facultative ant-plants associated with many ant species simultaneously (*Vachellia constricta* (Fabaceae; Nicklen & Wagner 2006), *Acacia myrtifolia*, (Acacia sensu stricto, Fabaceae; Martínez-Bauer *et al.* 2015), and *Heteropterys pteropetala* (Malpighiaceae; Assunção *et al.* 2014)). My results add *Turnera velutina* to the list of facultative myrmecophiles with synchronised ant and pollinator activity (Fig. 2). This synchronous myrmecophile vs. segregated myrmecophyte pattern is consistent with evidence that ants in obligate mutualisms are more aggressive and better defenders (Chamberlain & Holland 2009; Rosumek *et al.* 2009; Trager *et al.* 2010), but may impose greater ecological costs on host pollination. I suggest that temporal segregation of mutualists and/or ant repellent floral volatiles are alternative strategies that reduce such costs. Further studies on the timing of pollinator, ant visitation and ant aggressivity in a wider range of systems are required to test the temporal component of this hypothesis.

**Direct conflict**

I showed that dead ants inside the flowers of *T. velutina* have an impact on honeybee behaviour (Fig. 3). Ant presence was correlated with shorter honeybee flower visits (Fig. 3c), an increase in the proportion of visitors displaying inspection behaviours, and increased duration of inspection behaviours per visiting bout (Fig. 3). I interpret longer inspection behavior to indicate increased caution in the bees (as previously assumed by: Altshuler 1999; Ness 2006; Junker *et al.* 2007; Assunção *et al.* 2014; Cembrowski *et al.* 2014). My findings are consistent with work on *Heteropterys pteropetala* in which plastic ants inside flowers negatively affected pollination (Assunção *et al.* 2014). Results for *H. pteropetala* differ from ours in that the bees pollinating *H. pteropetala* showed significantly reduced visitation rates to flowers containing plastic ants. In
Chapter 3: Ant-Pollinator conflict

contrast, honeybees in *T. velutina* did not visit less frequently flowers containing ant corpses, compared to control flowers (Fig. 3a). In both systems, ants feeding at extrafloral nectaries did not hinder pollination (Assunção *et al.* 2014) or *T. velutina* (Villamil *et al*., unpublished data). This suggests that while pollinators avoid ants in flowers, plants may have evolved other mechanisms to prevent ants from intruding flowers, resulting in ant-pollinators encounters being rare.

Although experiments that place ant cues on flowers can tell us about the response of pollinators to ants, they must be interpreted with caution as an indicator of current ant-pollinator conflict. Firstly, because ants may rarely enter flowers (Villamil *et al.* submitted). Secondly, by placing such ant cues in flowers I may be violating existing ant-excluding or ant-repelling plant mechanisms (Willmer 2011). Thirdly, in contrast to such experimental treatments, ants do not naturally remain constantly in the flowers for long periods (Assunção *et al.* 2014), and only a low proportion of flowers may be occupied at any one time. For instance, in *T. velutina* only 10% of the flowers are occupied by ants (Villamil *et al*., submitted).

The effect of ants’ deterrence can vary: Does anti-herbivore match anti-pollinator? Although some studies have documented variation among ant species in aggression towards herbivores (Ness 2006; Miller 2007; Ohm & Miller 2014), little is known about the effect of different patrolling ant species with varying levels of aggressivity on pollinator visitation (Ness 2006; Miller 2007; LeVan *et al.* 2014; Ohm & Miller 2014). Nonetheless, a positive correlation between the level of defence provided and the level of pollinator deterrence they exert has often been assumed since ant traits involved in defence (patrolling activity and aggressivity) are likely to be the same as those involved in pollinator deterrence (Ohm & Miller 2014). Bees, like any animal, tend to forage in a way that maximises the net benefit of each foraging trip (Stephens & Krebs 1986; Jones 2010; Cembrowski *et al.* 2014). When foraging in ant-plants, this benefit might
be maximised if foragers avoid flowers or patches where predation risk is high (Dukas 2001; Dukas & Morse 2003; Ness 2006; Jones & Dornhaus 2011; Assunção et al. 2014), as could be the case when encountering ant species that attack pollinators. Some ants also consume FN and pollen, and such plants may represent high risk foraging environments with low net rewards for pollinators (Ness 2006). Shorter or fewer visits to such flowers may be a pollinator strategy to maximise foraging efficiency by avoiding flowers, plants, or patches with high predation risk (Jones & Dornhaus 2011).

In *T. velutina*, the most aggressive ant guard, *Dorymyrmex bicolor*, had the strongest effect on pollinator behaviour (Fig. 3), while *Brachymyrmex* sp. ants inside the flowers did not reduce the duration of pollinator visits. The least effective anti-herbivore ant species, *Paratrechina longicornis* (Villamil unpublished data), halved the duration of pollinator visits (Figs. 3). In *Ferocactus wislizeni*, plants tended by *Solenopsis xyloni*, the most aggressive ant species, had fewer and shorter pollinator visits (Ness 2006). Such differences are consistent with pollinator sensitivity to ant aggressiveness. In contrast, although ant exclusion in *Opuntia imbricata* significantly increased pollinator visitation, there were no differences in impacts associated with different ant species (Ohm & Miller 2014), and no evidence that the more aggressive guard (*Liometopum apiculatum*) had a stronger deterring effect on pollinators (Ohm & Miller 2014). Mapping whether the level of ant aggressivity towards herbivores correlates positively with the ecological costs on pollination via pollinator deterrence remains unknown (but see: Ness 2006; Miller 2007; LeVan et al. 2014; Ohm & Miller 2014), and should be tested, not assumed.

**Indirect conflict**

My experimental approach found no evidence for a trade-off in sugar investment in extrafloral and floral nectar in *T. velutina*. I conclude that there is no indirect nectar-mediated conflict between guarding ants and pollinators in
Chapter 3: Ant-Pollinator conflict

*Turnera velutina*, and that pollinators do not obtain greater rewards when rewards for patrolling ants are eliminated.

I found only two previous studies testing indirect, nectar-mediated ant-pollinator conflict by quantifying sugary rewards (FN and EFN) to both mutualists (Chamberlain & Rudgers 2012; Dutton *et al.* 2016). Previous work on other *Turnera* species by Dutton *et al.* (2016) found evidence of a trade-off in two of the three *Turnera* species tested; removing EFN decreased FN and *vice versa* in *T. ulmifolia* and *T. subulata*, but not in *T. joelii*. Interestingly, both *Turnera* species in which trade-offs were detected by Dutton *et al.* (2016) invested equally in FN and EFN, whilst *T. joelli* (which showed no trade-off) invested more in EFN (Dutton *et al.* 2016). The same pattern holds for *T. velutina*, a species with an asymmetric investment towards EFN, which accounts for 95% of the sugar allocation per leaf-flower module, and where I found no trade-off or resource reallocation from EFN to FN (Fig. 4). Unfortunately data on FN and EFN volume and sugar content was not reported for the cotton species (Chamberlain & Rudgers 2012).

One possible reason for lack of a trade-off is that sugar is not a limiting resource for the plant. If so, there would be no reason to expect dynamic reallocation. Estimates of the metabolic costs of nectar secretion vary, and while some studies suggest low metabolic costs (O’Dowd 1979: EFN accounts for 1% of the total energy invested per leaf), others indicate investment of up to 37% of daily photosynthesis in floral nectar (Southwick 1984; Pyke 1991). A second reason, which applies in particular to comparative cross-species analyses rather than experimental manipulations, is that investment in both forms of nectar may be influenced by other aspects of life history strategy. Chamberlain and Rudgers (2012) found no significant negative correlations between extrafloral nectary and floral traits in a comparative analysis across cotton (*Gossypium*) species, and correlations were significantly positive in 11 of 37 cotton species. Foliar extrafloral nectary volume was positively associated with plant...
investment in floral nectar, rejecting the hypothesis of trade-offs among investments in pollinators versus bodyguards in *Gossypium*. Several potential mechanisms underlying the positive correlations between FN and EFN have been proposed, including pleiotropy, genetic, physiological or ecological linkage (Chamberlain & Rudgers 2012). The pleiotropy or genetic linkage hypothesis could be tested using genome sequencing (Chamberlain & Rudgers 2012). Positive correlations could also arise from physiological or ecological linkage. Traits such as FN and EFN may be physiologically linked. However, the fact that in *Gossypium* FN volume was most strongly correlated with foliar EFN volume, but FN was weakly correlated with bracteal EFN volume (Chamberlain & Rudgers 2012) questions the physiological linkage hypothesis since bracteal and floral nectaries are spatially closer than floral and extrafloral nectaries, but they are not strongly correlated. I suggest that lack of any trade-off could also indicate that FN and EFN may be phenotypically integrated as a functional module for mutualist attraction. Although formal analyses are required to test this hypothesis, I think it is a strong possibility since *T. velutina* leaves are phenotypically integrated modules in which leaf economics, defensive and morphological traits covary and are ecologically linked (Damián et al. 2018). Whatever the drivers of these positive correlations may be, available evidence suggests that plants may experience fewer investment trade-offs among different functional traits than previously assumed.

**CONCLUSIONS**

To my knowledge, trade-offs between extrafloral and floral nectar traits have been studied in 41 species from two genera: 37 *Gossypium* species (Chamberlain & Rudgers 2012), and four *Turnera* species, including this study (Dutton et al. 2016; Villamil 2017, Fig. 2). Negative correlations or evidence for trade-offs have been found in only two of these species: *T. ulmifolia* and *T. subulata* (Dutton et al. 2016), representing less than 5% of the species studied. Although many more studies are required to shed light on quantitative trends of floral and extrafloral
investment in plants, trade-offs between floral and extrafloral seem infrequent. On the other hand, evidence of direct conflict with patrolling ants reducing pollinator visitation frequency and duration, inducing inspection behaviours and increasing foraging time has been widely reported (Rudgers & Gardener 2004; Ness 2006; Chamberlain & Rudgers 2012; Malé et al. 2012; Assunção et al. 2014; Koptur et al. 2015; Malé et al. 2015; Martínez-Bauer et al. 2015). I suggest that these two issues are not isolated, and hypothesise that positive correlations between FN and EFN investment in ant-plants may be a plant strategy to compensate or lure pollinators to apparently risky flowers.

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This chapter has been published:
CHAPTER 4

ECOLOGICAL AND EVOLUTIONARY COSTS OF ANT PATROLLING

How costly are guarding ants for pollination?

“Bees and humans alike have their criteria for selection: symmetry and sweetness in the case of the bee, heft and nutritional value in the case of the potato-eating human.”

“The bees! The bees will let themselves be lured into the most ridiculous positions, avidly nosing their way like pigs through the thick purple brush of a thistle, rolling around helplessly in a single peony’s blonde Medusa thatch of stamens - they remind me of Odysseus’s crew in thrall to Circe.”

Michael Pollan
The Botany of desire
INTRODUCTION

Mutualisms are traditionally defined as interactions from which both partners benefit. However, mutualisms involve a combination of costs and benefits (Begon et al. 1986, Bronstein 2001, Herrera and Pellmyr 2009). Hence, mutualisms should be viewed as reciprocal exploitation relationships where the benefits outweigh the costs (Begon et al. 1986). Identifying and quantifying costs and benefits are essential to understanding the magnitude and direction of the selective pressures acting upon the participants (Herre et al. 1999, Ohm and Miller 2014) and the evolutionary stability of the interaction (Palmer et al. 2010).

In order to address these costs, we need to tackle mutualisms with a broader approach, moving away from pairwise species interactions and into a multispecies community approach, considering several interactions simultaneously (Strauss 1997, Herrera 2000, Dátillo et al. 2016, Del-Claro et al. 2018).

Patrolling ants in ant-plant mutualisms defend plants from herbivores, pathogens, fungi, or encroaching vegetation (Bentley 1977a, Beattie 1985, Martin and Doyle 2003). However, engaging with patrolling ants may also impose costs on the plant, especially if these guards deter other mutualists (Koptur et al. 2015), although the net outcome has been demonstrated as beneficial in the subset of species in which this had been quantified (Romero and Koricheva 2011). Ant-plant mutualisms represent an ideal system to explore the costs and benefits of mutualisms from a multispecies approach because, on one hand ants are mutualists that benefit plants by decreasing herbivory (Chamberlain and Holland 2009, Rosumek et al. 2009, Trager et al. 2010, Romero and Koricheva 2011), and on the other ants can act as antagonists by consuming floral structures (Stanton et al. 1999, Gaume et al. 2005, Frederickson 2009), deterring pollinators (Assunção et al. 2014, Villamil et al. 2018) or other predatory arthropods (such as ladybirds, spiders, or wasps) that also benefit the plant (Jones and Koptur 2015).
The negative effects that competition between different pollinators for floral resources may impose on plant fitness are smaller compared to the negative effects of competition between pollinators and other plant mutualists that are not pollinators, such as bodyguards, may have on plant fitness (Cembrowski et al. 2014, Dutton et al. 2016). Negative interactions between predator ants and pollinators are theoretically expected, and have been confirmed in several species as an evolutionary stable strategy (Rico-Gray and Oliveira 2007b, Frederickson 2009, Palmer et al. 2010). Plant trade-offs in resource allocation to reproduction or defence may be one cause of conflicting interactions between pollinators and ants (Dutton et al. 2016, Villamil et al. 2018). Ant aggressivity and the predation risk it represents for pollinators is another source of conflict (Romero and Koricheva 2011, Villamil et al. 2018).

Aggressivity to herbivores and reductions in herbivory rates are the most commonly used criteria to evaluate the benefits and protection efficiency (herbivore removal and damage reduction rates) of ant mutualists (Chamberlain and Holland 2009, Rosumek et al. 2009, Trager et al. 2010, Romero and Koricheva 2011). However, these indicators do not reveal the ecological costs of such defensive mutualists, and in fact considering only these criteria can underestimate costs associated with myrmecophily that have been demonstrated by several studies (Dukas 2001, Gaume et al. 2005, Ness 2006, Goncalves-Souza et al. 2008, Frederickson 2009, Romero and Koricheva 2011, Stanton and Palmer 2011, Dutton and Frederickson 2012, Ohm and Miller 2014, Jones and Koptur 2015, Malé et al. 2015). In fact, ant aggressivity may be a double-edged sword that underlies the core ecological costs and benefits of myrmecophily. More aggressive ants are likely to be better defenders against herbivores, but also pose a higher predation risk to other mutualistic guilds such non-ant predators of herbivores or pollinators due either to direct attacks or
Non-consumptive effects of predation are defined as changes in prey traits or behaviours when under predation risk (Preisser et al. 2005, Sheriff and Thaler 2014) and their magnitude can be similar to, or higher than, that of direct consumptive effects (Preisser et al. 2005, Clinchy et al. 2013, Sheriff and Thaler 2014). Even low levels of predation risk may impose strong selection on pollinators to develop detection mechanisms and anti-predatory behaviours, given that the cost of failing to detect predators includes death (Dukas 2001, Abbott and Dukas 2009). Several mechanisms of predator detection have been documented in pollinators. Pollinators recognise parts of predator bodies, such as crab spider forelimbs (Goncalves-Souza et al. 2008), detect past predation events through remains of previously preyed insects (Romero et al. 2011) or, in the case of *Apis mellifera*, communicate predation zones with waggle dance communication (Abbott and Dukas 2009). Predation risk also has strong effects influencing pollinator behaviour as shown by a meta-analysis (Romero et al. 2011).

A meta-analyses showed that although anti-predatory responses in pollinators depend on a combination of both pollinator and predators traits (Romero et al. 2011), non-consumptive effects of predators on pollinators can have important consequences for plant-pollinator interactions and the reproductive success of both partners (Romero et al. 2011, Clinchy et al. 2013). When predators interfered in plant-pollinator mutualism, they decreased plant fitness by 17% as reported in a meta-analyses by Romero and Koricheva (2011). Yet, predator-pollinator interactions in flowers are still poorly known and heavily understudied compared to predator-herbivore interactions in plants (Romero and Koricheva 2011).
Many studies have investigated the effects of guarding ants on the reproductive outcome of the host plant. Four meta-analyses have compiled this information (Chamberlain and Holland 2009, Rosumek et al. 2009, Trager et al. 2010, Romero and Koricheva 2011), revealing that only few studies have addressed the ecological costs of ants. Most of the studies on the effects on ants on plant fitness have focussed on their effects via herbivore deterrence, overlooking the effects via pollinator deterrence (Romero and Koricheva 2011). In fact, up to 2011, only four studies had addressed the indirect interactions between ants and pollinators (Romero and Koricheva 2011) compared to over 31 papers on their effects via herbivore deterrence (Trager et al. 2010). And so, we emphasize the importance and need for a multispecies approach in ecological interactions research.

Although ants can increase plant fitness by decreasing herbivory and florivory, ants may also affect plant fitness by disrupting plant-pollinator mutualisms (Ness 2006, Goncalves-Souza et al. 2008, Ohm and Miller 2014). However, mutualisms are only maintained throughout evolutionary time if their fitness benefits are greater than their costs (Herre et al. 1999, Stanton and Palmer 2011). The overall net effect of predators, such as ants, on plant fitness was positive, increasing plant fitness by 32%, indicating that effects via herbivores were stronger than effects via pollinators (Romero and Koricheva 2011).

Due to ant threats, pollinators may respond in different ways which will have different consequences on the host plant mating system and fitness. Those studies that have addressed ant-pollinator conflict show contrasting evidence regarding the effects of ants on pollinator visitation and their consequences on plant mating systems. Through direct attacks or non-consumptive effects, aggressive ants can reduce pollinator visitation frequency and duration (Ness 2006), and reduce plant fitness by eating flower buds or making pollen inviable.
(Stanton et al. 1999, Frederickson 2009, Stanton and Palmer 2011, Dutton and Frederickson 2012, Malé et al. 2015), leading to decrease the number of fruits, seeds or seed mass (Ness 2006). Ness (2006) attributes lower seed set to a three-fold reduction in visitation frequency of *Ferocactus wislizenii* plants tended by aggressive ants, however he did not test this hypothesis. Guarding ants can also increase plant fitness despite disrupting plant-pollinator mutualisms. Due to ant threats, pollinators may spend less time per flower and could potentially visit more flowers, promoting more efficient pollen transfer (Altshuler 1999). Increased fruit set in the presence of ants in *Psychotria limonensis* was interpreted as a beneficial outcome of ant-pollinator interactions, due to a higher rates of movement between flowers, leading to enhanced outcrossing (Altshuler 1999), although this has not either been tested. Previous experiments on the ant-plant *T. velutina* have shown that ant corpses placed inside flowers reduce pollinator visit duration (Villamil et al. 2018). However, such an experimental setup may differ from natural circumstances since 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, changes in pollinator assemblage composition, visit frequency and duration have been hypothesized to drive positive or negative impacts of ant-pollinator interactions on plant reproduction (Altshuler 1999, Ness 2006). As yet, none of these have been specifically tested or observed in an adequate experiment where pollinator visitation, frequency, and duration of the visits is recorded along with the fitness of plants with and without defensive ant patrolling.

**QUESTIONS & AIMS**

We estimated the ecological and fitness consequences of myrmecophily on the pollination biology of *Turnera velutina* (Passifloraceae) by contrasting plants patrolled by free roaming, living ants with ant-excluded plants. We quantified the effects of ant patrolling on pollinator community composition, visitation
rates, behavioural consequences on floral visitors, and their consequences on the plant mating system, pollen transferring rates and dynamics, and plant fitness. We addressed the following questions:

(i) What is the effect of ant patrolling on pollinator visitation? (pollinator assemblage 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?

**HYPOTHESES AND PREDICTIONS**

*Pollinator composition and behaviour*

Pollinator responses to predation risk can vary between taxa. Pollinator body size and lifestyle (social or solitary) have been proposed as a source of variation. Smaller pollinators have been predicted to be more vulnerable to predation risk (Dukas and Morse 2003), whilst contrasting predictions have been made regarding lifestyle. On one hand, social hymenopterans can influence naïve colony members to forage away from dangerous flowers, such as *Apis mellifera* does by waggle dance communications (Abbott and Dukas 2009), leading to predictions of stronger avoidance behaviours in social species compared to solitary bees. But, social hymenopterans may also be under weaker selection pressures to evolve avoidance behaviours since death of a non-reproductive worker has lower inclusive fitness consequences than the death of a solitary bee female (Clark and Dukas 1994).

We predicted ant patrolling would induce changes in pollinator composition by deterring non-aggressive, solitary pollinators resulting in more visits by non-aggressive pollinators to plants without ants.
Pollinator visitation and plant mating system

Ant patrolling can affect pollinator dynamics and plant mating systems in a variety of ways as described in the following envisioned scenarios and the predictions associated with them.

1) Pollinators are completely deterred by the presence of ants and avoid these plants leading to reduced visitation frequency, pollinator limitation, and reduced seed set and plant fitness.

2) Pollinators are partially deterred and are forced to relocate frequently to avoid being attacked by ants, leading to a reduction in visit duration. If pollinators spend less time per flower they could potentially visit more flowers, promoting more efficient pollen transfer and increasing male fitness. If this scenario is true, there are two possible outcomes for the plant mating system:

   a. If pollinators relocate to another plant, this would promote higher outcrossing rates, increasing the genetic diversity of the seeds and enhancing the advantages of sexual reproduction for the host plant.

   b. If pollinators relocate to another flower within the same plant, this would promote geitonogamy (intra-plant pollination), which is a form of selfing that does not enhance genetic diversity, representing an intermediate cost for pollination of the host plant.

Plant fitness

The effects of patrolling ants on host plant fitness have been shown to vary. Romero and Koricheva (2011) detected several factors explaining the variation in the effect of predators on plant fitness via pollinator deterrence. We highlight three of these factors relevant to the ecology of T. velutina: predator hunting mode, habitat domain, and plant rewards.

1. Active hunters, such as ants, increased plant fitness whilst sit-and-wait predators, such as spiders, did not.
2. Predators with a broader territory, such as wasps and ants, had negative effects on plant fitness, whereas those with a narrow habitat domain, such as bugs, ladybirds and parasitoids had positive effects.

3. Predator effects were positive on plants that offered rewards such as extrafloral nectar, and negative for plants without rewards.

Based on these findings, the predictions for the effect of patrolling ants on *T. velutina*’s fitness via pollinator deterrence are contrasting. On the one hand, the active hunting mode of ants and the presence of EFN rewards in *T. velutina* predict ants would have a positive effect on fitness. But on the other hand, the wide territorial domain of ants predicts a negative effect. Patrolling ants in *T. velutina* increase plant fitness via herbivory suppression (Cuautle and Rico-Gray 2003), and we predicted that their effects on plant fitness via pollinator deterrence would be negative, decreasing fitness.

**MATERIALS AND METHODS**

**Study site**

Field experiments were conducted at Troncones, Guerrero on the southern Pacific coastline of Mexico (17°47’ N, 101°44’ W, elevation < 50 m). The climate is warm sub-humid, with a rainy season during the summer (mid-May to November), 1025 mm annual precipitation, and a mean annual temperature range of 25.4-28.0 °C (Zedillo-Avelleyra 2017). The habitat has been described as secondary vegetation of tropical low forest (Zedillo-Avelleyra 2017), which becomes coastal sand scrub vegetation as it approaches the sea.

**Study system**

*Turnera velutina* (Passifloraceae) is a Mexican endemic perennial shrub (Arbo 2005) that is also a myrmecophile (Cuautle and Rico-Gray 2003) which grows in lowland, coastal environments. It establishes a facultative mutualism with up to
13 ant species (Cuautle et al. 2005, Zedillo-Avelleyra 2017), 10 of which are present in Troncones (Zedillo-Avelleyra 2017). Extrafloral nectar is provided in paired cup-shaped glands located at the bottom of the leaf blade or on the leaf petiole, on the underside of the leaves. *T. velutina* is a self-compatible, monomorphic herkogamous species that requires pollinators for adequate seed production (Sosenski et al. 2016). Although it flowers year-round, flowering peaks during summer (Cuautle et al. 2005) and flowers are bisexual, last one day, and are insect-pollinated (Sosenski et al. 2016). As rewards for pollinators, flowers offer pollen and floral nectar that is easily accessible at the base of the yellow, pentamerous, bell-shaped corolla. At Troncones, native butterflies are the dominant flower visitors of *T. velutina*, followed by the introduced European honeybee (*Apis mellifera*). Native bees, wasps, and occasionally flies also visit flowers at Troncones. *Apis mellifera* has been confirmed as a pollinator for *T. velutina* (Ramos-Castro 2013, Sosenski et al. 2016) but the pollination efficiency of other floral visitors still remains to be confirmed.

**Ants exclusions and experimental setup**

Twelve large plants fitting the following criteria were haphazardly chosen: plants producing six or more flowers per day, surrounded by other *T. velutina* plants, and semi-isolated plants (not within dense patches) and with few vegetation links to other plants. Within these twelve plants, pairs of nearby plants with similar height and foliage abundance were chosen and marked as either control (N=6) or experimental (N=6). The vegetation surrounding these plant pairs was trimmed or cleared in order to isolate these plants from direct contact with any surrounding vegetation. Plants within a pair were located < 2 m apart, and each pair of plants was separated from any other pair by at least 10 m, to reduce the chances of dyed pollen from a neighbour pair confounding our results. These plant pairs were used to assess the effect of ant patrolling on pollinator visitation, plant mating system and fitness.
Ants were excluded from experimental plants using Tanglefoot™, a highly sticky insect-trapping resin, whilst control plants had no ant exclusions. A band of masking tape with the sticky side outwards was wrapped around the base of the trunk or branch from which ants will be excluded. A ring of Tanglefoot™ was applied only on the masking tape band, carefully avoiding spreading it onto plant tissue as it blocks stomata, killing the plant. A 2 cm wide masking tape band is enough for small twigs; thicker bands were required for larger branches. In shrubs with multiple stem branching from the base, exclusions were applied on every stem. All vegetation links to other plants were trimmed, and if required, parts of the bush were tied with string to contain and effectively isolate them, destroying any points of contact that ants could use to climb onto the plant. Tanglefoot™ was also applied to string ties to prevent ants from accessing the plants by crawling up the ties. Exclusions were checked daily and Tanglefoot™ was replenished if required.

To assess the effect of ant patrolling on pollen transfer and its consequences on the rate of selfing, geitonogamy, and outcrossing, the anthers of plants with and without ant patrolling were differentially dyed. We used a focal-plant, focal-flower experimental design. Each focal plant, either control or ant-excluded, was surrounded by other neighbouring plants. The anthers of focal plants were dyed (red, blue, green or purple), and flowers in neighbouring (non-focal) plants remained undyed (naturally yellowish-orange). Within a focal plant, a focal flower was selected and its anthers were dyed differently from the anthers in the rest of the flowers from that plant, hereafter referred to as satellite flowers.

Every morning at 0700 before flowers opened, six flower buds per plant (a focal bud surrounded by five satellite buds) were bagged using tulle bags to exclude visitors. All additional pre-anthesis buds were removed to standardise floral display across experimental plants. Once the corollas were fully open,
anthers were dyed and the flowers were re-bagged until the dye dried and anthers had dehisced. Pollinator observations were conducted on all flowers (focal and satellite) and stigmas from focal flowers were collected at the end of the anthesis period to count pollen grains received. To assure a minimum common supply of allogamous pollen across all flower pairs, 10-12 flowers from other plants around each control-exclusion plant pair were also bagged before anthesis and remained bagged until the focal and satellite flowers were un-bagged for observation.

All 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).
Figure 1. Experimental design and methods. a) diagram of the focal-plant, focal-flower experimental design. Empty pentagons represent focal plants with or without ants, coloured circles represent focal and satellite flowers with dyed anthers, and yellow pentagons represent neighbouring plants with undyed anthers (naturally yellow); b) syringes with the dyes, dyed anthers, stigmas bearing dyed pollen grains, and stigma squash slides; c) pollen flow diagram.
Visitation

Pollinator visitation to all flowers (1 focal + 5 satellites) on control and ant excluded focal plants was recorded to estimate the effect of ant patrolling on pollinator visitation. Once the dyed anthers had dried and dehisced, bags were removed from all flowers from one focal plant and its surrounding un-dyed flowers. Every focal plant was observed for 40 min split into two rounds of 20 min. The first round was conducted immediately after bag removal when flowers had a full pollen and nectar load. The second round started ~90 min after the first round was completed. Unobserved flowers remained bagged until their first observation round started to ensure all flowers had a full pollen and nectar load during the first round of observations. We recorded the identity, frequency, duration, and behaviour of floral visitors and visits, as detailed below.

Composition

To estimate the overall abundance of flower visitors by each taxonomic group, observations from control and ant-excluded plants were pooled together and the percentage of visits by each taxon was calculated. Ants and beetles were recorded as floral visitors but were not regarded as potential pollinators (see Results for further details). Potential pollinators (hereafter pollinators) comprised the following taxonomic groups: Apis mellifera, native bees, Lepidoptera, Diptera, and wasps. Bees were split into Apis mellifera and native bees because we were interested in contrasting the visitation patterns of the native bee species with those of the introduced European honeybee (Apis mellifera). 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. Since Apis mellifera and Lepidoptera jointly accounted for 94% of all visitors (Table 1) we considered only these taxonomic groups for all further analyses, excluding native bees, wasps and flies.
Visitation frequency was defined as the number of interactions between a floral
visitor or potential pollinator and any of the flowers from a focal plant during
the 40 min of observation (visits/40 min/plant). We recorded visitor identity
and so the same visitor could account for more than one visit if, for instance, it
landed, hovered, and landed again in a flower. Visitors were not followed, and
visits to other surrounding plants were not recorded. We stopped recording a
visitor whenever it left the plant and escaped our vision field. Visitors
abundance was estimated as the number of individual visitors per taxa
(individuals per plant).

The effect of ant patrolling on visitation frequency was tested using a
Poisson mixed model, fitting the number of visits per plant as the response
variable. Ant exclusion treatment, pollinator type (Apis mellifera or
Lepidoptera), and the interaction between both factors were fitted as fixed
effects. Plant identity and day were fitted as a random effect, along with an
observation level random effect (OLRE) to account for overdispersion (Hinde
1982). Post hoc Tukey model comparisons were used to detect statistically
significant differences between the two types of pollinators under control or ant
exclusion treatments.

Visit duration is the time a pollinator (Apis mellifera or butterflies) spent
interacting with a flower. The effect of ant patrolling on visit duration was tested
using a Poisson mixed model, fitting the mean visit duration per individual
visitor as the response variable. Ant exclusion treatment, pollinator type (Apis
mellifera or Lepidoptera), and the interaction between both factors were fitted
as fixed effects. Plant identity, day, and an OLRE were fitted as random effects.
Post hoc Tukey model comparisons were used to detect statistically significant
differences between the two types of pollinators under control or ant exclusion
treatments.
Pollinator behaviour

Two types of pollinator behaviours were identified: ‘inspection’ and ‘contact’, following (Villamil et al. 2018, Chapter 3). Inspection behaviour is defined as a pollinator approaching to, or hovering over, a flower without landing on it. Contact behaviours are defined as all behaviours made within the flower between landing and take-off, and included foraging on pollen or nectar, or resting, basking or grooming on the petals, anthers or stigmas.

The effect of ant patrolling on the likelihood of pollinators displaying inspection behaviours as defined above was tested using a binomial mixed model. For all *Apis mellifera* and butterflies observed, the presence or absence of inspection behaviours was fitted as the response variable. Ant exclusion treatment, pollinator type (*Apis mellifera* or Lepidoptera), and the interaction between both factors were fitted as fixed effects. Plant identity, pair identity, and day were fitted as random effects. Post hoc Tukey comparisons were used to detect statistically significant differences between all four groups (*Apis mellifera* or butterflies, at control or ant exclusion plants).

The effect of ant patrolling on pollinator deterrence was tested using a binomial mixed model. Pollinator deterrence is here defined as the absence of contact behaviours following an inspection behaviour, where pollinators displayed only inspection behaviours, approaching or hovering over, but without landing inside the flower. For every pollinator observed displaying inspection behaviours, the presence or absence of contact behaviours was fitted as the response variable. Ant exclusion treatment, pollinator type (*Apis mellifera* or Lepidoptera), and the interaction between both factors were fitted as fixed effects. Plant identity, pair identity, and day were fitted as random effects. Post hoc Tukey comparisons were used to detect statistically significant differences between all four groups (*Apis mellifera* or butterflies, at control or ant exclusion plants).
Chapter 4: Ecological costs

The effect of ant patrolling on the duration of each type of behaviour was tested using a Poisson mixed model, splitting visitor observations into inspection or contact behaviours. The total duration of each behaviour (inspection or contact) per visitor was fitted as the response variable. Ant exclusion treatment, pollinator type, (Apis mellifera or Lepidoptera), behaviour (inspection or contact) and paired interactions between these variables (Treatment*Type of pollinator, Treatment*Behaviour, Type of pollinator*Behaviour) were fitted as fixed effects. Plant identity, pair identity, day, and an OLRE were fitted as random effects. Post hoc Tukey model comparisons were fitted to test statistically significant differences between all eight groups (inspection and contact behaviours in Apis mellifera or butterflies, under control or ant exclusion plants).

Pollen dyes
Anthers were dyed once the corolla opened completely (~0800-0815), but before pollen was released (anther dehiscence). Anthers, one by one, were embedded in a droplet of dye until soaked. This was done carefully, to avoid staining any other floral structures, such as the filaments, pistils or petals. Once dyed, flowers were bagged again until anthers dehisced and the pollen released was dry. Purple dye was prepared by diluting commercially available methyl violet with ethanol 70% [50:50], the green dye used was commercially available Green S without dilution. Red (safranin) and blue (methylene blue) were donated by Manuel Ochoa from LIPA, UNAM. These dyes were prepared by diluting 1 g of either dye in 15.5 ml of distilled water, and then mixing this solution with ethanol 95% [50:50]. Previous studies showed that dyeing Turnera velutina anthers in these colours had no effect on pollinator visitation (Ochoa Sánchez 2016).

Towards the end of the anthesis period (11:30), pistils from focal flowers were collected in Eppendorf tubes and mounted on a slide as a glycerine squash. Pistils were collected before corollas closed in order to prevent petals touching
the stigma, which could damage and collapse the stigmatic hairs, interfering with the squashes pollen counts. Slides were sealed using nail polish and kept in a fresh and dry environment, with controlled room temperature (22°C) (Kearns and Inouye 1993, Ochoa Sánchez 2016). Selfing, outcrossing, and geitonogamy rates on plants with and without ant patrolling were estimated by counting red, blue, green, purple, and yellow (un-dyed) pollen grains from focal flower stigmas under a light microscope at 10x or 40x using phase contrasts Ph3 and Ph4 for colour discrimination.

**Plant mating system**

Pollen load, the total number of pollen grains received per stigma, was counted from stigma squash slides. The effect of ant patrolling on stigma pollen load was tested using a Poisson mixed model, fitting the total number of pollen grains per stigma as the response variable. Ant exclusion treatment was fitted as a fixed effect, plant identity, pair identity, day, and an OLRE were fitted as random effects.

The effect of ant patrolling on pollen transfer and its consequences on plant mating system (selfing, geitonogamy, and outcrossing) was assessed by counting differentially dyed pollen grains. Pollen grains received from the same flower (selfing), from another flower within the same plant (geitonogamy), from the reciprocal pair plant, or another plant (outcrossing) were counted on focal-flower stigmas. The number of pollen grains from each origin (selfing, geitonogamy or outcrossing) was divided by the pollen load per stigma to determine the proportion of pollen from each mating system. Proportional data were transformed to normality using the logit transformation as follows: Logit pollen proportion = \log\left(\frac{\text{pollen proportion}}{1-\text{pollen proportion}}\right). Infinite numbers resulting from impossible quotients were replaced by zeros. The effect of ant patrolling on the mating system was tested using a linear mixed model, fitting the proportion of pollen as the response variable. Ant exclusion treatment, mating
system (outcrossing, geitonogamy or selfing), and the interaction between both factors were fitted as fixed effects. Plant identity, pair identity, and day were fitted as random effects. Post hoc Tukey comparisons were used to test statistically significant differences between the proportion of pollen grains from each type of mating system in plants with and without ants.

The pollen flow dynamics were analysed using six categories to describe the mating system and the pollen origin (MSPO), dictating from where to where the pollen was moved. The effect of ant patrolling on pollen flow dynamics was tested using a Poisson mixed model, fitting as the response variable the number of pollen grains received and donated to and from every possible pollen source identifiable in this experiment (same flower selfing, other flower from the same plant: geitonogamy, reciprocal pair plant (outcrossing pair), or another plant from the same species (outcrossing unknown)). Ant exclusion treatment, pollen origin-destination, and the interaction between both factors were fitted as fixed effects. Plant identity, pair identity, day and an OLRE were fitted as random effects. Post hoc Tukey comparisons were used to test statistically significant differences between these groups.

**Male plant fitness**

The number of pollen grains donated per flower was used as an estimate for male plant fitness and quantified based on pollen grains from each flower/colour deposited on focal stigmas. The total number of pollen grains from satellite flowers was divided by five, to obtain the mean number of pollen grains donated by each satellite flower within the plant. The total number of pollen grains from the reciprocal pair plant was divided by six to obtain the mean number of pollen grains donated by each flower from the reciprocal plant pair.
Chapter 4: Ecological costs

The effect of ant patrolling 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. Ant exclusion treatment (control or ant exclusion) was fitted as a fixed effect and plant identity, pair identity, day, and an OLRE were fitted random effects. *Post hoc* Tukey comparisons were used to test statistically significant differences between these groups.

The effect of ant patrolling on the destination of the pollen grains donated per flower was tested using a Poisson mixed model. The number of pollen grains was fitted as the response variable. Ant exclusion treatment, pollen donor-destination, and the interaction between both factors were fitted as fixed effects. We only contrasted the number of pollen grains donated by focal or satellite flowers, as every plant had only six flowers, since we controlled floral display in our experimental design. Pollen donated by unknown plants was excluded as the number of donor flowers was unknown, and hence cannot estimate pollen grains donated per flower. Plant identity, pair identity, day, and an OLRE were fitted as random effects. *Post hoc* Tukey comparisons were used to test statistically significant differences between these groups.

RESULTS

**Floral visitors and potential pollinators**

We recorded 40 h of observations of 360 flowers on 12 plants over five days. These flowers received 967 floral visitors over the observation period, which included *Apis mellifera*, several morphospecies of butterflies, native bees, beetles, ants, wasps, and flies (Table 1). Only one individual ant was observed inside one of the flowers in the ant exclusion treatment, and three individual ants were observed inside flowers on control plants. A total of 108 beetles were
recorded inside flowers, with 54 in each of control and ant-excluded flowers (see Supplementary Natural History Notes). Nonetheless, ants and beetles were excluded from the list of potential pollinators and from any further analyses because their role as pollinators of *T. velutina* is dubious (for ants see: Rico-Gray and Oliveira 2007b, Dutton and Frederickson 2012) (for beetles see: Kerner 1878, Krupnick and Weis 1999, Rudgers and Gardener 2004, Takayuki 2005). Beetles were excluded as they are usually regarded as florivores since they eat pollen, anthers (Baldock et al. 2011), and are nectar robbers in *T. velutina* (see Supplementary Natural History Notes). Beetles are also commonly excluded from pollination studies because their visit durations are extremely long and would otherwise inflate and confound the behavioural patterns of other floral visitors. All of these factors are true in *T. velutina* where the duration of beetle visits ranged from 30 – 1200 sec, whilst the duration for other visitors ranged from 1 - 95 sec.

We recorded 853 individual visitors from taxa regarded as potential pollinators (hereafter pollinators), because they were observed contacting male and female plant sexual organs, although further experiments testing their efficacy as pollen vectors are still required. Visitors so defined comprised Lepidoptera, *Apis mellifera*, native bees, wasps, and Diptera (Table 1). Because lepidopterans and *Apis mellifera* accounted for more than 80% of all floral visitors and > 94% of potential pollinators (Table 1), we considered only these two taxa in further analyses regarding pollinator frequency, duration, and behaviour.

**Pollinator visitation: composition**

The total number of pollinators from each taxonomic group visiting *T. velutina* flowers did not differ significantly between control and ant-excluded plants ($X^2 = 1.42, df = 4, P = 0.84$; Fig. 2).
Table 1. Relative abundance of floral visits by and visitors by floral visitors and potential pollinators. Visits consider the number of times a visitors contacted the floral organs, whilst visitors consider the number of individuals contacting flowers.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Number</th>
<th>Visits (%)</th>
<th>Pollinators (%)</th>
<th>Number</th>
<th>Visits (%)</th>
<th>Pollinators (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Floral visitors</td>
<td></td>
<td></td>
<td>Floral visitors</td>
<td></td>
</tr>
<tr>
<td>Lepidoptera</td>
<td>436</td>
<td>45.09</td>
<td>51.11</td>
<td>192</td>
<td>42.01</td>
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<tr>
<td>Apis mellifera</td>
<td>370</td>
<td>38.26</td>
<td>43.38</td>
<td>132</td>
<td>28.88</td>
<td>38.26</td>
</tr>
<tr>
<td>Native bees</td>
<td>31</td>
<td>3.21</td>
<td>3.63</td>
<td>8</td>
<td>1.75</td>
<td>2.31</td>
</tr>
<tr>
<td>Wasps</td>
<td>10</td>
<td>1.03</td>
<td>1.17</td>
<td>8</td>
<td>1.75</td>
<td>2.31</td>
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<tr>
<td>Diptera</td>
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<td>0.70</td>
<td>5</td>
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<td>1.44</td>
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<tr>
<td>Coleoptera</td>
<td>110</td>
<td>11.38</td>
<td></td>
<td>108</td>
<td>23.63</td>
<td></td>
</tr>
<tr>
<td>Ants</td>
<td>4</td>
<td>0.41</td>
<td></td>
<td>4</td>
<td>0.87</td>
<td></td>
</tr>
<tr>
<td>TOTAL</td>
<td>967</td>
<td>100%</td>
<td>100%</td>
<td>457</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>

Figure 2. Composition of floral visitors visiting *Turnera velutina*. (a) Overall number of visitors per taxa in control (blue) and ant excluded (red) plants; and (b) community composition comparisons of floral visitors to plants with and without ant patrolling.
**Pollinator visitation: frequency**

Visitation frequency did not differ significantly between pollinator types, and ant exclusion treatment had no significant effect on visitation frequency, regardless of the pollinator taxa considered as shown by the non-significant interaction term (Fig 3a, Table 1).

**Pollinator visitation: visit duration**

Pollinator taxa differed in visit duration, but there was no significant effect of ant exclusion treatment on visit duration. The ant exclusion x pollinator taxon interaction on visit duration was significant (Fig. 4b, Table 1). The duration of *Apis mellifera* visits had a significant two-fold increase in ant-excluded plants ($Z = 2.45, P = 0.05; \text{Table 1}; \text{Fig. 4b}$), whilst butterfly visit duration was not significantly affected by ant exclusion ($Z = 1.07, P = 0.70; \text{Table 1}; \text{Fig. 4b}$).

**Pollinator visitation: behaviour**

The likelihood of displaying inspection behaviours differed significantly between pollinator taxa (Table 1, Fig. 3c), with butterflies being on average 15% more likely to display inspection behaviours than *Apis mellifera* (Fig. 3c). However, the ant exclusion treatment did not affect the likelihood of pollinators displaying inspection behaviours, regardless of the pollinator taxon (*Apis mellifera* or butterflies), as indicated by the non-significant interaction term (Table 1, Fig. 3c).

The percentage of pollinators deterred from a plant differed significantly between pollinator taxa, with, on average, three times more butterflies deterred than *Apis mellifera* (butterflies: 27%, *Apis mellifera*: 8.5%; Fig. 3d, Table 1). Ant exclusion treatment had no significant effect (Fig. 3d, Table 1). The ant exclusion x pollinator taxon interaction was significant (Table 1), with exclusion having a
positive effect on butterflies, increasing deterrence, but a negative effect on *Apis mellifera*, decreasing deterrence (Table 1, Fig. 3d).

When the duration of a given pollinator’s visit was split between inspection and contact behaviours, we found that the effect of ant exclusion treatment on visit duration differed between pollinator taxa, and behaviours (Fig. 3e, Table 1), as shown by both significant interaction terms (Ant exclusion treatment x pollinator taxon; pollinator taxon x behaviour; Table 1). However, the effect of ant exclusion treatment did not differ between behaviours as shown by the non-significant interaction between ant exclusion treatment and behaviour (Table 1, Fig. 3d). Ant exclusion significantly increased the duration of *Apis mellifera* contact visits ($Z = 2.96, P = 0.05$), increasing the time bees spent inside flowers. However, ant exclusion did not significantly affect the time butterflies spent inside the flowers (contact behaviours: $Z = -2.34, P = 0.25$), or the duration of inspection behaviours by either pollinator (*Apis mellifera*: $Z = -0.48, P = 0.99$; butterflies: $Z = -1.73, P = 0.65$). Pollinators spent longer displaying contact behaviours than inspection behaviours, regardless of the ant exclusion treatment. This trend did not differ between *Apis mellifera* and butterflies, as demonstrated by the post-hoc Tukey contrasts (Fig. 3e).

**Plant mating system**

Pollen load per stigma was significantly increased in stigmas from ant-excluded plants, (Fig. 4a, Table 1), with ant excluded stigmas receiving 150 additional pollen grains compared to stigmas from ant-patrolled flowers (Control: 85 ± 12; Exclusion: 240 ± 32).
Figure 3. Effects of ant patrolling on (a) pollinator visitation frequency, (b) visit duration, and pollinator behaviours (c-e) affecting (c) the display of alert behaviours, (d) the likelihood of deterrence, and (e) the time spent displaying alert (triangles) or contact behaviours (circles) by *Apis mellifera* (Am) and native butterflies on *Turnera velutina* flowers on control plants with ant patrolling (blue), and ant excluded plants (red).
Chapter 4: Ecological costs

The proportion of pollen grains in each mating system category differed significantly both within and between plant treatments (Fig. 4b, Table 1). The effect of ant exclusion on the proportion of pollen grains differed between mating systems, as shown by the significant ant exclusion x mating system interaction (Fig. 5, Table 1). In fact, excluding ants from plants significantly reduced outcrossing by half and geitonogamy by 33-fold, whilst tripling selfing rates (Fig. 5).

The effect of ant exclusion on pollen flow differed significantly between the MSPO (categories used to describe pollen flow dynamics), as shown by the significant interactions between exclusion treatment and MSPO. Ant exclusion increased by four times the number of selfing pollen grains received by stigmas, doubled the number of allogamous pollen grains received from neighbouring, un-dyed flowers, and reduced to a third the number of geitonogamous pollen grains.

Flowers received significantly more pollen grains than the number of pollen than we could trace as donated by each of the dyed flowers to the focal stigmas. However, our donation counts are under-estimated in a ratio of 1:22 as we only counted donations to focal stigmas, and not to any other flowers, whilst accounting for ≥ 22 donors (one selfing flower + five geitonogamous flowers + six flowers from the reciprocal pair + ≥ 10 neighbouring, un-dyed flowers). Therefore, the only symmetrical estimate of received:donated pollen grains is the comparison between reciprocal pair plants, as in that case the ratio is equal 1:6, with six flowers donating pollen to one stigma in both directions. However, 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).
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 (blue) and ant-excluded (red) plants.
Chapter 4: Ecological costs

Plant fitness

Male fitness, assessed as the number of pollen grains donated per flower, was positively and significantly affected by ant exclusion, increasing the number of pollen grains donated per flower from 27.2 ± 6.54 in ant-patrolled plants to 163 ± 23.9 in ant-excluded plants. Ant exclusion, mating system, and the interaction between these factors all had significant effects on the number of pollen grains donated per flower to different destinations (Table 1). The vast majority of the pollen donated per flower arrived on the same flower's stigma as selfing pollen, regardless of the exclusion treatment (Fig. 5b). The effect of ant exclusion on the number of pollen grains donated differed between mating systems and pollen destinations. Ant exclusion significantly increased the number of pollen grains donated to the same flower's stigma and significantly decreased the number of pollen grains donated to other flowers of the same plant, but had no significant effect on the number of pollen grains donated to the reciprocal pair plant.

![Figure 5](image)

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 (blue) and ant excluded (red) 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.

<table>
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<th>Response</th>
<th>Fixed effects</th>
<th>N</th>
<th>LRT</th>
<th>P-value</th>
<th>Random effects</th>
<th>Variance</th>
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<tr>
<td>Visitation frequency</td>
<td>Ant exclusion</td>
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<td>0.64</td>
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### Behavioural consequences

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<td><strong>4.54-08</strong></td>
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### Mating system consequences

| Pollen load | Ant exclusion | 72 | **9.19** | **0.002** | * | | |
| Mating system | Ant exclusion | 336 | 0.08 | 0.77 | | | |
| | Mating system | 24.67 | 4.37-06 | *** | | | |
| | Ant exclusion x Mating system | 75.92 | 2.2-16 | *** | | | |

| Pollen flow | Ant exclusion | 336 | 0.58 | 0.44 | | | |
| | Pollen origin | **108.95** | **2-16** | *** | | | |
(mating system, received and donated)

Ant exclusion x Pollen origin

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<th>LRT</th>
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Day OLRE 0.69 0.47
OLRE 4.25 2.06
DISCUSSION

This study provides a comprehensive picture of the interaction between myrmecophily and pollination by showing the ecological and behavioural effects of ant patrolling and associated changes in plant mating systems and fitness consequences. Experimental evidence in this system had shown potential for direct ant-pollinator conflict (Villamil et al. 2018). Despite this, and contrary to our expectations, excluding ants from plants did not affect pollinator composition (Fig. 2) or visitation frequency (Fig. 3a). Ant exclusion did not affect either the probability of pollinators being deterred (Fig. 3d), the probability of pollinators displaying inspection behaviours (Fig. 3c), or the duration of inspection behaviours (Fig. 3e). However, ant exclusion had a positive effect on visit duration (Fig. 3e), pollen load, outcrossing rates (Fig. 4), and male fitness (Fig. 5). Ant exclusion increased the time *Apis mellifera* spent inside flowers (Fig. 3b). Furthermore, ant exclusion increased the pollen load on stigmas by 150%, switched the mating system from outcrossing to selfing (Fig. 4b), and increased by 150% the number of pollen grains sired per flower. The increase in the time *Apis mellifera* spent inside flowers may be the mechanism underlying the increased selfing rates observed in plants under the ant exclusion treatment. The positive effect of ant exclusion on the duration of contact visits by *Apis mellifera* may also be responsible for the increase in male fitness if longer visits allow *Apis mellifera* to collect and transport more pollen grains. Although ant patrolling had negative effects on pollinator visitation behaviours and foraging efficiency, such behavioural changes seems to result in positive effects on plant fitness promoting outcrossing and increasing male fitness.

Effects of ant patrolling on pollinator visitation dynamics: Composition, visitation frequency and duration

In Troncones, butterflies were slightly more frequent visitors than *Apis mellifera* (Fig. 3a), accounting for 51% of the pollinator visits, whilst *Apis mellifera* accounted for 43% of the visits (Table 1). Our results are consistent with
previous studies suggesting that butterflies are more frequent visitors, and bees are better pollinators (Barrios et al. 2016), although the pollinator efficiency of each taxon in *T. velutina* remains to be investigated. Such differences in the abundance of native butterflies and *A. mellifera* are an interesting contrast with other populations of *T. velutina* where *A. mellifera* is the dominant pollinator accounting for 94% of floral visits (Sosenski et al. 2016, Villamil et al. 2018). Such contrast and the higher frequency of native pollinators make Troncones an ideal population to study the evolutionary ecology of plant-ant-pollinator interactions around *T. velutina*.

Other non-pollinator animals interacting with flowers can reduce flower attractiveness for pollinators and alter pollinator visitation dynamics, resulting in lower pollen donation, lower pollen deposition and reduced seed set (Krupnick and Weis 1999). In ant-plant systems patrolling ants can deter pollinators, decreasing visitation frequency and duration (Ness 2006). The predation risk that patrolling ants represent for pollinators can also reduce flower attractiveness, increase flower avoidance rates and decrease pollinator visitation frequency and duration (Romero et al. 2011). Anti-predatory responses in pollinators vary depending on the pollinator and predator taxa involved (Romero et al. 2011) but few studies have documented how different floral visitors respond to ant patrolling (Ness 2006, Ohm and Miller 2014, Carper et al. 2016) and how different ant partners affect pollinators (Ness 2006, Miller 2007, Ohm and Miller 2014, Villamil et al. 2018).

We are only aware of three systems in which the effect of different ant partners on pollinators has been examined: two Cactaceae species and *Turnera velutina* (Passifloraceae). In *Opuntia imbricata* ant patrolling significantly decreased pollinator visitation, but there were no differences between ant species (Ness 2006, Miller 2007, Ohm and Miller 2014). In *Ferocactus wislizeni* plants patrolled by the most aggressive bodyguards received less and shorter
pollinator visits and produced less and lighter seeds (Ness 2006). In *T. velutina* the identity of the ant partners had no effect on visitation frequency, but the presence of the most aggressive bodyguard inside flowers increased by 20% the likelihood of pollinators displaying inspection behaviours, halved the time *Apis mellifera* spent inside flowers, and reduced pollinator foraging efficiency by increasing by 12% the proportion of time spent displaying inspection behaviours (Villamil et al. 2018).

Several hypotheses have been made regarding the effect of different ant partners on pollinators, and many more studies have addressed this question (Romero et al. 2011). Pollinator body size and lifestyle (social or solitary) have been proposed as a source of variation, predicting smaller and solitary pollinators as more vulnerable to predation risk (Clark and Dukas 1994, Abbott and Dukas 2009). In Troncones, native bee and butterfly species are solitary, non-aggressive floral visitors, whilst *Apis mellifera* are introduced, colonial and aggressive visitors. We predicted social and aggressive pollinators would be less susceptible to ant patrolling. However, these predictions were only partially supported by our results. Contrary to our initial predictions, we found no changes in pollinator composition between control and ant excluded plants (Fig. 2). Contrary to our initial predictions, only the introduced, aggressive, social pollinators (*Apis mellifera*) showed behavioural changes by increasing visit duration in ant excluded plants (Fig. 3). However, consistent with our predictions, non-aggressive solitary pollinators showed higher likelihood of anti-predatory behaviours. We found significant differences between solitary and social pollinators in avoidance and inspection behaviours rates, although no significant differences were found between pollinator taxa in response to ant exclusion (Fig. 3, Table 1). *Apis mellifera* and butterflies differed in their likelihood of avoidance and inspection behaviours, with butterflies more likely to avoid flowers (e.g. approach or hover over a flower without landing in it) (Fig.
3d), and also more likely to display inspection behaviour (Fig. 3c), regardless of the ant exclusion treatment.

Our results on pollinator composition are consistent with the general pattern revealed in a meta-analysis by Romero et al. (2011) showing that pollinator lifestyle (social vs. solitary) is not a good predictor of anti-predatory sensitivity. Overall, our findings suggest that the predation risk represented by ant patrolling in *T. velutina* is not strong enough to affect pollinator composition or increase the natural avoidance and inspection rates of pollinators, but provides evidence highlighting the different responses between pollinator’s lifestyles. Solitary pollinators are more inclined to display inspection behaviours and avoid flowers more often, regardless of ant patrolling. This suggests they are more sensitive and perhaps more susceptible to predation risk than social pollinators.

In *T. velutina* we found that ant patrolling halved the time *Apis mellifera* spent inside flowers, from 7.98 ± 1.69 sec in control plants to 16.04 ± 2.83 sec in ant-excluded plants. Although the effect of ant exclusion on visit duration differed between pollinator taxa since ant patrolling did not affect the time butterflies spend inside flowers (Table 1, Fig. 3b). Our results are comparable with those for *F. wislizeni* plants, in which ant patrolling decreased the duration of the visits, with foraging bouts being 20 s longer on plants without ants (Ness 2006).

We found no significant effect of ant exclusion on pollinator visitation in either butterflies or *Apis mellifera* (Fig. 3a). A meta-analysis on the effects of predation risk on pollinators found a non-significant trend for reduced visitation frequency in solitary pollinators, and reduced duration in social species (Romero et al. 2011). This result was consistent with our findings in *T. velutina*, where ant patrolling had a non-significant negative trend on the
visitation frequency of butterflies (solitary pollinators) (Fig. 3a, Table 1) and a significant negative effect on the time *Apis mellifera* bees spent inside flowers (Fig. 3b, Table 1). These results suggest that both pollinators are capable of detecting ant patrolling as a predation risk but differ in their anti-predatory response behaviours. The non-significant trend on visitation frequencies of solitary pollinators in our data is consistent with the overall data pattern suggesting that the effect of ant patrolling on solitary pollinators may be smaller than the effect on social pollinators. Perhaps because solitary pollinators are more risk-averse, the effect of ant exclusion is smaller than the effect on social pollinators who seem to be more risk-prone. We believe this reflects a real biological pattern, rather than a spurious result although larger sample sizes may be required to detect a significant effect.

**Effects of ant patrolling on plant mating system**

Although the deleterious effects of selfing and geitonogamy are well known (Waser and Price 1991, de Jong et al. 1992, Lloyd 1992), it is less well known the extent to which bisexual flowers suffer from them because it requires partitioning pollination events into intraflower (hereafter selfing), intraplant (hereafter geitonogamy), and interplant (hereafter outcrossing) (de Jong et al. 1992, Schoen and Lloyd 1992, de Jong et al. 1993, Eckert 2000, Wu et al. 2018), a methodological approach rarely undertaken (Wu et al. 2018). Furthermore, the full decomposition of pollen on stigmas into donor components (intraflower, intraplant, interplant) has rarely been performed, and its importance remains underappreciated (de Jong et al. 1993, Wu et al. 2018). For these reasons in this study we assessed the effects of ant patrolling on plant mating systems and male fitness decomposing pollen flow by origin and fate and considering selfing, geitonogamy, and outcrossing.

In this self-compatible species, ant exclusion shifted the plant mating system from predominantly outcrossing to predominantly selfing, and reduced
geitonogamy (Fig. 4b). In bisexual flowers self-pollination can be mediated by pollinators (Wu et al. 2018). We suggest that ant patrolling reduced selfing rates in the bisexual flowers of *T. velutina* by affecting pollinator visitation behaviour, reducing the time bees spent inside the flowers.

Ant exclusion in *T. velutina* significantly increased the stigmatic pollen load by 150%, (Fig. 4a) and had strong impacts on the direction of pollen transfer, affecting the donor and destination of pollen grains being transferred (Fig. 4c). The number of pollen grains exchanged (donated and received) between the reciprocal pair plants was not significantly different between control and ant excluded plants (Fig. 4c, Table 1). Although ant patrolling significantly decreased the number of pollen grains sired per flower (Fig. 5a), it did not affect the transfer ratio of received:donated pollen grains (Fig. 4c). This is probably because ant patrolling also decreased pollen load, and so, the received:donated pollen ratio was maintained.

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 flow (showing counts of pollen grains from different origins) it becomes evident that the increase in pollen load under ant exclusion is driven by selfing and allogamous pollen from other undyed, surrounding, *T. velutina* plants. This comparison also reveals that the number of selfing pollen grains on ant excluded stigmas is much higher, and this drives the change in the mating system from outcrossing to selfing. Yet, ant exclusion also increased the absolute number of allogamous pollen grains received by the stigmas.

Higher numbers of selfing pollen grains on focal stigmas from ant excluded plants (Fig. 4c) can be explained by bees staying longer inside the flower (Figs. 3d), foraging on pollen and nectar and hence increasing the transfer of pollen from the anthers to the stigmas of the same flower. Longer
visit durations to ant excluded plants also increase the chances of bees depositing allogamous pollen grains (interplant or outcrossing) attached to their bodies. This would explain the increased number of outcrossing pollen grains from unknown, undyed, donors that we observed on ant excluded plants (Fig. 4c). 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 and Koricheva 2011).

Our results suggest that ants contribute to maintain an outcrossing mating system in this self-compatible species, which has a beneficial effect on the quality of the progeny by promoting genetic diversity, especially given that this is a self-compatible species (Sosenski et al. 2016). The magnitude of the effect of ant patrolling on outcrossing that we found in *T. velutina* (Fig. 4) was comparable to the effect of distyly on outcrossing in *Fagopyrum* (Wu et al. 2018). Ant patrolling in *T. velutina* and distyly in *Fagopyrum* promoted a significant two-fold increase in outcrossing rates. Selfing accounted for 71.3% of the pollen load on the homostylous buckwheat *Fagopyrum tataricum*, which was twice as much as the proportion of self-pollen found on stigmas of the distylos congener *Fagopyrum esculentum* (35.3% - 39.6%), as expected from the greater degree of herkogamy in the distylos species (*F. esculentum*) (Wu et al. 2018). Plants are known to have a diversity of physical, mechanical, and chemical traits to avoid selfing and promote outcrossing, but we suggest that ant patrolling may by another mechanism to promote outcrossing in ant-plants, although further research on the mating systems of ant-plants is required.
Effects of ant patrolling on plant fitness

In order to fully understand the effect of biotic interactions on plant fitness, both female and male fitness components should be evaluated since the magnitude and direction of the effects of mutualists and antagonists may differ between plant male and female reproductive functions (Schaeffer et al. 2013, Carper et al. 2016). Male fitness is expected to increase as the number of mates reached by the pollen increases. Pollinator behaviour has strong impacts on pollen transfer, with longer visits increasing pollen export and pollen deposition on the stigma (Carper et al. 2016). Consequently, since male fitness is more constrained by the number of mates than female fitness, we expect male fitness to be more susceptible to changes in pollinator behaviour (Krupnick and 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 and male reproductive functions. Our experimental design allowed us to trace the consequences of ant patrolling on pollen transfer dynamics, estimate pollen transfer on a per flower basis, and estimate the effect of ant patrolling on male reproductive function on a per flower and per plant basis.

In *T. velutina* ant exclusion significantly increased the number of pollen grains donated per flower by a six-fold (Fig. 5a) suggesting guarding ants may be hindering male fitness. The full decomposition of donated pollen grains by fate revealed that most of this trend is driven by pollen donated towards selfing (Fig. 5b), which contrasts with stigmas from ant patrolled flowers which were dominated by outcrossing pollen (Fig. 5b). Our results are consistent with other ant-plant case studies. Ant patrolling had negative consequences on the female fitness component in *Opuntia imbricata*, reducing seed count per fruit by 30% and seed mass by 16%, although these effects varied between ant species (Ohm and Miller 2014). Plants tended by *Crematogaster opuntiae* ants had lower seeds per fruit, but not those tended by *Liometopum apiculatum* ants, suggesting that *L. apiculatum* benefits plant fitness by decreasing the proportion of fruits infested by the pre-dispersal seed predator (Ohm and Miller 2014). Ant
patrolling in *H. physophora* had negative effects on female and male plant fitness: first, by castrating the host by consuming floral buds, and second, by deterring pollinators, reducing pollen transfer and causing pollinator limitation which reduced fruit set in those buds that escaped ant predation (Malé et al. 2012).

Overall, the effect of ant patrolling on *T. velutina*’s fitness differs between male and female functions. On the one hand, guarding ants reduced male fitness by reducing the number of pollen grains donated per flower, but on the other hand they may increase female fitness by promoting outbred seeds and so increasing the quality of the offspring. These findings exemplify how other non-pollinators insects interacting with plants and its pollinators may have different effects on male and female plant reproduction, and highlight the importance of considering both fitness components.

**Pollinator efficiency and net ant effects**
The contribution of a given pollinator to plant fitness is determined by its visitation frequency and its efficiency at carrying and depositing pollen grains on conspecific stigmas (Ne’eman et al. 2010, Willmer 2011 pp. 279-287, King et al. 2013). Pollination efficiency is a function of several morphological and behavioural traits (Ollerton et al. 2007, Ne’eman et al. 2010, Willmer 2011 pp. 279-287, Willmer and Finlayson 2014, Barrios et al. 2016, Willmer et al. 2017). The relationship between size and structure of floral visitors’ body and mouthparts with floral morphology are crucial factors determining their efficiency as pollinators (Armbruster 1985, 1988, Ordano et al. 2008, Barrios et al. 2016). In the self-incompatible plant *Angadenia berteroi* (Apocynaceae) which relies on pollinators to set fruit, lepidopterans were the most common visitors although very inefficient pollinators that seldom carry pollen because their long, thin tongues reach the nectar but miss the plant sexual organs and consequently their visits did not lead to fruit set (Barrios et al. 2016). This contrasted with long-tongued bees who were less frequent visitors, but better pollen carriers.
whose visits lead to fruit set because they push their mouthparts against the anthers to reach for nectar, collecting more pollen on their wide and shorter tongues (Barrios et al. 2016). In fact, butterflies have been reported as poor pollinators that act mostly as nectar thieves in other systems (Venales and Barrows 1985). We observed a similar pattern in *T. velutina* with butterflies being more abundant than bees, but where changes in the behavioural patterns of bees in ant excluded plants (Fig. 3) seem to be driving changes in plant mating systems (Fig. 4) and fitness (Fig. 5). Based on these observations we suggest that butterflies may be less efficient pollinators than *Apis mellifera* in *T. velutina*, although further studies are required to test this hypothesis.

The effect of defensive mutualists on the host plant pollination and fitness can vary depending on whether predators deter efficient pollinators or a subset of inefficient visitors (Romero and Koricheva 2011). Guarding ants had negative effects on plant fitness when they attacked efficient pollinators in *Ficus pertusa* (Moraceae: Bronstein 1991) and *Opuntia imbricata* (Cactaceae: Ohm and Miller 2014), but had positive effects in *Banistriopsis malifolia* (Malpighiaceae: Alves-Silva et al. 2013) with wasps efficiently protecting flowers from herbivory without interfering with pollinators. Yet, there are still many cases in which their effect is unknown because deterrence has not been assessed for all pollinators, as in *Heteropterys pteropetala* (Malpighiaceae), where only large bees (*Centris* and *Epicharis*) are efficient pollinators, whilst small bees are mostly thieves; but it still remains unknown whether patrolling ants deter all or only a certain subset of these bees (Assunção et al. 2014). Even when deterrence is known for different floral visitors, the effects of patrolling ants on plant fitness may be unclear if the pollinator efficiency of those floral visitors has not been determined, as is the case of *T. velutina*.

Contrary to our initial prediction, the effect of ant patrolling on *T. velutina* via pollinator deterrence may benefit plant fitness by reducing
pollinator visit duration, which promoted pollinator relocation and lead to reduced selfing rates and higher outcrossing rates. Although *T. velutina* has self-compatible hermaphroditic flowers, it has a suite of traits to favour outcrossing such as being a monomorphic, herkogamous species with pollinator rewards and attraction features (Sosenski et al. 2016). Although ant patrolling does reduce pollen load and male fitness, far from having an ecological cost on the host plant pollination and mating system, ant patrolling seems to be another mechanism contributing to promote outcrossing in *T. velutina*.

**Conclusions**

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 that affect plant mating systems and lead to fitness consequences. Ant patrolling in *T. velutina* decreased pollinator visit duration, increased pollen deposition to stigmas, maintained outcrossing as the predominant mating system, and decreased male fitness, but assured outbred progeny. However, its effects on plant male and female fitness varied. Overall, we conclude ant patrolling is not an ecological cost for the host plant reproductive outcome as it promotes outcrossing in this self-compatible species, which has a beneficial effect on the progeny by promoting genetic diversity. This study contributes towards understanding the potential of plant mating systems to be shaped and evolve in response to ant-pollinator interactions and considers pollination from a complex multispecies approach, as product of interactions several simultaneous interactions.

Plant mating system seems to be the main determinant of population genetic structure (Loveless and Hamrick 1984, Vekemans and Hardy 2004) and changes in pollinator availability are likely to affect the evolution of plant mating systems and hence, population genetic structures (Opedal et al. 2017).
Chapter 4: Ecological costs

Differences in ant aggressivity between *T. velutina* populations (Zedillo-Avelleyra 2017) could be affecting plant mating systems differently. Consequently, it would be worth exploring the effect of ant aggressivity on shaping long-term mating system traits such as herkogamy and the genetic structure of plant populations.
CHAPTER 5

THE DISTRACTION HYPOTHESIS OF EXTRAFLORAL NECTAR

Does EFN distract ants from entering flowers and reduce ant-pollinator conflict?
INTRODUCTION

Ant-plants recruit ants by providing nesting sites and/food resources, and benefit from ant-mediated reduction in damage by herbivores and pathogens (Janzen 1966, Bentley 1977a, Chamberlain and Holland 2009, Herrera and Pellmyr 2009, Rosumek et al. 2009, Trager et al. 2010). The most widespread reward produced by ant-plants is extrafloral nectar (EFN), a key food resource for ants (Rudgers and Gardener 2004, Ochoa Sánchez 2016) that increases individual survivorship (Fisher et al. 1990), colony growth rate and reproductive output (Byk and Del-Claro 2011). Guarded plants in turn show increased somatic growth (biomass or leaf production) and reproductive output (Heil et al. 2001a, Chamberlain and Holland 2009, Rosumek et al. 2009, Trager et al. 2010, Escalante-Pérez and Heil 2012).

Most ant-plants are angiosperms (Keeler 2014), and many require the services of animal pollen vectors for seed set (Bentley 1977b, Torres-Hernández and Rico-Gray 2000, Raine et al. 2002, Díaz-Castelazo et al. 2005, Rico-Gray and Oliveira 2007b, Ballantyne and Willmer 2012, Dutton et al. 2016, Villamil et al. 2018, amongst many other studies documenting animal-pollinated ant-plants), making ants and pollinators likely to co-occur on a given host plant. This raises the possibility of several types of direct and/or indirect conflicts between ants and pollinators. First, indirect conflict can arise if there is a trade-off between plant allocation of resources to reproduction (which benefits pollinators) versus investment in growth and defence (which benefits ant guards) (Bazzaz et al. 1987). Plants that do not reproduce grow faster and develop larger resource-acquiring and producing organs (roots and leaves) (Frederickson 2009), leading to indirect conflict between ants and pollinators over plant resources and rewards (Afkhami et al. 2014, Dutton et al. 2016). In extreme cases of ant-pollinator conflict, ants actively increase plant investment towards growth and defence by castrating their host plant through consumption of floral meristems (Frederickson 2009, Palmer et al. 2010) or mature inflorescences (Izzo and
Chapter 5: The Distraction Hypothesis of EFN

Vasconcelos 2002). Second, ants may enter flowers and consume floral nectar without providing pollination services, providing no benefit to the plant and potentially reducing the attractiveness of flowers to effective pollinators (Rico-Gray and Oliveira 2007b). Third, ant visits to flowers may reduce pollen viability by depositing antimicrobial substances that decrease pollen germination rates, and hence decrease male fitness for the plant (Wagner 2000, Dutton and Frederickson 2012). Finally, ants may attack or intimidate pollinators directly (Wagner and Kay 2002, Willmer et al. 2009, Villamil et al. 2018), reducing flower visitation rates (Ness 2006, Lach 2008) or duration (Villamil et al. 2018). One hundred and forty years ago, Anton Joseph Kerner, an Austro-Hungarian botanist, wrote:

“Of all the wingless insects it is the widely dispersed ants that are most unwelcome guests to flowers. And yet are they the very ones which have the greatest longing for the nectar, as numberless observations sufficiently show.” (Kerner 1878, p21)

Whilst ants may be unbidden floral visitors, they are also effective bodyguards (Bentley 1977a, Chamberlain and Holland 2009, Rosumek et al. 2009, Trager et al. 2010), which may represent an ecological trade-off for ant-plants. Given that ant guards can have both costs and benefits for different aspects of plant fitness, we expect natural selection to have acted on ant-plant traits to minimise the negative impacts of ants relative to the protection they provide, ameliorating the negative consequences of ant-pollinator antagonism for plant fitness (Raine et al. 2002). A wide range of mechanisms have been interpreted as achieving this by reducing ant visitation to flowers during anthesis, including physical barriers (Harley 1991, Galen 1999, Galen and Cuba 2001, Carlson and Harms 2007, Willmer 2011), chemical repellents (Willmer and Stone 1997, Junker et al. 2007, Agarwal and Rastogi 2008, Junker and Blüthgen...
Chapter 5: The Distraction Hypothesis of EFN  

2008, Willmer et al. 2009, Ballantyne and Willmer 2012) or bribes (Kerner 1878, Willmer 2011, Martínez-Bauer et al. 2015). Physical barriers include spiny or hairy surfaces on the outside of the corolla or on floral pedicels that prevent tarsi from gripping and so hinder ant walking (Willmer 2011), and waxy or sticky plant secretions that prevent ants from climbing (Harley 1991). Bracts around the calyx can act as a water trap, creating a pool of water or mucilage that prevents ants and other small insects from crawling into the flowers (Carlson and Harms 2007). The shape of the flower may itself stop ants from entering flowers: pendant, thin, and constricted corollas are effective ant-excluding morphologies (Galen 1999, Galen and Cuba 2001, Willmer et al. 2009). Several species produce ant-repelling flowers (Junker et al. 2007, Agarwal and Rastogi 2008, Junker and Blüthgen 2008, Willmer et al. 2009) and furthermore, ant repellence may be concentrated in specific floral parts such as petals (Ness 2006, Ballantyne and Willmer 2012), or pollen and anthers (Willmer and Stone 1997, Ghazoul 2001, Raine et al. 2002, Willmer et al. 2009, Ballantyne and Willmer 2012). Finally, some species may entice ants away from flowers by offering alternative sugary rewards outside the flowers, using EFN as a distraction or bribe (Wagner and Kay 2002, Galen 2005, Chamberlain and Holland 2008, Willmer 2011). During the nineteenth century, Kerner (1878) suggested that EFN in plants with floral nectar might serve to distract ants from visiting the flowers.

“Any insects that creep along the stem must, if they would get at the flower, of necessity pass over this disk with its drop of nectar; thus what they would have found, in the flower, is already offered to them here in rich abundance. The creeping insects are not fastidious. They are content with that which is first offered, and so do not trouble themselves to climb farther up to the flowers. […] I do not therefore hesitate to interpret all nectar-glands that are found on leaves
Chapter 5: The Distraction Hypothesis of EFN as means of protection against the unwelcome, because unprofitable, visits of creeping insects.” (Kerner 1878, p137-139)

The idea that EFN could distract non-pollinating insects away from flowers and so reduce any disruption of pollination is known as the Distraction Hypothesis (Wagner and Kay 2002, Chamberlain and Holland 2008, Holland et al. 2011). In many ant-plants, flowers and extrafloral nectaries are in close proximity (Weber and Keeler 2013, Keeler 2014) and several species secrete extrafloral nectar only or predominantly during the flowering period (Bentley 1977b, Chamberlain and Holland 2008, Holland et al. 2010, Villamil-Buenrostro 2012, Villamil et al. 2013, Falcão et al. 2014, Dutton et al. 2016, Villamil 2017). For example, extrafloral nectaries on leaves associated with flowers of the Mexican ant-plant *Turnera velutina* (Passifloraceae) secrete more nectar with higher sugar content than extrafloral nectaries on leaves bearing buds and fruits (Villamil 2017). This increase in EFN secretion during anthesis is compatible with the Distraction Hypothesis, in that EFN secretion near flowers could lure ants that might otherwise enter flowers seeking floral nectar. However, the same floral behaviour and the frequent proximity of extrafloral nectaries to reproductive structures can also be explained by the Optimal Defence Theory (ODT), which predicts that plants should focus defensive investment on highly vulnerable and valuable tissues for plant fitness, such as flowers, fruits and seeds (Stamp 2003). Finally, it is possible that high EFN secretion on flowering shoots in myrmecophiles could fulfil both distracting and protective roles, simultaneously keeping ants out of flowers but promoting their patrolling around reproductive tissues to deter herbivores.

While the defensive role of ant recruitment through EFN secretion has been widely demonstrated (Chamberlain and Holland 2009, Rosumek et al. 2009, Trager et al. 2010), the Distraction Hypothesis has not been adequately
Chapter 5: The Distraction Hypothesis of EFN tested. To our knowledge, since Kerner proposed it in 1878 only three experimental studies have been performed and all have rejected it (Wagner and Kay 2002, Galen 2005, Chamberlain and Holland 2008). However, none of these studies were carried out in an ecologically realistic setting (a point that we address further in the Discussion).

QUESTIONS & AIMS

Here we use experimental manipulation of EFN secretion during anthesis in a Mexican endemic plant, *Turnera velutina*, to test the Distraction Hypothesis under natural conditions, improving on previously reported experimental designs. We evaluated the potential ecological and fitness consequences of the Distraction Hypothesis addressing the following questions:

(i) How often are flowers occupied by ants and how many ants are found in them?

(ii) Does preventing EFN secretion affect the number of ants patrolling extrafloral nectaries, the number of ants inside the flowers or the number of pollinators visiting flowers?

(iii) Does preventing EFN secretion increase the probability of a flower being occupied by ants?

(iv) Does the number of ants at extrafloral nectaries or inside flowers affect pollinator visitation?

(v) Does preventing EFN secretion affect plant fitness?
HYPOTHESES AND PREDICTIONS

If the Distraction Hypothesis is true, we predict that experimental elimination of extrafloral nectar secretion should: 1) reduce ant visitation to extrafloral nectaries, 2) increase the numbers of ants inside flowers, 3) increase the proportion of flowers occupied by ants, 4) leading to decreased levels of floral visitation by pollinators, and 5) a reduction in plant fitness.

MATERIALS AND METHODS

Study site and system
All experiments and observations were conducted in the stabilised coastal sand dunes at the CICOLMA Field Station in La Mancha, Veracruz, in the Gulf of Mexico. Within this population, we selected four sites with high densities of *Turnera velutina* (Passifloraceae). At La Mancha, *Turnera velutina* establishes facultative mutualisms with at least thirteen ant species (Cuautle et al. 2005, Zedillo-Avelleyra 2017) and its main herbivores are caterpillars of a butterfly, *Euptoieta hegesia* (Nymphalidae). Extrafloral nectar is provided in paired cup-shaped glands located on the underside of either the leaf blade and petiole (Fig. 1). Although it flowers year-round, flowering peaks during summer (Cuautle et al. 2005). Flowers last one day, are insect-pollinated (Sosenski et al. 2016) and have a yellow, pentameric, campanulate corolla with nectar easily accessible at the base. Honeybees are the dominant pollinators (*Apis mellifera*) at La Mancha, accounting for 94% of visits (Sosenski et al. 2016, Villamil et al. 2018).

FIELDWORK METHODS

Surveys of ants inside flowers
We quantified ant occupancy in flowers of *T. velutina* by surveying 1604 flowers across four sites within CICOLMA in November 2014. Flowers at each site were
Chapter 5: The Distraction Hypothesis of EFN observed for 2 minutes every hour throughout the whole anthesis period (0830–1230 hours), with one observer at each site. We estimated the proportion of flowers occupied by ants, and the total number of ants across occupied flowers within a site. Flowers were sampled at the same site over multiple days, for 10 site-and-day combinations. Since these are one-day flowers, we considered each site-day as a replicate \((n = 10\) site-days), with site-and-day effects incorporated into our statistical modelling (see below).

**Experimental manipulation of EFN secretion**

To test the Distraction Hypothesis we experimentally clogged extrafloral nectaries to prevent nectar secretion and compared ant and pollinator behaviours on paired shoots with and without EFN secretion. This experiment was conducted over 5 days during November 2014. Early on each day of the experiment, a pair of neighbouring, unopened floral buds within a plant were marked as either control or clogged treatments \((n = 216\) flowers; \(n = 108\) pairs, \(n = 108\) plants). EFN secretion on clogged treatment leaves was eliminated by sealing the nectary cup with a droplet of transparent acrylic textile paint (Mylin dimensional, Mexico). On control treatment leaves we applied similarly sized droplets of the same textile paint a couple of millimetres above the gland (Fig. 1), controlling for any effects of the acrylic paint itself. Pilot tests confirmed that the paint totally prevented EFN secretion and also that the paint did not deter ants or pollinators. We recorded the frequency and identity of ants (to genera or species level following Zedillo-Avelleyra 2017) and other insects visiting each flower pair and the associated extrafloral nectaries for two minutes every hour during anthesis (0830–1230 hours). Simultaneous observations were performed at each of three sites by different observers. For brevity, we refer to non-ant flower visitors as pollinators, while recognising that the efficacy of visits by all species mentioned in contributing to seed set in *T. velutina* remains to be demonstrated.
Chapter 5: The Distraction Hypothesis of EFN

Based on the results from the clogging experiment described above (from now on referred to as the short-term experiment), we conducted a follow-up experiment in which treatment duration and spatial scale were both increased by a factor of 10, using paired branches and focusing on one flower on each control or clogged branch, rather than paired flowers on the same branch. We refer to this experiment from now on as the long-term experiment (See Supplementary material 1 for further details). The extrafloral nectaries of all ten leaves on the clogged treatment branches were sealed as described above, and the treatment was maintained for ten days (Fig. 1). Our hypothesis was that increasing both the temporal and spatial scales of our treatment would result in a larger experimental effect size. However, a comparison of the results from the short-term and long-term experiments showed that ants respond at a smaller scale (Supplementary material 1: Table S2), and we therefore focus on the results of the short-term experiment and highlight differences in results for the longer-term, larger-scale experiment where these are relevant to the Distraction Hypothesis. Full results for the long-term experiment are provided in the Supplementary material 1.

Impacts of EFN secretion on fitness

To quantify the impact of clogging EFN secretion and the Distraction Hypothesis on plant fitness we collected the fruits resulting from experimental flowers (control and clogged) at which pollinator visitation was observed. We recorded whether those flowers developed into fruits with seeds or whether they were aborted, and counted the number of seeds per fruit. All fruits were collected at least one week post-anthesis, at which stage retained fruits can be distinguished from aborted fruits, and developing seeds can be counted distinguishing viable from unviable seeds, even if still immature.
Figure 1. Images showing a) an apex of *Turnera velutina* bearing an apical flower bud and two lateral fruits, b) the location of extrafloral nectaries on the underside of a leaf, c) a comparison of clogged and control leaves, and d) the spatial arrangement of the short-term and long-term experiments.

**STATISTICAL ANALYSES**

All statistical analyses were conducted in R version 3.23 (R Core Team 2016). Mixed effects models were fitted using `lme4` (Bates et al. 2016) or `MCMCglmm` (Hadfield 2010) R packages.

**Surveys of ants in flowers**

To test if the proportion of flowers with ants inside them changed over the anthesis period, we fitted a binomial mixed model with time of day as a fixed effect. Flowers of *T. velutina* last for a single day, and because multiple flowers
were sampled on a given site on a given day, we fitted site identity as a random effect to account for differences between site and day variation in variables that could influence ant abundance, such as resource availability, ant diversity, or the abundance of ant nests. Tukey post hoc comparisons were used to test differences between hours using the ‘multcomp’ R package (Hothorn et al. 2008).

To test if the number of ants inside occupied flowers changed over the anthesis period we fitted a Poisson mixed model, using the number of ants inside flowers per site as the response variable and fitted as fixed effects time of day as a linear and as a quadratic term. The number of flowers occupied by ants was fitted as a log-transformed offset to control for ant density in flowers, which is likely to decrease in sites with more flowers occupied by ants, since we recorded counts per site rather than counts per individual flower (see fieldwork methods). Time of day was fitted as a linear and as a quadratic term to investigate the shape of the activity pattern of ants in flowers relationship between the number of ants inside flowers through the day. We fitted site identity as a random effect to account for variation that could influence ant abundance (as detailed above). We also included an observation-level random effect where each data point receives a unique level of a random effect to control for overdispersion (Hinde 1982). Tukey post hoc comparisons were used to test differences between hours using the ‘multcomp’ R package (Hothorn et al. 2008).

**Ecological consequences of EFN secretion**

Five mixed effects models (i-v) were fitted to test the ecological consequences of the Distraction Hypothesis. Because all of these models had the same random effects structure unless otherwise specified, we detail the random effects first and then describe the fixed effects for each model. Flower identity was fitted as a random effect to account for repeated hourly
observations. Because this experiment had a paired experimental design we fitted flower pair identity as a random effect to control for between-pair variation in floral and extrafloral investment. We also included an observation-level random effect where each data point received a unique level of a random effect to control for overdispersion. We fitted the following models, and have structured our results following the same order:

(i) To test the effect of nectary clogging on the number of ants we fitted a Poisson mixed effects model using number of ants as the response variable. Ant location (at extrafloral nectaries or in flowers), treatment, and the interaction between these two factors were fitted as fixed effects. Tukey tests were conducted to test differences between the number of ants at extrafloral nectaries or flowers under control or clogged gland conditions.

(ii) To test whether preventing EFN secretion by clogging the glands increased the probability of flower occupancy by ants, we fitted a binomial mixed effect model with the presence or absence of ants in a flower as a response variable. Clogging treatment was fitted as a fixed effect. The observation-level random effect was omitted.

(iii) To test the effect of clogging EFN secretion on pollinator visitation we fitted a Poisson mixed model using the number of pollinators as the response variable and treatment as the only fixed effect.

(iv) To test the effect that the total number ants had on pollinator visitation (regardless of their location in flowers or at extrafloral nectaries), we fitted a Poisson mixed model using number of pollinators as the response variable. As fixed effects we fitted the total number of ants, and treatment to test whether treatment affected pollinator visitation in a way that was unlinked to the number of ants.
To test if the location (inside flowers or at extrafloral nectaries) and number of ants had an effect on pollinator visitation, we fitted a Poisson mixed model. The number of pollinators was fitted as the response variable, whilst treatment, number of ants in flowers, and number of ants at extrafloral nectaries were fitted as fixed effects.

Data from the long-term experiment were analysed following a similar model structure reported for the ecological consequences models (S.i-v) (see Supplementary material).

**Impacts of EFN secretion on plant fitness**

To test the effect of clogging on fruit abortion rates, we fitted a binomial mixed model, with clogging treatment as the fixed effect and pair identity as a random effect.

For those fruits that developed seeds, we tested the effect of clogging on the number of seeds by fitting a Poisson mixed model. Clogging was fitted as a fixed effect and as random effects we fitted pair identity and an observation-level random effect to account for overdispersion.

**Exploring the responses and effects of different ant species**

We fitted additional models aiming to explore differences between ant species in their response to clogging and in their effects on pollinator visitation. We investigated whether ant species differed in their response to clogging (see model (S.vi) in Supplementary material), and whether different ant species patrolling the plants or inside the flowers differed in their effect on pollinator visitation (see models (S.vii, S.viii) in Supplementary material). These models allowed us to: (1) estimate the effects of individual ant species on pollinator visitation; (2) account for plants occupied by multiple ant species, and (3) capture the variation in ant abundance within a given ant species.
Cohen $d$ effect sizes for all models were calculated using the likelihood ratio tests (LRT) statistics from each model. To test whether increasing the duration and scale of the clogging treatment by a factor of 10 had a larger effect on the number of ants and pollinators, we estimated the ratio of change in the effect size between the short- and long-term experiment for each type of visitor (See Supplementary material).

RESULTS

Surveys of ants in flowers
We observed 10 ant species from four subfamilies interacting with *T. velutina*: *Dorymyrmex bicolor* (Dolichoderinae), *Camponotus planatus*, *Camponotus mucronatus*, *Camponotus novogranadensis*, *Brachymyrmex* sp., and *Paractrechina longicornis* (Formicinae), *Cephalotes* sp., *Crematogaster* sp., and *Monomorium ebenimum* (Myrmicinae), and *Pseudomyrmex gracilis* (Pseudomyrmicinae). We observed ants associated with plants to vary spatially, we assume due to variation in the proximity of nests of different species. Though not formally quantified, we observed apparent differences in ant behaviour among species. Some species patrolled individually, such as *Camponotus planatus*, *C. mucronatus* and *C. novo-granadensis*, while others were gregarious, such as *Dorymyrmex bicolor*, *Brachymyrmex* sp., and *Paratrechina longicornis*. *Monomorium ebenimum* probably provides no guarding services to *T. velutina* since they have only been observed consuming floral nectar and not patrolling elsewhere. In addition, this species belongs to a worldwide genus of floral nectar thieves (Ettershank 1966, Bolton 1987). Feeding preferences also vary among ant species, from opportunistic carnivores such as *Pseudomyrmex gracilis* found inside flowers hunting for thrips and beetles, to omnivores such as
Chapter 5: The Distraction Hypothesis of EFN

*Crematogaster* sp. that harvest elaiosomes attached to *T. velutina*’s seeds (S. Ochoa-López pers. comm., Dec. 2014).

Across all four sites and over all time intervals, surveys of the frequency and abundance of ants inside flowers revealed that 9.30 ± 0.19 % of the flowers within a site were occupied by ants, with an average density of 2 ± 0.28 ants/occupied flower. The low proportion of flowers (Fig. 2a) with low numbers of ants (Fig. 2b) was constant throughout the anthesis period (Tables 1,2). The number of ants inside flowers did not vary significantly through daily time and we found no statistical support for any quadratic effect (Table 2).

**Floral visitors**

All but one of 202 visits to *T. velutina* flowers by non-ant visitors were made by other insects (Table 1). The only exception was a single visit by a hummingbird (Trochilidae). Of the insect visits, 90.5% were by honeybees, *Apis mellifera*, with most of the remainder by native bees and butterflies.
Figure 2. a) Proportion of flowers with ants inside them and b) number of ants per flower throughout the anthesis period (mean ± SE per site) in hourly observations ($n = 42$ observations, from 10 sites).
Table 1. Taxonomic identities of floral visitors recorded in the short-term clogging experiment. Taxa with the epithet ‘sp.’ were identified only to genus, but all the individuals belong to the same morphospecies.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Number of visitors</th>
<th>Subfamily</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>At EFN</td>
<td>In flowers</td>
</tr>
<tr>
<td>Ants at EFN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dorymyrmex bicolor</td>
<td>373</td>
<td>10</td>
</tr>
<tr>
<td>Brachymyrmex sp.</td>
<td>342</td>
<td>74</td>
</tr>
<tr>
<td>Paratrechina longicornis</td>
<td>166</td>
<td>3</td>
</tr>
<tr>
<td>Camponotus planatus</td>
<td>128</td>
<td>1</td>
</tr>
<tr>
<td>Camponotus mucronatus</td>
<td>41</td>
<td>4</td>
</tr>
<tr>
<td>Camponotus sp.</td>
<td>49</td>
<td>5</td>
</tr>
<tr>
<td>Camponotus novogranadensis</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Crematogaster sp.</td>
<td>26</td>
<td>1</td>
</tr>
<tr>
<td>Cephalotes sp.</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>Monomorium ebenium</td>
<td>58</td>
<td>11</td>
</tr>
<tr>
<td>Pseudomyrmex gracilis</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>Unidentified ants</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>Floral visitors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apis mellifera</td>
<td>183</td>
<td></td>
</tr>
<tr>
<td>Native bees (Apoidea)</td>
<td>12</td>
<td></td>
</tr>
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<td>Diptera</td>
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<td>Wasps</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Hummingbird</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 5: The Distraction Hypothesis of EFN

**Ecological consequences of EFN removal**

Numbers of patrolling ants were significantly affected by EFN treatment (clogged versus control), ant location, and the interaction between these factors (Fig. 3a; Table 2). Ten times more ants were found patrolling extrafloral nectaries (1.49 ± 0.079 ants) than were found inside flowers (0.14 ±0.02 ants) (Fig. 3a; Table 2), regardless of treatment (control: $Z = -17.23$, $P < 0.001$; clogged: $Z = -14.03$, $P < 0.001$). The effect of eliminating EFN secretion on the number of ants differed between extrafloral nectaries and flowers, resulting in a significant decline in numbers of ants patrolling extrafloral nectaries ($Z = -4.22$, $P < 0.001$), but no significant change in the numbers of ants observed inside flowers ($Z = 1.05$, $P = 0.705$; Fig. 3a; Table 2). The percentage of flowers occupied by ants increased significantly from 6.1% under the control treatment to 9.7% when extrafloral nectaries were clogged (Table 2).

Numbers of flower visitors were not significantly affected by the elimination of EFN secretion (Fig. 3a; Table 2), nor was there any significant interaction between visitor numbers and the total number of ants (Table 2). When ant abundance was partitioned by location on the plant (at extrafloral nectaries or in flowers), neither the number of ants patrolling extrafloral nectaries nor the number of ants inside a flower had a significant effect on the number of flower visitors (Table 2).

In all five models used to analyse the short-term experiment (one leaf, one day), differences between individual plants (captured by the pair random effect) explained the largest proportion of variation in the numbers of ants and pollinators (Table 2). Differences between individual flowers (captured by the flower random effect) or random variation between observations (captured by the OLRE random effect) explained smaller proportions of variation in the numbers of ants or pollinators (Table 2).
Chapter 5: The Distraction Hypothesis of EFN

Figure 3. Mean numbers of visitors to flowers of *Turnera velutina* (mean ±1 SE) recorded in hourly surveys during 2 minutes of observation per flower for the short-term experiment. Clogged treatment flowers had secretion of EFN prevented by clogging the associated extrafloral nectaries. The short-term experiment involved prevention of EFN secretion associated with one flower for one day (see Fig.1). Red circles represent ants at extrafloral nectaries; blue triangles represent ant in flowers, and green squares represent pollinators.

Impact of EFN secretion on plant fitness

Clogging had a marginally significant effect (*P = 0.059*) on fruit abortion, increasing by 12% the probability of abortion in flowers associated with leaves in which EFN had been clogged (Fig. 4a, Table 2). Despite the *P*-value being marginally significant, clogging had a considerable Cohen *d* effect size (Cohen 1988) on fruit abortion (Tables 2, 3). However, clogging had no effect on the number of seeds per fruit (Fig. 4b, Table 2) with a small Cohen *d* effect size between treatments (Cohen 1988) (Table 3).
Figure 4. Effects of clogging the extrafloral nectaries on a) the probability of fruit abortion (mean ± se), and b) the number of seeds (mean ± se) produced by *Turnera velutina.*
Table 2. Estimates and likelihood ratio test results for statistical models used to test ant occupation of flowers, and the ecological and plant fitness consequences of clogging EFN secretion on *Turnera velutina*. Ant location stands for the number of ants patrolling EFN or inside flowers. The values highlighted in bold are statistically significant (* = \( P < 0.05 \); ** = \( P < 0.01 \); *** = \( P < 0.001 \); NS = non-significant, \( P > 0.05 \)), OLRE stands for observation-level random effect.

<table>
<thead>
<tr>
<th>Experiment</th>
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<th>Fixed effects</th>
<th>Estimate</th>
<th>LRT</th>
<th>( P )-value</th>
<th>Random effects</th>
<th>Variance</th>
<th>SD</th>
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<td></td>
<td></td>
<td></td>
<td>log (Flowers with ants)</td>
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<td>18.55</td>
<td>1.64(^{-05}) ***</td>
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<td>Flower</td>
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<td></td>
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<td>Ant location</td>
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<td>647.09</td>
<td>2.2(^{-16}) ***</td>
<td>Pair</td>
<td>1.04</td>
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<td>Clogging × Ant location</td>
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<td>0.0002 ***</td>
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<td></td>
<td></td>
<td>Pair</td>
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<td>OLRE</td>
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Chapter 5: The Distraction Hypothesis of EFN
Chapter 5: The Distraction Hypothesis of EFN

<table>
<thead>
<tr>
<th>iv)</th>
<th>Number of ants inside flowers</th>
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<th>0.4413</th>
<th>3.41</th>
<th><strong>0.06</strong></th>
<th>~</th>
<th>Flower Pair</th>
<th>2.02^{-08}</th>
<th>1.42^{-05}</th>
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<td>Flower Pair</td>
<td>1.53^{-8}</td>
<td>1.23^{-05}</td>
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<td>Ants at EFN</td>
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<td>4.20</td>
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<td>*</td>
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<td>0.83</td>
<td>0.91</td>
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<td>Ants in flowers</td>
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<tr>
<td>Clogging: Impacts on plant fitness</td>
<td>Fruit abortion</td>
<td>Clogging</td>
<td>0.6059</td>
<td>3.54</td>
<td><strong>0.05</strong></td>
<td>*</td>
<td>Pair</td>
<td>4^{-14}</td>
<td>2^{-7}</td>
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<td>vi)</td>
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<td>0.35</td>
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<tr>
<td>vii)</td>
<td></td>
<td></td>
<td></td>
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<td>OLRE</td>
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<td>0.92</td>
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</table>
Comparison of patterns across spatio-temporal scales
In contrast to our prediction, increasing the duration and spatial scale of the clogging treatment by a factor of 10 did not result in larger effect sizes on ant behaviours (Table 3). In fact, the long-term clogging experiment had less impact on ant patrolling than the short-term clogging experiment, resulting in smaller effect sizes on numbers of ants at extrafloral nectaries, numbers of ants inside flowers, and the proportion of flower occupancy by ants (Table 3). The impact of preventing EFN secretion on the number of inside flowers changed from positive at a short-term, local scale to negative in the long-term, branch-scale experiment (10 leaves, 10 days) (Table 3).

Ant species-specific responses to clogging and effects on pollinators
Although ant species explained only 0.21% of the variation in the number of ants inside flowers (Model S.vi in Table S3), there was variation between ant species in responses to clogging (Fig. S4a). *Brachymyrmex* sp. ants were the most abundant ants found inside flowers, as shown by ant surveys (Table 1) and predicted by the non-zero-overlapping effects in the model (Fig. S4a). While effect estimates vary for other ant species, confidence intervals for all taxa other than *Brachymyrmex* sp. overlap with zero (see Table 1 for rank order of abundance inside flowers and Fig. S4a for likelihood of response to clogging; see Supplementary material for further details). Activity by individual ant taxa at extrafloral nectaries had very small effects on pollinator visitation, as shown by the small estimates (model S.vii, Table S4), although their effects were precisely estimated by our models, as indicated by narrow variation around these estimates (Fig. S4b). In contrast, the effects of activity by individual ant taxa inside flowers on pollinator visitation of individual ant species inside flowers could not be precisely estimated from our data, as indicated by the large variation associated with these estimates (model S.viii, Fig. S4c).
Table 3. Comparison of the effect sizes of the short and long-term clogging experiments on the number of visits per visitor type.

<table>
<thead>
<tr>
<th>Model</th>
<th>Short term experiment: Clog 1 day 1 leaf</th>
<th>Long term experiment: Clog 10 days 10 leaves</th>
<th>Effect size difference ratio</th>
</tr>
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<tbody>
<tr>
<td>Fixed effect</td>
<td>d</td>
<td>Effect size</td>
<td>Fixed effect</td>
</tr>
<tr>
<td>i) Ants in EFN</td>
<td>-0.2865</td>
<td>Small</td>
<td>Ants in EFN</td>
</tr>
<tr>
<td>i) Ants in flowers</td>
<td>+0.070</td>
<td>Small</td>
<td>Ants in flowers</td>
</tr>
<tr>
<td>ii) Flowers occupied by ants</td>
<td>+0.146</td>
<td>Small</td>
<td>Flowers occupied by ants</td>
</tr>
<tr>
<td>iii) Pollinators</td>
<td>+0.0503</td>
<td>ns</td>
<td>Pollinators</td>
</tr>
<tr>
<td>iv) Clogging</td>
<td>-0.0488</td>
<td>ns</td>
<td>Clogging</td>
</tr>
<tr>
<td>iv) Total ants</td>
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<td>ns</td>
<td>Total ants</td>
</tr>
<tr>
<td>v) Clogging</td>
<td>-0.0676</td>
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<td>Clogging</td>
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<td>v) Ants in EFN</td>
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<tr>
<td>v) Ants in flowers</td>
<td>-0.0893</td>
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<td>Ants in flowers</td>
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</table>
DISCUSSION

The Distraction Hypothesis

Plants face a potential trade-off between the benefits they receive from ants patrolling their leaves and flowers and the costs associated with this (Altshuler 1999, Assunção et al. 2014, Dutton et al. 2016). In *Turnera velutina* the presence of the most aggressive ants inside flowers increases the likelihood of pollinators displaying alert behaviours and reduces the time honeybees spend inside the flowers (Villamil et al. 2018). To reduce the costs without waiving the protective benefits, several authors have hypothesised that plants should evolve mechanisms that minimise ant access to floral structures and pollinators, whilst recruiting them to the vicinity in order to reduce herbivore damage (Willmer and Stone 1997, Martínez-Bauer et al. 2015). Two current theories – the Distraction Hypothesis and Optimal Defence Theory (ODT) - are compatible with the commonly observed location of extrafloral nectaries close to valuable and vulnerable reproductive structures. The Distraction Hypothesis specifically predicts that EFN secretion draws ant guards away from flowers in such a way that ant-pollinator conflict is reduced (Kerner 1878). The Distraction Hypothesis has been widely overlooked, with only three studies addressing it since its proposal in 1878. We briefly outline these studies below, highlighting aspects of their experimental design that contrast with our approach, and we summarise the extent to which our results match predictions of the Distraction Hypothesis and ODT.

Wagner and Kay (2002) tested the Distraction Hypothesis using sticks as artificial plants, and identical plastic caps as artificial floral (primary) or extrafloral (additional) nectaries. Sticks with additional nectar sources did not attract more ants, but reduced the number of ants at primary sources. They concluded that additional (extrafloral) nectar sources did not increase ant recruitment, but distracted ants from the primary, floral nectar sources (Wagner and Kay 2002). These results differ from studies conducted on natural
Chapter 5: The Distraction Hypothesis of EFN plants (Bentley 1976, Shenoy et al. 2012, Villamil et al. 2013) and from our findings (Fig. 3), which show that increased EFN results in increased ant visitation. Furthermore, the plastic caps used by Wagner and Kay (2002) to simulate floral (primary) and extrafloral (additional) nectaries were morphologically identical and equally accessible, but neither assumption is met in natural EFN-bearing species (Escalante-Pérez and Heil 2012, Keeler 2014). Therefore, no robust conclusions about the Distraction Hypothesis can be drawn from this experimental design.

In 2005, Galen tested the Distraction Hypothesis on Polemonium viscosum, a plant species without extrafloral nectaries. Extrafloral nectaries were simulated by trimming the petals, anthers and pistils from some flowers, leaving only the calyx and toral disk that bears the floral nectaries to simulate extrafloral nectaries (Galen 2005). Control inflorescences contained only intact flowers, whilst inflorescences with simulated extrafloral nectaries contained intact flowers plus trimmed flowers simulating extrafloral nectaries (Galen 2005). Intact flowers in the EFN-simulation inflorescences had higher ant visitation than flowers from control inflorescences, and Galen saw this result as rejecting the Distraction Hypothesis. However, rather than testing the Distraction Hypothesis, we suggest that this experiment tested the effect of total floral nectar availability on ant recruitment, and the effect of removing floral parts on ant visitation to flowers. By trimming the corolla and sexual organs, Galen facilitated ant access to the flower. Previous studies on P. viscosum demonstrated that corolla morphology effectively excludes ants from flowers (Galen 1999, Galen and Cuba 2001). Furthermore, artificial damage (trimming) is a confounding factor because it triggers plant induced defences (Heil et al. 2001b, Ness 2003, Heil 2008, Ballaré 2011) that strongly affect floral and extrafloral nectar secretion (Ness 2003, Radhika et al. 2008, Heil 2011, 2015). Consequently, higher ant visitation to intact flowers in the EFN-simulation
Chapter 5: The Distraction Hypothesis of EFN

Inflorescences may have been a response to the trimming of neighbouring flowers.

Finally, Chamberlain and Holland (2008) tested the Distraction Hypothesis on *Pachycereus schottii*, a senita cactus bearing extrafloral nectaries. They found higher rates of ant visitation on flowers from plants where EFN had been experimentally removed, as the Distraction Hypothesis would predict. However, in contrast to our clogging treatment in *T. velutina*, Chamberlain and Holland’s EFN-elimination treatment consisted of removing EFN secreting structures (buds, flowers and fruits). Hence, as in Galen’s (2005) study, the increase in flower-ant interactions observed on EFN-removal plants could be an ant response to the artificial damage inflicted by removing reproductive structures.

We tested the Distraction Hypothesis in a field population of *T. velutina*, a species bearing extrafloral nectaries, by experimentally manipulating EFN availability without inducing artificial damage to plant structures. If the Distraction Hypothesis is true and EFN distracts ants from entering the flowers, we predicted that elimination of EFN by clogging extrafloral nectaries should result in: 1) decreased numbers of ants patrolling extrafloral nectaries; 2) increased numbers of ants inside flowers; 3) an increase in the proportion of flowers occupied by ants, leading to 4) a reduction in the numbers of pollinators visiting the flowers, and 5) a reduction in plant fitness. If EFN secretion has evolved to reduce herbivore damage to flowers by increasing ant activity in their proximity, as predicted by ODT, then we expect elimination of EFN to result in patterns compatible with predictions 1 and 5 above, with the difference that reduced plant fitness should be caused by increased floral herbivory rather than ant-associated reduction of visitation. However, ODT does not make predictions 2, 3 and 4.
Chapter 5: The Distraction Hypothesis of EFN

Our results support the Distraction Hypothesis with predictions one, three, and five being met. We found that clogging EFN secretion reduced the number of ants patrolling extrafloral nectaries by 30 % (prediction 1), increased the likelihood of flower occupation by ants by 3.6 % (prediction 3), and increased the likelihood of fruit abortion by 12 % (prediction 5). However, we found no significant increase in the number of ants inside flowers (prediction 2), or reduction in pollinator visitation (prediction 4) when extrafloral nectaries were clogged (Tables 1-2). Support for prediction 3 (increased flower occupation by ants), and reduction in plant fitness through increased rates of fruit abortion (rather than damage to flowers; Fig. 4) are both specific to the Distraction Hypothesis. We therefore conclude that our results represent the first experimental support for this hypothesis obtained under field conditions.

Exploring ant species-specific effects

We found ten ant species interacting with T. velutina, representing a diverse mosaic of partners that may differ in their response to clogging and on their effects on pollinators. Evolution of plant mechanisms that reduce plant pollinator conflict could be driven by interactions with one or more of these species. We expect plants to evolve phenotypes that favour ant taxa that are both effective guards and that have minimal net negative impacts, including interference with pollinators. Despite the potential for variation in effects across ant taxa, we found that ant species and the interaction between clogging and ant species had a negligible effect on the number of ants found inside flowers (Table S4) and on pollinator visitation (Table S5).

The estimates of the effects that individual ant species inside flowers have on pollinator visitation in model (S.vii) are imprecise (Fig. S4c) for two main reasons: first, ants rarely occupy flowers (Fig. 2, Table 2), and second, the scarce variation in ant species composition between flowers, caused by
Brachymyrmex sp. being the dominant ant taxa inside flowers, resulting in few observations for other taxa (Table 1). Because of these two reasons, our data has little variation and model estimates are thus driven largely by uncertainty and further experiments are required to test the effect different ant species inside flowers have on pollinator visitation. (Villamil et al. 2018). Finally, although models S.vi – S.viii do not elucidate the effects of specific ant species, they demonstrate quantitatively that lumping ant species together and testing the Distraction Hypothesis on the ant community associated with T. velutina is an adequate approach given the constraints of our dataset imposed by the biology of this system (see Supplementary material for further details).

Little is known about the extent to which positive and negative impacts of ant taxa are correlated and whether ant species that are threatening for herbivores (hence, highly defensive species) are also threatening for pollinators (hence, ecologically costly via pollinator deterrence) (but see: Ness 2006, Miller 2007, LeVan et al. 2014, Villamil et. al. 2018 in press, Ohm and Miller 2014, Villamil et al. 2018). Large-bodied and eusocial pollinators such as Apis mellifera have been assumed to be less susceptible than smaller solitary bees or other non-eusocial pollinators to ant attacks and more prone to visit flowers patrolled by aggressive ants (Queller 1989, Gadagkar 1990, Brechbühl et al. 2010a, Brechbühl et al. 2010b, Romero et al. 2011).

Our findings suggest that ant species vary in their deterrent effect on Apis mellifera bees, (Fig. S4). Qualitative patterns show that the presence of the most aggressive ant species, Dorymyrmex bicolor, inside flowers and at extrafloral nectaries have, on average, a negative effect on pollinators. These results are consistent with experimental findings demonstrating that placing dead Dorymyrmex bicolor ants inside flowers of T. velutina induced alert behaviours in Apis mellifera, reduced visit duration, and increased handling time per flower leading to a decreased in pollinator foraging efficiency (Villamil et al. 2018).
However, *A. mellifera* honeybees are introduced pollinators, and further work is required to assess the effect of the ant community on pollinator assemblages dominated by native, smaller-bodied, solitary pollinators.

The spatio-temporal scale of the Distraction Hypothesis

Based on the relatively small effect sizes of the short-term leaf-scale experiment (Tables 1-2), we hypothesised that clogging the glands of only one leaf for one day was perhaps too local and short-term a treatment to detect a measurable effect. In the long-term experiment we therefore increased both the spatial scale and duration of the EFN-removal treatment by a factor of ten, expecting to obtain larger effect sizes overall. However, in contrast to our prediction, the long-term clogging experiments had smaller effect sizes on ant patrolling (Table S2). For example, the short-term clogging experiment had a in 13 % greater effect size in reducing numbers of ants patrolling extrafloral nectaries, 155 % greater effect size increasing the numbers of ants inside flowers, and 132 % greater increase of ant occupancy of flowers than the long-term experiment (Fig. 3b; Tables 1-2). Hence, we can robustly conclude that clogging the glands of only one leaf for one day is not too local and short-term a treatment. In fact, leaf-day is the scale at which we detected an effect of clogging and our experimental evidence showed that ant foraging behaviour responds to reward availability over a this spatio-temporal scale. The non-provision of a whole branch for ten days is a rather unnatural setting for ants, or may resemble a low-rewarding plant (Lemus Domínguez 2014).

Our results suggest that in *T. velutina* EFN-mediated ant distraction is a mutualist management strategy that acts at a local and short-term scale. This makes adaptive sense because plant structures vary in their vulnerability to herbivores and sensitivity to both benefits and costs of ant guards over similar local and short-term scales (Bentley 1977b, Willmer and Stone 1997, Falcão et al.
Chapter 5: The Distraction Hypothesis of EFN secretion

2014, Villamil 2017). From the plant’s perspective, protection needs changes at a very small spatial and temporal scale (Bentley 1977b, Willmer and Stone 1997, Falcão et al. 2014, Villamil 2017) because in *T. velutina*, buds, flowers and fruits indeed occur in close proximity on the same shoot, and develop from bud to young fruit in only three days. Flowers are suggested to be the most vulnerable structure due to their soft and exposed water-rich tissues, whilst buds and fruits are protected by the sepals or the exocarp, respectively (Villamil 2017). Previous work has shown that EFN secretion in *T. velutina* is greatest at the flower stage, with glands in the associated leaf secreting ten times more sugar than glands associated with fruit, and 40% more sugar than glands associated with buds (Villamil 2017). This pattern of investment is compatible with both ODT (McKey 1979, Rhoades 1979, Stamp 2003, Ochoa-López et al. 2015, Ochoa-López et al. 2018) and the Distraction Hypothesis (for reduction of negative ant-pollinator interactions).

From the ant’s perspective, adjustment of foraging patterns at a local scale could maximise net sugar gain (Schilman and Roces 2006). The rapid transition from bud to fruit in *T. velutina* means that secretion by individual glands can vary substantially over consecutive days since EFN secretion varies greatly throughout this transition (Villamil 2017). Consequently, for the ants, missing the extrafloral nectaries of leaves associated with flowers means missing a bountiful reward.

Implications for ant and pollinator foraging strategies

We suggest that ants associated with *T. velutina* learn the location of highly rewarding EFN glands by monitoring variation in rewards within a single day, rather than relying on cues from previous days – a pattern compatible with demonstrated ability of ants to learn spatial and temporal scales of food rewards (Jackson and Morgan 1993, Robinson et al. 2005, Jackson and Ratnieks 2006).
There is also evidence that at least some pollinating insects can respond to similarly local variation in ant activity. Bees in other systems are known to use ant scents to discriminate and avoid heavily patrolled flowers, preventing harassment (Cembrowski et al. 2014) and we suggest that bees visiting *T. velutina* may also use olfactory cues to reduce their visitation of ant-occupied flowers.

The local foraging decisions we propose and the effects of within-plant variation in EFN availability on ants and pollinators should be seen as occurring against a backdrop of significant between-plant variation in EFN rewards. Differences between individual plants, and not between branches or flowers, explained a large part of the variance in both numbers of ants and pollinators at both experimental scales (Tables 1, S1). It is possible that plant-level variation in nectar availability underlies the positive correlation between numbers of patrolling ants and pollinator visitation observed in the long-term clogging treatment (Table S2), with each mutualist guild independently selecting more rewarding plants. Plant level variation in EFN rewards could have many causes, including phenotypic plasticity (Ochoa-López et al. 2018), genetic variation in floral (Ramos-Castro 2013) and extrafloral nectar (Ochoa-López 2013, Ochoa-López et al. 2015, Ochoa-López et al. 2018), and other variables such as plant age (Villamil et al. 2013, Ochoa-López et al. 2018), size, floral display (Ramos-Castro 2013), proximity to a nest or hive (Cuautle and Rico-Gray 2003, Cuautle et al. 2005), plant vigour or soil fertility (Yamawo et al. 2012, Dattilo et al. 2013).

**Conclusions**

Our findings on flower occupancy by ants at a leaf-day scale support the Distraction Hypothesis suggesting extrafloral nectar secretion during anthesis can bribe ants away from flowers and reduce ant occupancy of flowers. However, clogging EFN secretion did not result in a significant increase in ant abundance within flowers. This suggests that distraction via EFN secretion is
Chapter 5: The Distraction Hypothesis of EFN

neither the only nor the strongest mechanism in mutualist management by *T. velutina*. Further research is required to understand why ants rarely visit the flowers of *T. velutina*, and which mechanisms may be keeping ants outside these accessible, nectar producing flowers. Perhaps other mechanisms such as floral ant repellents (Willmer et al. 2009, Ballantyne and Willmer 2012) reinforce ant exclusion. Differences in chemical composition or sugar concentration between floral and extrafloral nectars may also underlie observed ant foraging preferences. Further studies on a range of ant-plants are required to assess the wider significance of EFN-mediated ant distraction in amelioration of ant-pollinator conflict.

I am the sole author of all the text used here, with comments on earlier drafts from Graham Stone, Karina Boege, Sarah Heath and Richard Whittet. Cristina Rosique kindly drew the illustration for Figure 5.1. Jarrod Hadfield contributed valuable statistical advice.

This chapter has been accepted at Journal of Ecology.
SUPPLEMENTARY MATERIAL 1

Long-term clogging experiment
(10 leaves, 10 days)

FIELDWORK METHODS

Based on the results from the short-term experiment, we conducted a follow-up experiment in which treatment duration and spatial scale were both increased by a factor of 10, using paired branches and focusing on one flower on each control or clogged branch, rather than paired flowers on the same branch (Fig. 1). In each of three sites, we selected 15 T. velutina plants bearing at least four apical flowering branches, each with ten leaves and reproductive structures \((n = 45\) plants). Two branches per plant were marked as control and two as clogged treatments using different string colours. We chose two branches to increase the probability of having one pair of focal flowers with each of the experimental treatments per plant per day. This experiment was conducted over 5 days during September 2015 (08 - 12 Sept.) for a total of 80 pairs of control and blocked flowers \((n = 160\) flowers). The extrafloral nectaries of all ten leaves on the clogged treatment branches were clogged as described in the methods for the short-term experiment, and the treatment was maintained for ten days. Glands
were checked each day, and the paint was replenished if required, especially after heavy rains overnight. If new leaves emerged on a branch during the experiment, paint droplets were applied on or above their extrafloral nectaries according to their respective treatment (Fig. 1). Every morning all plants were checked and those bearing at least one pair of flowers with control or clogged treatment were observed following the same observational protocol as for the short-term experiment. Simultaneous observations were performed at each of three sites by different observers.

STATISTICAL ANALYSES

Five mixed effects models with the same structure as those used to analyse the results from the short-term clogging experiment were fitted to analyse the long-term experiment data. Because all of these models had the same random effects structure, unless otherwise specified, we detail the random effects first and then describe the fixed effects for each model. In the long-term experiment, branch identity was fitted as a random effect to account for repeated hourly observations (Table S1). Because this experiment had a paired experimental design we fitted branch pair identity as a random effect to control for between-pair variation in floral and extrafloral investment. We also included an observation-level random effect where each data point received a unique level
Chapter 5: The Distraction Hypothesis of EFN

of a random effect to control for overdispersion. We fitted the following models, and have structured our results following this order:

(S.i) To test the effect of nectary clogging on the number of ants we fitted a Poisson mixed effects model using number of ants as the response variable. Ant location (at extrafloral nectaries or in flowers), treatment, and the interaction between these two factors were fitted as fixed effects. Tukey post-hoc tests were conducted in this model to find differences between the number of ants at extrafloral nectaries or flowers under control or clogged gland conditions.

(S.ii) To test whether preventing EFN secretion by clogging the glands increased the probability of a flower being occupied by ants, we fitted a binomial mixed effect model. We used the presence or absence of ants in a flower as a response variable, and fitted treatment as a fixed effect. The random effects remained the same, except for the observation-level random effect which we omitted.

(S.iii) To test the effect of clogging EFN secretion on pollinator visitation we fitted a Poisson mixed model using the number of pollinators as the response variable and treatment as the only fixed effect.

(S.iv) To test the whether the total number ants, regardless of their location, had an effect on pollinator visitation we fitted a Poisson mixed model using number of pollinators as the response variable. As fixed effects we fitted the
Chapter 5: The Distraction Hypothesis of EFN

To test if the location (inside flowers or at extrafloral nectaries) and number of ants affect pollinator visitation, we fitted a Poisson mixed model. The number of pollinators was fitted as the response variable, whilst treatment, number of ants in flowers, and number of ants at extrafloral nectaries were fitted as fixed effects.

Cohen $d$ effect sizes for all models were calculated using the LRT statistics from each model. To test whether increasing the duration and scale of the clogging treatment by a factor of 10 had a larger effect on the number of ants and pollinators, we estimated the ratio of change in the effect size between the short- and long-term experiment for each type of visitor (Table S2).

RESULTS AND DISCUSSION

Reduction of EFN secretion

EFN treatment (clogged versus control) and ant location both had significant effects on the number of ants, but with no significant interaction between these factors (Fig. S1b; Table S1). There were significant differences between the numbers of ants patrolling the nectaries and those inside flowers over both EFN treatments (Table S1). Almost seven times more ants were found patrolling extrafloral nectaries ($1.53 \pm 0.087$) than were found inside the flowers.
Chapter 5: The Distraction Hypothesis of EFN (0.22 ± 0.034) (Fig. S1b; Table S1), regardless of EFN treatment (control: $Z = -14.04, P < 0.001$; clogged: $Z = -11.41, P < 0.001$). In contrast to the results from the short-term experiment, eliminating EFN secretion had a negative effect on the number of ants at both locations (Fig. S1b; Table S3). Clogging significantly reduced the number of ants at extrafloral nectaries ($Z = -3.043, P = 0.011$; Table S2), but had no significant effect on the number of ants in flowers ($Z = -0.477, P = 0.96$; Table S2) (Fig. S1b).

(S.ii) The percentage of flowers occupied by ants was 11% in both treatments, hence eliminating EFN secretion from all leaves did not increase ant visitation to flowers (Table S1).

(S.iii) Eliminating EFN secretion had no significant effect on the number of pollinators (Fig. S1b; Table S1).

(S.iv) The number of pollinators was significantly and positively correlated with the total number of ants found patrolling either the extrafloral nectaries or inside the flowers, regardless of the treatment (Tables S1, S2).

(S.v) Furthermore, when ant abundance was partitioned by their location on the plants between extrafloral nectaries and flowers, the number of ants patrolling extrafloral nectaries was significantly positively correlated with the number of pollinators (Table S1), whilst the number of ants inside a flower was significantly
negatively correlated with the number of pollinators (Table S2). These results contrast strongly with patterns at the single flower scale.

In models (S.i)-(S.iii) used to analyse the long-term experiment data, differences between plants (captured by the plant random effect) explained more of the variation in the numbers of ants or pollinators than differences between branches (captured by the branch random effect) or random variation between observations (captured by the OLRE random effect) (Table S1). In models (S.iv, S.v) random variation between observations explained a larger proportion of the variation in the numbers of ants or pollinators, followed by plant identity (Table S1). Branch identity was the random effect that consistently explained least variation in the numbers of ants or pollinators (Table S1).

**Effect sizes**

Increasing the duration and spatial scale of the clogging treatment by a factor of 10 did not result in larger overall effect sizes as we had predicted (Table S2). In fact, the direction of some effects was reversed when the scale was increased. For example, the impact of preventing EFN secretion on the number of ants inside flowers changed from positive at a short-term, local scale to negative in the long-term, branch-scale experiment (Table S2). The effect size of total ant numbers on pollinator activity changed from negative to positive when we increased the scale of the treatment, as expected. The same pattern was observed for the effect size of ants patrolling extrafloral nectaries on pollinator...
activity. However, the effect size of ants inside flowers on pollinator activity was reduced when we increased the scale of the treatment.

**Comparing effect sizes and spatio-temporal scales**
Here we provide further discussion regarding other comparisons between the short and long-term clogging experiments that are beyond the scope of Distraction Hypothesis.

The effect of the total number of ants and the number of ants at EFN on pollinators was three times greater in the long-term scale experiment, as expected. Clogging extrafloral nectaries from a whole branch caused a 13% greater decrease in the number of ants patrolling the EFN glands than clogging EFN glands from only one leaf. Since most ants are found patrolling EFN glands, this decreased the total ant numbers, and such reduction in ant patrolling may be driving the increase in pollinator visitation. However, the negative effect of ants inside flowers on pollinator visitation was reduced in the long-term experiment, supporting the idea that the Distraction Hypothesis operates at a leaf, daily scale.

The long-term experiment showed that numbers of ants inside flowers have a significant negative effect on the number of pollinators, as predicted by the Distraction hypothesis (Tables S1, S2). But the same experiment revealed two positive effects that are not predicted by the Distraction Hypothesis:
Chapter 5: The Distraction Hypothesis of EFN between the total number of ants and pollinators, and between the number of ants patrolling extrafloral nectaries and pollinators (Tables S1-S2).

The positive effect of the number of ants on pollinator visitation may well represent the outcome of reward-based foraging decisions at the plant scale by both mutualists, leading to selection of more rewarding plants. Having all extrafloral nectaries within a branch clogged may resemble a low-rewarding plant, as explained in the main text. The positive relationship between ants and pollinators at a plant-scale (Table S2), may imply that plants of *T. velutina* able to recruit large numbers of ants do not experience a trade-off in pollinator visitation. Further experiments are required to test whether the ecological costs of ant patrolling on pollinator visitation differ between plants with high or low reward secretion.
Figure S1. Effect of clogging the extrafloral nectaries on the number of visitors recorded in hourly surveys during 2 min of observation per flower (mean ± se) a) for the short-term experiment where one leaf was clogged for one day; and b) for the long-term experiment where ten leaves within a branch were clogged for ten days. Circles represent ants at extrafloral nectaries; triangles represent ant in flowers, and squares represent pollinators. Plot a) is also reported in the main text but has been repeated here to facilitate comparison with b).
Chapter 5: The Distraction Hypothesis of EFN

Figure S2. Hourly observation showing the effect of clogging of extrafloral nectaries on the number of visitors (mean ±se) a) for the short-term experiment in which one leaf was clogged for one day, and b) for the long-term experiment in which ten leaves within a branch were clogged for ten days.

[Graph showing data]
Table S1. Likelihood ratio test results for statistical models used to test different response variables in the experiments. The values highlighted in bold are statistically significant ($P > 0.05$).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Model</th>
<th>Response</th>
<th>Fixed effects</th>
<th>df</th>
<th>LRT</th>
<th>$P$-value</th>
<th>Random effects</th>
<th>Variance</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long-term Clogging experiment</td>
<td>S.i)</td>
<td>Number of ants</td>
<td>Clogging</td>
<td>1</td>
<td>7.28</td>
<td><strong>0.0069</strong></td>
<td>Branch</td>
<td>0.20</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ant location (EFN or flowers)</td>
<td>1</td>
<td>395.5</td>
<td><strong>2.2-16</strong></td>
<td>Plant</td>
<td>0.30</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Clogging × Ant location</td>
<td>1</td>
<td>1.79</td>
<td>0.18</td>
<td>OLRE</td>
<td>0.60</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td>S.ii)</td>
<td>Proportion of flowers occupied by ants</td>
<td>Clogging</td>
<td>1</td>
<td>16.49</td>
<td><strong>0.98</strong></td>
<td>Branch</td>
<td>9.87×10</td>
<td>3.14×10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Plant</td>
<td>1.07</td>
<td>1.03</td>
</tr>
<tr>
<td></td>
<td>S.iii)</td>
<td>Number of pollinators</td>
<td>Clogging</td>
<td>1</td>
<td>0.63</td>
<td>0.42</td>
<td>Branch</td>
<td>1.04×9</td>
<td>3.23×05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Plant</td>
<td>0.39</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>OLRE</td>
<td>0.54</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td>S.iv)</td>
<td>Number of pollinators</td>
<td>Clogging</td>
<td>1</td>
<td>1.43</td>
<td>0.23</td>
<td>Branch</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Plant</td>
<td>0.39</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>OLRE</td>
<td>0.48</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td>S.v)</td>
<td>Number of pollinators</td>
<td>Clogging</td>
<td>1</td>
<td>2.00</td>
<td>0.15</td>
<td>Branch</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ants in EFN</td>
<td>1</td>
<td>10.85</td>
<td><strong>0.0009</strong></td>
<td>Plant</td>
<td>0.38</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ants in flowers</td>
<td>1</td>
<td>3.78</td>
<td><strong>0.05</strong></td>
<td>OLRE</td>
<td>0.41</td>
<td>0.64</td>
</tr>
</tbody>
</table>
Table S2. Comparison of the effects sizes of the short and long-term clogging experiments on the number of visits per visitor type.

<table>
<thead>
<tr>
<th>Model</th>
<th>Short term experiment: Clog 1 day 1 leaf</th>
<th>Long term experiment: Clog 10 days 10 leaves</th>
<th>Effect size difference ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fixed effect</td>
<td>d</td>
<td>Effect size</td>
</tr>
<tr>
<td>i)</td>
<td>Ants in EFN</td>
<td>-0.2865</td>
<td>Small</td>
</tr>
<tr>
<td>i)</td>
<td>Ants in flowers</td>
<td>+0.070</td>
<td>Small</td>
</tr>
<tr>
<td>ii)</td>
<td>Flowers occupied by ants</td>
<td>+0.146</td>
<td>Small</td>
</tr>
<tr>
<td>iii)</td>
<td>Pollinators</td>
<td>+0.0503</td>
<td>ns</td>
</tr>
<tr>
<td>iv)</td>
<td>Clogging</td>
<td>-0.0488</td>
<td>ns</td>
</tr>
<tr>
<td>iv)</td>
<td>Total ants</td>
<td>-0.0915</td>
<td>ns</td>
</tr>
<tr>
<td>v)</td>
<td>Clogging</td>
<td>-0.0676</td>
<td>ns</td>
</tr>
<tr>
<td>v)</td>
<td>Ants in EFN</td>
<td>-0.1314</td>
<td>ns</td>
</tr>
<tr>
<td>v)</td>
<td>Ants in flowers</td>
<td>-0.0893</td>
<td>ns</td>
</tr>
</tbody>
</table>
SUPPLEMENTARY MATERIAL 2

Ant species-specific responses to clogging and effects on pollinators

STATISTICAL ANALYSES

Effects of clogging on ant species
(S.vi) To investigate whether ant species differed in their response to clogging we fitted a Poisson mixed model using the number of ants inside the flower as the response variable, and treatment as a fixed effect. As random effects we fitted flower identity, pair identity, an observation-level random effect to deal with overdispersion, ant species, and the interaction between treatment and ant species. This model allowed us to estimate the amount of variance in the number of ants inside flowers explained by ant species, and estimate the effect of clogging on the abundance of each ant species inside flowers.

Although the ant species and the interaction between clogging and ant species explained a negligible amount of variation in the abundance of ants inside flowers, we extracted the random effect estimates for our “species” and “species x clogging” random effects. For the random effect “ant species” in model (S.vi) we extracted the estimates for every level within this random effect and its standard error (Fig. S3a).
Effects of ant species on pollinators

To investigate whether different ant species patrolling the plants or inside the flowers differed in their effect on pollinator visitation, we fitted multiple membership mixed models using MCMCglmm (Hadfield 2010). In these models, we fitted the abundance of ants from each species as covariates and treated the regression coefficients as random. This allowed us to estimate the effects that different species of ant have on the number of pollinators.

These statistical tools allow us to account for plants occupied by multiple ant species, and capture the variation in ant abundance within a given species. This model also prevents us from making a priori assumptions of ant attributes that may make certain species more prone to responding to clogging or ant attributes that may have an effect on pollinator visitation. Such a priori assumptions may be flawed due to the lack of previous studies testing this. Instead, these models allow us to detect the magnitude and direction (positive or negative) of the effect each ant species has on pollinator visitation. Using this approach allows us to generate robust evidence-based assumptions of ant species attributes that may enhance or hinder pollinator visitation.

To investigate if the number of ants of different species inside the flowers affected pollinator visitation, we fitted an ordinal multi-membership mixed model. Although the data are count data we chose to treat the number of pollinator visits as ordinal because the number of visits fell into a few categories (0, 1, 2, or 3) and the mean and variance of the counts were equal suggesting that the data would be underdispersed with respect to the Poisson if there was real heterogeneity in pollinator visitation rates. The number of pollinators was fitted as the response variable, whilst treatment, number of ants in flowers, and number of ants at extrafloral nectaries were fitted as fixed effects. As random effects we fitted flower, pair identity, and an observation-level random effect to account for overdispersion. The regression coefficients of the number of ants inside flowers from each species were treated as random additive effects. Fitting
these additive random effects allowed us to estimate the effects that different ant species inside flowers had on pollinator visitation.

(S.viii) To investigate whether the number of ants of different species patrolling the extrafloral nectaries differed in their effect on pollinator visitation, we fitted an ordinal multi-membership mixed model. The number of pollinators was fitted as the response variable, whilst treatment, number of ants in flowers, and number of ants at extrafloral nectaries were fitted as fixed effects. As random effects we fitted flower and pair identity, and an observation-level random effect to account for overdispersion. We also fitted the abundance of ants from each species at the extrafloral nectaries as random additive effects which allowed us to estimate the amount of variance in the number of pollinators explained by ant species, and estimate the effects of patrolling by each ant species on pollinator visitation.

RESULTS AND DISCUSSION

Effects of clogging on ant species
(S.vi) We found no significant effect of clogging on the number of ants inside flowers (Table S3), which confirmed the results from model (i) in the short-term experiment. The interaction between clogging and ant species explained no variation in the number of ants inside flowers. Most of the variation was explained by the differences between observations (OLRE: 97 %), followed by pair identity (2.3 %), and finally ant species explaining only 0.21 % of the variation. The estimates of the between-species effects are low and imprecise (Fig. S3a). The imprecision is for two reasons; more common species are sampled more often, whilst rare species are seldom sampled, hence their confidence intervals are larger and estimates less precise. Secondly, because
Chapter 5: The Distraction Hypothesis of EFN

Overall ants inside the flowers is a rare phenomenon (Fig. S1). The qualitative patterns obtained from the “ants species x clogging” effects helped us detect ant species with the greatest differences between control and clogged (Fig. S3a) which would be worth exploring in a controlled experimental way. These species are: *Brachymyrmex, Paratrechina longicornis, C. planatus*, and *C. mucronatus*.

**Effects of ant species on pollinators**

(S.vii) The effects of different ant species at extrafloral nectaries on pollinator visitation are precisely estimated by our models, (see SE in Fig. S3b). All effects overlap with zero, revealing a negligible effect of ant species at extrafloral nectaries on pollinator visitation (Table S4). Despite this, we could detect qualitative patterns in this data: *Dorymyrmex bicolor* ants have a on average, negative effect on pollinator visitation, whilst *Camponotus mucronatus* and *Paratrechina longicornis* have, on average, a positive effect on pollinator visitation (Fig. S3b).

(S.viii) The effects of different ant species inside flowers have on pollinator visitation are imprecisely estimated by our models, as indicated by the large SE values associated with these estimates (Fig. S3c). Qualitative patterns in this data suggest that when inside flowers, *Dorymyrmex bicolor* ants may have a negative effect on pollinators. Such patterns also help us detect ant species whose positive or negative effects on pollinators would be worth exploring in further experimental work. Furthermore, these results are consistent with experimental findings demonstrating that *Dorymyrmex bicolor* ants placed inside flowers of *T. velutina* induce alert behaviours in pollinators, reduce visit duration, increasing handling time per flower and reducing foraging efficiency (Villamil et al. 2018).
Figure S3. a) Effect of clogging the extrafloral nectaries on different ant species inside flowers and effects of different ant species on pollinators, and their precision. Effect of ant species (b) patrolling extrafloral nectaries, or (c) inside the flowers on pollinator visitation (mode ± SE).
Table S3. Mean effect of each ant species patrolling at EFN glands on pollinator visitation using a multiple membership mixed model.

<table>
<thead>
<tr>
<th>Model</th>
<th>Response</th>
<th>Fixed effects</th>
<th>Estimate</th>
<th>LRT</th>
<th>P-value</th>
<th>Random effects</th>
<th>Variance</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.vi)</td>
<td>Ants inside flowers</td>
<td>Clogging</td>
<td>0.55</td>
<td>0.61</td>
<td>0.43</td>
<td>Species</td>
<td>0.20</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Species x Clogging</td>
<td>6.14</td>
<td>2.47&lt;sup&gt;0.05&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Flower id</td>
<td>2.20</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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Chapter 5: The Distraction Hypothesis of EFN
Table S4. Mean effect of each ant species patrolling at EFN glands on pollinator visitation using a multiple membership mixed model.

<table>
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<tr>
<th>Model</th>
<th>Response</th>
<th>Fixed effect</th>
<th>Post mean</th>
<th>95% CI lwr</th>
<th>95% CI upr</th>
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<th>Variance</th>
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CHAPTER 6

GENERAL DISCUSSION

Key messages and loose ends
SUMMARY OF KEY FINDINGS

Chapter 2. Ants aggressivity

- I quantified the aggressivity of six ant species interacting with *T. velutina*, described their behavioural ecology and used qualitative and quantitative components to assess their protection effectiveness.
- *Cephalotes* sp. ants were detected as parasitic non-defenders.
- The rest of the species were ranked according to their protection effectiveness as: *Capmponotus planatus* < *Crematogaster* sp. < *Paratrechina longicornis* < *Brachymyrmex* sp. < *Dorymyrmex bicolor*.

Chapter 3. Direct and indirect ant-pollinator conflict

- I explored ant-pollinator conflict in *Turnera velutina*, which rewards ants with extrafloral nectar (EFN) and pollinators with floral nectar (FN).
- Secretion of FN and EFN, activity of ants and pollinators, and pollen deposition all overlapped in time.
- I found evidence of direct conflict, since ants inside flowers altered pollinator behaviour and reduced visit duration, and more aggressive ants had stronger effects on pollinator visitation.
- I found no evidence for indirect conflict with no significant difference in the volume or sugar content of FN between control plants and those in which EFN secretion was prevented.

Chapter 4. Ecological costs

- I experimentally tested the effects of ant patrolling on pollinator community composition, visitation frequency, duration and, behaviour of floral visitors, and their cascading effects on the plant mating system, pollen transfer dynamics, and plant fitness.
- Patrolling did not affect pollinator composition, visitation frequency, or inspection/alert behaviours in pollinators.
- Ant patrolling had a significantly decreased visit duration, pollen load on stigmas and male fitness; and significantly increased outcrossing rates.
Chapter 6: General discussion

- The effects of ant patrolling on pollinator visitation lead to negative effects on pollinator foraging efficiency, but such behavioural changes had positive effects for plant progeny promoting outcrossing.

Chapter 5. EFN as ant distractors

- I experimentally tested the Distraction Hypothesis for EFN, which posits that rewarding ants with extrafloral nectar could reduce their visitation of flowers, reducing ant-pollinator conflict while retaining protection of other structures.
- Ant activity inside flowers was low throughout all anthesis period.
- Preventing EFN secretion reduced ant patrolling at extrafloral nectaries, increased the proportion of flowers occupied by ants, and reduced plant reproductive output through an increase in fruit abortion, but did not affect the numbers of ants or pollinators inside flowers.
- These results support the Distraction Hypothesis, suggesting that EFN secretion may bribe ants away from reproductive structures during the crucial pollination period, reducing ant-occupation of flowers.
SOME LOOSE ENDS

Natural history

The pollination biology of *Turnera velutina* at Troncones, where visitors are mostly native butterfly species, should be further investigated. So far, the only studies on the pollination biology of this species was conducted at La Mancha (Ramos-Castro 2013, Sosenski et al. 2016), where the introduced honeybee *Apis mellifera* is the dominant pollinator. Beetles were excluded from the analyses because their role as pollinators is dubious, however, my observations reveal interesting ecological dynamics worthy of further investigation, such as:

- The role of beetles as florivores and their impacts on plant fitness
- The effect of their presence on pollinator visitation
- Their mating behaviour and whether they obtain attractive pheromones from these flowers
- Beetle-ant interactions

Is the corolla a natural ant shield for pollinators?

In *Heteropterys pteropetala* EFN are located at the base of the pedicel that supports several flowers, and corollas are small, less than a fifth of the size of the bees that pollinate it (Assunção et al. 2014). Hence, bees cling to the entire stalk supporting several flowers and their bodies are in close proximity to the EFN, leaving plenty of opportunity for patrolling ants to attack them. In contrast, *Turnera* flowers are cup-like structures at least three times bigger than honeybees and large enough to fit an entire butterfly inside. Consequently, pollinators can feed on floral resources, and ants on extrafloral resources simultaneously without contacting each other. Pollinators and ants need not be in close proximity whilst foraging, unless ants intrude the flowers. Hence, I
hypothesise the corolla acts as a natural shield to protect the pollinators, whom are spatially separated from the EFN.

This hypothesis could be experimentally tested by trimming and manipulating corolla shape, without damaging its structural integrity. Although artificial damage is a confounding factor. A potential experiment could have four treatments: (1) Control: with intact flowers; (2) Procedures control: cutting the edge of the petals to induce artificial damage without hugely affecting the corolla’s shape, symmetry, structural integrity or providing an entry for ants (as punch-holes would do); (3) Plucking: plucking the whole petals out from the toral disc, to completely deploy the corolla structure, but aiming to reduce artificial damage and volatile emission; (4) Trimming: trimming the petals just above the sepals to deploy the corolla structure, whilst inducing artificial damage and releasing volatiles. Additionally, it would be worth exploring the pollen volatiles for ant-deterring odours.

Networks

Observational data collected on ant patrolling to different plants could be used to build networks of ant-pollinator species coexisting on a plant. These data would help unravel the effects of specific ants on specific pollinator taxa, building the network of two interactions simultaneously.

Using these same data, we can delve into the community dynamics of ants, studying which ant species can coexist and share a host plant, and which ones do not coexist. This information could shed light on competition dynamics between ant species.
Chapter 6: General discussion

BROADER IMPLICATIONS

**Herbivore vs. pollinator susceptibility to ants**

Ant aggressivity towards herbivores in *T. velutina* does not seem to correspond exactly to the threat level they represent for pollinators. The reduction in pollinator visit duration caused by different ant species varying in their aggressivity levels (Chapter 3) was not proportional to the level ant aggressivity against herbivores (Chapter 2). Honeybees spent the same time visiting control flowers without ants as they spent visiting flowers with *Brachymyrmex* ants. *Brachymyrmex* sp. ants were the second most aggressive ant species, and more aggressive to herbivores than *P. longicornis*. Yet, the presence *P. longicornis* inside flowers had a similar effect size on pollinator visit duration as that of the most aggressive ant species, *D. bicolor*. This suggests that herbivores and pollinators are not equally vulnerable to all ant species, adding the challenge of considering species-specific ant effects when estimating of the net effect of ant patrolling on plant fitness via herbivore suppression and pollinator deterrence.

In the cholla cacti *Opuntia imbricata*, Ohm and Miller (2014) found that the weaker defender, imposed more pollination costs than those of an aggressive defender. But these results contrast with previous findings on *Ferocactus wislizeni* reporting that better guards also had high anti-pollinator effects (Ness 2006). Our results in *T. velutina* and those of Ohm and Miller (2014) in cholla cacti contrast with the general hypothesis that more aggressive bodyguards will also have stronger pollinator deterring effects, imposing larger costs for the host plant pollination. This contrasting evidence suggests herbivores and pollinators may not be equally vulnerable to all ant species. Morphological traits such cuticle thickness, and behavioural ones such as foraging locations may affect visitors (herbivores or pollinators) susceptibility to ant attacks. Ant attacking mode may also make certain visitors more susceptible than others. But on the other hand, highlights the need for more
studies assessing the costs of myrmecophily. Especially those with a community approach since a given ant species may differ in aggressivity display and protection effectiveness depending on the host plant (Fagundes et al. 2017).

Are *Brachymyrmex* ants the best guards by being pollinator friendly?

I expected the probability of finding different ant species inside the flowers to vary between species, with the least aggressive ant species more often found inside flowers. I predicted that the most aggressive ant species would be patrolling at the extrafloral nectaries, whilst least aggressive ant species would be the ones more often found inside flowers. However, this prediction was not supported by the data. The ant community inside flowers is not a representative sample of the ant community on the EFNs (Chapter 4, 5). *Brachymyrmex* ants are overrepresented inside the flowers (Chapter 5), despite being the second most aggressive ant species (Chapter 2). Additionally, the effect of *Brachymyrmex* ants on pollinator visitation were not significantly different from those of control flower without ants (Chapter 3).

This evidence suggests that if *T. velutina* has ant-repelling mechanisms, these are not deterring one of the most aggressive species from the flowers; alternatively, *Brachymyrmex* ants may be resistant to the floral repelling mechanisms. This may also imply some degree of specialisation in the *T. velutina-Brachymyrmex* association. Either ants that are aggressive towards herbivores may not be equally aggressive towards pollinators, resulting in good defensive traits without ecological costs for the host plant pollination. Or, if plants have ant-repelling compounds, *Brachymyrmex* ants may have evolved resistance against those signals, which allows them to enter flowers. But perhaps both scenarios are true, and restricting *Brachymyrmex* sp. access to flowers has no fitness benefits because *Brachymyrmex* sp. ants may not represent a cost for pollination. Despite being the second most aggressive ants species (Chapter 2),
the presence of *Bracymyrmex* sp. corpses inside flowers did not significantly reduce the time honeybees spent inside flowers, the percentage of visitors displaying inspection behaviours, or the proportion of time spent by pollinators displaying inspecting flowers per visiting bout, compared to control flowers without any ant corpses (Chapter 3). In contrast, the presence of *Dorymyrmex bicolor* corpses—the other highly aggressive ant species (Chapter 3)—did reduce visit duration and increased inspection behaviours.

It would be interesting to further explore the repellence response induced by flowers on different ant species. For instance, using wiping a petal onto half a Petri dish and placing an ant in the centre and observing the amount of time spent on the flower-wiped vs non-flower-wiped Petri dish section.

**Ant patrolling, outcrossing rates, and plant selective fruit abortion**

I found evidence for direct ant-pollinator conflict in *T. velutina* since the presence of dead ants inside flowers induced alert behaviours in pollinators and reduced the duration of visits, under an experimental setting (Chapter 3). The ant exclusion experiment confirmed this in a more realistic setting with live ants instead of ant corpses: ant exclusion resulted in longer pollinator visits and higher selfing rates (Chapter 4). Clogging extrafloral nectaries increased fruit abortion by 12% and decreased ant patrolling (Chapter 5). Hence, it is likely that fruits developed from flowers where extrafloral nectaries were clogged had a higher proportion of selfed seeds than those developed from control flowers.

If this was the case, selective fruit abortion may then explain the higher abortion rates in fruits developed from clogged flowers (predominantly selfing), compared to fruits in the same plant developed from control (predominantly
Chapter 6: General discussion

Plants can abort fruits with a higher proportion of selfed seeds, to increase resource allocation in fruits with a higher proportion of outcrossed seeds. Selective fruit abortion linked to pollen origin (selfing vs outcrossing) has been observed in a wide array of plant species (Stephenson 1981, Marshall and Ellstrand 1988, Niesenbaum 1999, Huth and Pellmyr 2000).

Selective abortion in *T. velutina* may also be linked to pre-dispersal seed predators (Parachnowitsch and Caruso 2008, Sanz-Veiga et al. 2017). There is evidence of pre-dispersal seed predation in *T. velutina* by the lepidopteran larvae *Crocidosema plebejana* (Ochoa-López pers. obs.). If the ant community in *T. velutina* protects plants against pre-dispersal seed predators, then fruits developing in ant excluded plants may have higher infestation levels. Selectively aborting damaged fruits and reallocating those resources into healthy fruits would be a beneficial plant strategy to enhance fitness. The increased rates of fruit abortion in plants with low ant patrolling levels could be due to pre-dispersal seed predators or plant selective abortion linked to pollen origin. Further studies are required to analyse pre-dispersal seed predation and selective fruit abortion in *T. velutina* in order to demonstrate the suggested mechanisms and its underlying cause.

The effect of ant patrolling on pre-dispersal seed predation could by clarified by characterising the rates of pre-dispersal seed predators in *T. velutina*, the role of ant patrolling in preventing it, and the rate of fruit abortion linked to pre-dispersal predators. This could be done by excluding ants from certain branches or plants and assess to assess the effect of ant exclusion on pre-dispersal seed predation.

The hypothesis of selective plant abortion linked to pollen origin could be experimentally tested selective fruit abortion in *T. velutina* by conducting an experiment with two types of manual crosses: Self-pollinating half the flowers
within a plant, and outcrossing the remaining flowers; and compare fruit abortion rates between treatments.

**Ant patrolling & dioecy**

In *T. velutina* ant patrolling had contrasting effects on male and female fitness components. Ant patrolling had a negative effect on male fitness reducing the number of pollen grains sired per flower, but a positive effect on female fitness by reducing the likelihood of fruit abortion and promoting outbred seeds and so increasing the quality of the offspring (Chapter 4). If this trend of opposite effects of ant patrolling on male and female fitness was maintained across most ant-plants, I would expect natural selection to favour different ant patrolling regimes or abundances in each sex on species with unisexual individuals (dioecious, androdioecious or gynodioecious).

I would expect natural selection to favour male plants that reduce or deplete ant patrolling to avoid the costs of ants of pollen exports. Whilst female plants would be under weaker or no selective pressure to deplete ant patrolling, assuming that EFN secretion or ant rewards are not very costly. Although, in dioecious plants, female individuals do not need ant patrolling to promote outcrossing, ant patrolling may reduce fruit abortion to florivores, and so ant patrolling may result in fewer fitness benefits. However, this contrast may be stronger in androdioecious plants, where individuals bearing bisexual flowers may benefit from ant patrolling promoting pollinator relocation, and so enhance outcrossing rates over geitonogamy. Exploring the effects of ant patrolling on male and female fitness components of dioecious ant-plants bearing EFN such as *Diospyros, Fraxinus, Ilex, Populus, Salix, Costus*, may uncover interesting findings on the role of myrmecophily as a selective pressure for the evolution of ant-plants of mating systems. However, herbivory levels also
vary between plant sexes (refs), and so these costs should also be considered when estimating the net effects of ant patrolling on the fitness of each plant sex.

**Herkogamy, outcrossing and ant aggressivity**

Pollinators are usually considered the main selective agents of floral traits (Mothershead and Marquis 2000, Parachnowitsch and Kessler 2010), however other agents interacting with plants have proven to also play important roles in shaping the floral and mating system traits of plants (Rudgers and Gardener 2004, Parachnowitsch and Caruso 2008, Hernández-Cumplido et al. 2010, Kessler et al. 2011, Hernandez-Cumplido et al. 2016). Ant patrolling has been suggested as a factor influencing pollinator visitation and plant fitness (Altshuler 1999, Ness 2006). In Chapter 4, I showed an example of ant patrolling affecting pollinator visit duration with cascading effects on *T. velutina*’s mating system and fitness. By affecting pollinator visitation patterns, ant patrolling may impose pollinator limitation or unreliability to the host plant, and so influence plants mating systems further than conceived so far.

Previous studies have demonstrated that outcrossing/selfing, self-compatibility and herkogamy (anther-stigma distance) respond to pollinator reliability (Opedal et al. 2016). Populations with unreliable pollinators showed higher compatibility, lower herkogamy and higher selfing rates, compared to populations with reliable pollinators (Opedal et al. 2016). These results are consistent with the reproductive assurance hypothesis, which suggests that plants with pollinator limitation may develop floral and mating system traits to promote self-pollination, as a means to assure seed set.

*T. velutina* is a self-compatible species incapable of autonomous pollination, and so requires pollinators to transport pollen grains onto the stigmas in order to set seed (Sosenski et al. 2016). Ant patrolling in *T. velutina*
significantly decreased stigmatic pollen load by 150%, (Chapter 4), which may imply negative effects of ant patrolling on pollinator reliability. However, ant patrolling also decreased selfing, which is not consistent with pollinator unreliability. Further studies testing changes in herkogamy in *T. velutina* populations with varying levels of ant aggressivity would shed light on the long-term evolutionary effects of ant patrolling on this host plant's mating system. Following the reproductive assurance hypothesis, I would expect populations with more aggressive ants to have lower pollinator reliability, and hence lower herkogamy to promote self-pollination.
Definition of Poetry
By Samuel Taylor Coleridge

“Poetry is not
the proper antithesis
to prose
but to science.
Poetry is opposed to science.”

On reading poetry

“If I had to live my life again I would have a rule to
read some poetry and listen to some music at least once
every week; for perhaps parts of my brain now atrophied
could thus have been kept active through use. The loss of
these tastes is a loss of happiness and may possibly be
injurious to the intellect, and more probably to the
moral character, by enfeebling the emotional part of our
nature.”

Charles Darwin
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NATURAL HISTORY
SUPPLEMENTARY NOTES

Ants

Behavioural observations catalogue

The behavioural catalogue presented here is a compilation of observations carried out throughout field experiments across years, particularly between 2014-2016, but also includes personal observations recorded prior to such period.

*Dorymyrmex bicolor*

- Actively patrolling in groups 5-6 per branch or apex. Usually observed climbing up from the stem, searching for nectar. Very alert and aggressive, quickly detecting plant disturbances and always responding to them by displaying aggressive behaviours. This species does not share a host plant with other plant species (we only have one record of a *Cephalotes* ants on a plant occupied by *D. bicolor*). *D. bicolor* ants are exclusive patrollers of groups of plants near their nests, usually dominating patches consistently over at least a whole field season, indicating they are highly territorial. Although their location may change between years, their abundance seems constant over years.
- Efficient recruiters with small response times, capable of recruiting over 30 ants in a few minutes.
- A few go inside the flowers and once inside they patrol petals, walking around the inside of the corolla. These ants deter pollinators, reducing visit duration and preventing visitors from entering the flowers by
rapidly patrolling the inside of the petals (video from Troncones available).

- Also seen walking on the outside of the corolla. Never seen at the bottom of the corolla consuming floral nectar. However, in Troncones, Guerrero, these ants used the holes bitten by the Curculionidae beetles on the corolla base to consume floral nectar from the outside of the corolla.
- When extrafloral nectaries lack visible secretion droplets, they do not bite the glands, but rather explore/touch them with their antennae.
- *D. bicolor* ants secrete high amount of some acid, probably formic acid, when stressed. This can be detected from its citric smell and sour taste.
- Not biologist-friendly, these ants attack humans and their bite is painful. Nests easily identifiable through the sit-and-ouch! method.

**Camponotus platanus**

- Active patrolling, climbing up from the stem and patrolling the upper and underside of the leaves. Very rarely seen inside the flowers, and when so, only seen walking on the petals, never seen consuming floral nectar.
- Solitary ants, usually one per branch or apex, or even one per plant. Sometimes seen standing immobile. Poor recruiters, seldom recruiting more than two other individuals, which is probably due to their solitary patrolling habit.
- Alert and very responsive towards plant disturbance, but do not attack biologists. Their attacking mode mixes bites and also spraying through their acidopore. These ants recoil the gaster, curving their abdomen forward, underneath their thorax, pointing the rear end with the acidopore towards the attack target. Although their main response is recoiling.
- When they feel under attack, these ants head towards the underside of the leaves and drop themselves off the plant.
• When EFN lack visible secretion droplets, they bite the glands and contact them with their mouthparts open.
• Often seen sharing a plant with their congeneric species: *C. mucronatus* and *C. novogranadensis*, as well as with *Crematogaster, Paratrechina longicornis, Cephalotes*, and *P. gracilis*.

**Camponotus novogranadensis**

• They do not actively patrol the plant and are usually solitary ants (1-2 per plant). They remain (hide) amongst other gregarious ant species. Very agile and quick. When under threat (trying to capture them) they quickly dissociate from the other ants and escape. They are seen foraging nectar, but are inefficient patrollers, recruiters or attackers, which display evasive behaviours when plants are disturbed and hence considered as parasitic species. Their occupation patterns are infrequent and erratic, and are always seen sharing a host plant, suggesting no territoriality.

**Camponotus mucronatus**

• These ants are not active patrollers and are usually seen standing immobile on the underside of petioles and leaf blades.
• Not alert or responsive towards plant disturbance, whose main attacking mode seems to be spraying, although seldom seen recoiling the gaster and pointing the rear end with the acidopore towards the attack target. Do not attack biologists.
• Solitary ants, usually one per branch or apex, or even one per plant. Very infrequent patrollers across sites and throughout years. Observed almost exclusively on site C, and occasionally site B. Usually located in a few areas per year, but highly unpredictable suggesting they are not territorial.
• Not seen foraging on nectar and rarely found inside the flowers.
• When they feel under attack, these ants head towards the underside of the leaves and drop themselves off the plant. They display this behaviour much more often than *C. planatus* and so were considered very shy ants.

• They often share a host plant with *Brachymyrmex* and *C. planatus*.

*Brachymyrmex* sp.

• Highly aggressive ants actively patrolling through branches, they climb up the stem and look for nectar in EFN associated with buds, flowers, and fruits. Seen on both leaf blade sides (upper side and underside).

• Gregarious patrolling, with several ants per branch or apex. They consume floral and extrafloral nectar.

• Efficient recruiters with very small response times, capable of recruiting over 30 ants in <120 sec. Although they have an acidopore, these ants were rarely seen recoiling their abdomens against the larvae or other herbivores in the typical spraying behaviour. Their main attacking mode is biting and directly harassing larvae. Docile to biologists, no human attacks recorded.

• They were observed inside the flowers deterring *Apis mellifera*: one ant was enough to prevent the bee from entering the flower, and causing it to overfly the flower in circles. When the ant detected the pollinator, it started patrolling towards the edge of the inner petal, and the bee stopped overflying and flew away (Xochitl Damián pers. obs. 19 Nov. 2014).

• These ants are exclusive patrollers, and do not share a host plant with other plant species. During the direct conflict experiment when we planted ant corpses from different species inside flowers, In plants patrolled by *Brachymyrmex* ants, these ants often removed the experimental ant corpses from other species placed by us inside the flowers to test direct ant-pollinator conflict (Chapter 3). Consequently,
these ants seem to be highly territorial, since they can reliably by found patrolling the same plants over several days or weeks.

- Although their location changes between years, their abundance seems constant over years.

**Monomorium ebenium**

- These ants seem to be parasitic, nectar thieves as were never observed patrolling, recruiting or attacking. Usually observed directly on the nectaries – floral and extrafloral. Their abundance fluctuates greatly between years, but when abundant, they are commonly spotted inside flowers, at the base of the corolla, consuming floral nectar from the junction between petals where floral nectaries are located.
- Small, gregarious ants, always seen in large groups (6-12 or more). Perhaps the ant with the smallest body size (<2 mm total length).
- They do not interfere with pollinator visitation. *Apis mellifera* and other native bees will visit the flowers *M. ebenimum* ants inside.
- Observed sharing a host plant or branch with *Paratrechina longicornis* and *Brachymyrmex*; but not with *C. planatus*.

**Cephalotes**

- These ants are not active patrollers and are usually seen standing immobile on the leaf blades.
- Not alert or responsive towards plant disturbance. Not aggressive, seen patrolling but rarely recruiting and never seen attacking or repelling any herbivore. They do not attack biologists.
- Solitary ants, usually one per branch or apex, or even one per plant. Very infrequent patrollers across sites and throughout years. Usually located in a few areas per year, but highly unpredictable suggesting they are not territorial.
• Rarely seen foraging on extrafloral nectar and almost never found inside the flowers.

• This ant species has the prototypical evasive behaviour: when detecting any sort of plant disturbance they will move away from it, without engaging with it at all.

• When they feel under attack, these ants lower themselves onto the leaves, by slightly flattening themselves, presumably through bending their legs in order to get a better grip onto the leaf blade. And sometimes head towards the underside of the leaves, where they also ground themselves to the leaf blade.

• Observed sharing a host plant with *M. ebenium*, *Crematogaster* sp., *C. planatus*, and *D. bicolor*.

• On a personal note, these were by far my personal favourite ant species, and the same opinion was shared almost by consensus with my fellow colleagues from the LIPA Lab in Mexico. Nicknamed ‘tanquecito’ due to its robust appearance and metallic dark grey colour, resembling a wee war tank. Under the microscope these ants are also amazing! Honouring even more their nickname, with the exoskeleton covered in spines and their thick plates accentuated. I was utterly disappointed to find out that this charismatic species is a well-known parasitic genus in facultative ant-plant mutualisms (Byk and Del-Claro 2010).

•

*Crematogaster*

• Actively patrolling the apex of the plants, especially those bearing mature fruits. Rarely seen inside the flowers, but frequently observed harvesting extrafloral nectar and seeds. This species has been associated with seed dispersion, seen collecting the seeds as soon as the fruit dehisces, harvesting seeds and bringing them down to the ground, and removing the elaiosome enabling the seed to germinate. Gregarious patrolling, usually 5-10 ants per apex and many more (30+) swarming around a
newly dehisced fruit. They are very quick at detecting dehisced fruits, usually faster than biologists and fruits must be bagged or we are outran by these ants.

- Their attacking mode mixes bites and also spraying through their acidopore. These ants recoil the gaster, curving their abdomen forward, underneath their thorax, pointing the rear end with the acidopore towards the attack target.

- Their abundance fluctuates between years, but these ants are reliably found within the same area throughout weeks or a whole field season, indicating territoriality.

- Observed sharing a host plant with *Cephalotes* ants.

**Pseudomyrmex gracilis**

- Only seen in specific areas within a dune site (Parche C). These ants seem to be opportunistic patrollers and show a very erratic plant occupation pattern. Ants may be found patrolling a few neighbouring plants for two or three consecutive days at most, but then will not be found in those or other plants for several days. They tend to occupy a very low number of plants, in fact, rarely more than four. They are lone patrollers, one ant per plant. They are usually seen patrolling the flowers, swiftly dashing in and out of them, hunting for thrips or beetles (Staphylinoidea) inside the flowers. Not seen consuming floral or extrafloral nectar, not seen either deterring pollinators or any other visitor. We are unsure about their status as defenders, commensals or parasitic. Species within this genus, including this species, are often obligate mutualists of ant-plants, nesting within the plant and displaying very aggressive behaviours towards any plant visitor (including humans). However, they were never observed displaying aggressive behaviours on *T. velutina* and were always rather shy, escaping the plant after the least disturbance. They are very infrequent and have remained infrequent throughout years.

- Often seen sharing a host plant with *C. planatus*. 
Beetles

Beetles in *T. velutina*'s flowers at Troncones, Guerrero were observed usually in paired numbers ranging from 1 to 10 beetles, with a mean abundance of $3.18 \pm 0.39$ beetles per flower. Beetles were observed engaging in several behaviours: piercing the base of the corolla and stealing floral nectar, walking constantly around the inner petal surface, sometimes climbing up the reproductive organs (often the anthers), and mating inside the flowers. As they walked around, beetles left visible traces in the inner surface of the petals, especially when they walked whilst mating. These petal marks could be used in further studies to trace the presence and behaviour of beetles in flowers, even if they are not directly observed.

Beetles spent very long periods inside the flowers with often the same individuals being there before our observation round started, and remaining there until the end of our observation round (range: 30 sec – 20 min).

Preliminary analyses show a positive correlation between the numbers of beetles and the numbers of ants. This could imply that ants are either recruited to deter beetles, or that ants are attracted to flowers with beetles. Based on observations of ants using the corolla holes created by beetles to drink floral nectar from outside the corolla, I think there may be potential for a facilitation loop between ants and beetles, where beetles open the flowers and ants later use these pre-made holes to access floral nectar. Since ants were rarely observed inside flowers and were never observed consuming floral nectar, I hypothesise *T. velutina* flowers may have ant-repelling compounds to deter ants from inside the corolla, which also prevent ants from consuming floral nectar from inside the corolla. However, floral nectar does not seem to be unpalatable to ants, although further experiments outwith the scope of this thesis are required to unravel these dynamics.
PUBLICATIONS ARISING FROM THIS THESIS
Las dunas costeras de Troncones, Guerrero, se encuentran 284 km al oeste de Acapulco y albergan un amplio repertorio de insectos nativos que polinizan la planta Turneria velutina, una prima del sabroso maracuyá a la que comencé a estudiar durante la carrera de biología en la UNAM. A pesar de su enorme valor biológico, Troncones está en grave peligro: dos letreros que enmarcan la región indican que “se vende”. Las dunas costeras son un ecosistema que, como su nombre lo indica, se localiza a unos cuantos pasos del mar. Como el suelo está formado principalmente de arena, la lluvia se filtra con rapidez al subsuelo, lo que reseca el terreno y hace del agua un recurso escaso, a pesar de que se ubican en regiones lluviosas y tropicales. Uno puede distinguir dónde comienza el ecosistema de las dunas costeras y termina la playa porque en él la arena está sujeta por las raíces de la vegetación, mientras que en la playa un viento intenso y persistente podría levantar una nube calante de arena que amenaza con cubrir cosas valiosas. Gracias a la cobertura vegetal, los cordones o crestas de las dunas costeras se estabilizan y éstas dejan de ser transformadas por la acción del viento, a diferencia de los caprichosos montículos de arena donde los vacacionistas se asolean.


Las primeras colonizadoras de estos cordones son plantas rastreras, como las del género Ipomoea, al que pertenece el camote. Poco a poco, los tímidos listones de camotes reclutan otras plantas sin leño (herbáceas y pastos) y luego arbustos, hasta formar pastizales que recubren por completo la arena. Los arbustos se reúnen en núcleos de matorrales que se enriquecen con más y más especies, avanzando tierra adentro hasta formar una selva baja conforme las dunas se alejan del mar. Hasta hace muy poco, el primero de marzo de 2018 para ser precisos, no existía ley o regulación alguna que protegiera estos ecosistemas costeros en la reputada Ley General del Equilibrio Ecológico y la Protección al Ambiente (LGEEPA). A pesar de ser un país con 808,711 hectáreas de dunas costeras las únicas legislaciones que teníamos con respecto a los desarrollos en playas mexicanas eran seis criterios contradictorios en varios aspectos y soslayados en una legislación menor, la Norma Oficial Mexicana (NOM).

En marzo de 2018 el Congreso finalmente aprobó la petición para incluir en el artículo 28 de la LGEEPA el concepto de “ecosistemas costeros”, como los manglares, humedales, franjas intermareales, dunas costeras, lagoones costeras, macroalgas, arrecifes de coral, pastos marinos, fondos marinos o bentos y las costas rocosas. Dado que estos espacios figuran entre los más lucrativos para el desarrollo turístico, es difícil creer que esta laguna en la legislación sea un descuido producido por la sobrecarga laboral de la Secretaría de Medio Ambiente y Recursos Naturales.
Algunos legisladores, en su rol como activistas, van más allá de las presiones políticas y de la visión mercantilista de las subastas de tierras costeras. En lugar de eso, buscan formas de conservar y preservar estos espacios naturales, incluso si ello significara una disminución en las ganancias económicas a corto plazo. La ley de Troncones fue aprobada por el Consejo de Estado en México, y hoy en día es un referente en el campo de la protección de la biodiversidad.

En Troncones, Quebrada, con los terrenos que pertenecen a la empresa, las dunas costeras están consideradas como una especie de santuario natural. La idea principal detrás de esta ley es proteger este ecosistema, que en el futuro podría ser declarado como un parque nacional. Entre los principales beneficios de esta ley, se destacan:

1. Protección de la biodiversidad: La ley busca preservar la gran variedad de plantas y animales que habitan en estas costas. Es especialmente relevante para las criaturas que dependen de las dunas costeras para su supervivencia.
2. Protección de la cultura: La ley también busca preservar la cultura y el patrimonio histórico de la zona, que incluye la arquitectura típica y las tradiciones locales.
3. Promoción del turismo sostenible: La ley busca promover el turismo de ecoturismo, que se basa en la conservación del medio ambiente y el respeto por la cultura local.

En conclusión, la ley de Troncones es un ejemplo de cómo los legisladores pueden trabajar para proteger la naturaleza y la cultura de un área, en lugar de simplemente permitir su explotación sin considerar los posibles costos a largo plazo. Es un escenario similar al de otros lugares en México, donde se están discutiendo propuestas de ley para proteger la biodiversidad costera.

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Ant-Pollinator Conflict Results in Pollinator Deterrence but no Nectar Trade-Offs

Villamil N, Boege K and Stone GN

INTRODUCTION

Extrafloral nectaries, domatia, and food bodies are all means by which ant-plants (comprising myrmecophytes and myrmecochytes) reward ants and support ants by providing nectar or food (Blaney and Overmeer, 2007; Román et al., 2009). In return, ants attack herbivores, prune climbing vines and prevent fungal and microbial infection, or protect flowers (Blaney and Overmeer, 2007; Román et al., 2009). The mutualistic interaction is usual myrmecophily.

Castration is an extreme example of direct ant-pollinator conflict, which reduces male reproductive success by preventing pollen transfer. Although this behavior is rare among plants, it has been documented in a few Mexican endemics. In particular, Turnera velutina, a myrmecophile, rewards ants with extrafloral nectar, but also causes an extreme example of direct ant-pollinator conflict (Blaney and Overmeer, 2007; Román et al., 2009). In this plant, the presence of ants in flowers alters pollinator behavior and reduced visitation, although visit frequency was unchanged. We found evidence for indirect conflict, with no significant difference in the volume or sugar content of floral nectar between control plants and those in which ant presence was prevented. The presence of ants on flowers alters pollinator behavior, which is likely to affect ant-pollinator dynamics, though there is no apparent trade-off between plant investment in ant rewards for pollinators and ant guards. Further studies are required to quantify the effect of the structural abundance of ants on flowers on pollinator behavior, and any associated impacts on plant reproductive success.

Keywords: Turnera velutina, ant, plant, food body, extrafloral nectar, ant-plants, conflict, myrmecophily, reward allocation, indirect interaction, myrmecophily.

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addressed the following specific questions: (i) What are the daily timings of nectar reward secretion, ant activity, and floral visitation? (ii) Does the presence of patrolling ants deter pollinators from the flowers? (iii) Do ant species vary in their deterrence for pollinators? (iv) Are plants able to regenerate, in 20 μM plants. Nectar volume was measured using a digital caliper (Mitutoyo Digimatic) and sugar concentration was determined in Apis mellifera, pollinator in this l of deionized water using a 0–5 μl aliquot. Nectar volume was measured in a single flower (Minicaps Disposable capillaries, Hirschmann vapors from influencing pollinator behavior. Flowers containing dead ants were observed for 20 min, recording pollinator identity, visit frequency and duration, and nectar and pollen behaviors. Observations were conducted during October-November 2016 with four simultaneous observations of data (from different plants).

When assessing the effects of ants inside the flowers on pollinator visitation we only considered visits by Apis mellifera, since this is the dominant T. velutina pollinator in the population (Gessner et al., 2016) and accounted for 91% of the visits in this experiment. We identified hoverfly behaviors as "inspection" or "contact." Inspection behaviors comprised either approaching or hovering over a particular flower without landing. Contact behaviors were those that occurred inside the flowers between landing and take-off and comprisedาง hugging on pollen or nectar means or standing on the petals, siliques or stigma.

Indirect Conflict To test possible trade-offs in plant resources between FN and EFN we conducted a greenhouse experiment on 72 plants, with a substrate of local soil (50% sand and 50% watered every other day (for nesting Apis mellifera, 2015). During the experiment, plants were watered every night, and detached nectaries were sprayed with water to wash away any nectar secretions from previous days and to prevent fungal infections from the flowers. The ant colonies were tasked only to forage for FN. The ant colonies were assessed in a shadehouse at CICOLMA. The extrafloral nectar is provided in paired...
**Statistical Methods**

All statistical analyses were conducted in R version 3.5.2 (R Core Team 2018). All mixed effects models were fitted using the "lme4" R package (Bates et al., 2015) and post hoc Tukey comparisons were fitted using the "multcomp" R package (Hothorn et al., 2008) unless stated otherwise.

**Mutualist Activity and Reward Secretion Curves**

To test whether ant or pollinator activity changed over daily time, we fitted a Poisson mixed model with either the number of inspection events or the number of floral-visits as the response variable. We fitted day as a fixed effect, with linear and quadratic terms to detect non-linear activity patterns overtime. The number of flowers per site was fitted as a logarithm-transformed offset to control for floral density, since we recorded visitor counts per site rather than counts per individual flower (see fieldwork methods). Flowers of *T. velutina* last for a single day, and because multiple flowers were sampled on a given site on a given day, we fitted site identity as a random effect to account for differences between sites and day variances in visitability that could enhance or reduce visitation, such as resource availability, visit diversity, or the abundance/availability of ant nests. We also included an observation-level random effect (sugar content) where such data point received a unique level of a random effect to control for non-independence (Diniz-Silva, 1992).

To test if EFN and PEN secretion changed over the anthesis period we fitted a Poisson mixed model with day (as a factor) and ant species (as a fixed effect) to test for differences in visitation behaviors. As random effects we fitted flower identity, and plant-day identity to independently explain variation in both nectar volume and sugar content.

**Timing of Daily Activity and Secretion Peaks**

We compared the time at which each mutualist activity reached its maximum by calculating the time at which the slope of the fitted model with respect to time was equal to zero and then solving for time, as follows:

\[ \beta_0 + \beta_1 \times \text{time} = 0 \]

**Direct Conflict**

The effect of different ant species on the visitation frequencies was tested using Poisson mixed effects models. We fitted the number of visitors as the response variable, and ant species as the predictor. For each plant, we estimated the difference in FN production before and after the extrafloral nectaries were clogged as follows:

\[ \text{Pre}_F N = \text{FN production before the extrafloral nectaries secretion was prevented} \]

\[ \Delta FN = \text{FN production after the extrafloral nectaries secretion was prevented} \]

To test if PEN and EFN secretion changed over the anthesis period we fitted a Poisson mixed model with day (as a factor) and ant species (as a fixed effect) to test for differences in visitation behaviors. As random effects we fitted flower identity, and plant-day identity to independently explain variation in both nectar volume and sugar content.

**RESULTS**

**Mutualist Activity, Reward Secretion, and Polen Deposition Curves**

Activity curves show that both pollinators and ants were most active within the first 2h post-anthesis (Figure 2A), although the variation peak by potential pollinators predicted from model estimates was on average even 4h before the preditive peak for ant activity (from post-anthesis for potential pollinators, Wright et al. 2015 for ant activity (Table 1)).

On average, a flower and its associated leaf secreted a total of 2,605 ± 197 μg of sugar via floral and extrafloral nectaries throughout the 4.5h anthesis period. The total sugar content in PEN was 295 ± 153 μg of sugar, while total extrafloral sugar was 2,310 ± 705 μg. Thus, the relative sugar contributions of floral and extrafloral nectaries for leaf flowers were 5.3 and 96.7% respectively. When the embryos are mature, 0.5% of the leaf's total sugar is used in storage and 99.5% is used for ovule development, and so the variation in nectar duration is limited.
not necessarily that for the constraints that harvest each resource. Pollen deposition occurred at the beginning of anthesis and steadily declined over time (Figure 2C). Hence, pollen deposition data were analysed using a linear model without blocking hour as a squared term and we did not examine timing of daily maxima using model diagnostics (Table 1).

We recorded 1,535 ant visitors from nine ant species patrolling extrafloral nectaries of T. velutina at CICOSOA (Table 2). Dorymyrmex bicolor, Paratrechina longicornis, and Brachymyrmex spp. accounted for 66.5% of the total ants observed, and 73.8% of the patrolling ants, after excluding Ameiomyrme species that received or observed displaying guarding behaviors on T. velutina and are mostly parasitic associations of FN and RBN (entomophagous) (Osterheld, 1998; Bolton, 1987).

**Direct Conflict**

We counted FN fluidizations, of which 467 (30.5%) were by the honeybee, Apis mellifera. Of the remaining 8.5%, 61 visits were by butterflies (Lepidoptera), 11 by Diptera, and one by a beetle (Coleoptera) and one by a wasp (Hymenoptera) (Figure 3A).

Neither the presence of ants inside flowers nor their identity had an affect on the number of honeybees visiting the flowers (Figure 3A, Table 3). The presence of the most aggressive ant species, Dorymyrmex bicolor, increased the likelihood of a pollinator displaying inspection behavior by 20% (Figure 3B, and increased by 13% the proportion of time per visit spent inspecting inspection behavior rather than foraging or pollinating the flower (Figure 3C). Finally, the presence of Dorymyrmex bicolor and Brachymyrmex longicornis inside the flowers halved the duration of contact visits, increased control treatment FN secretion after consumption (Pacini et al., 2003; Pacini and Nepi, 2007; Pacini et al., 2010). We suggest that rapid resupply is crucial in short-lived flowers, such as the one-day flowers of Turnera spp. placed inside flowers. Further studies are required to test (i) the effect of having guarding ants on pollinator visitation, and (ii) the impact of any such effects on plant fitness. The latter are crucial to understanding whether ants are ongoing.

**Indirect Conflict**

Clogging extrafloral nectaries on the leaves associated with mutualists that harvest each resource. Hence, FN volume (Table 2) and sugar content (Table 3) were significantly lower in plants under both control and clogged treatments (Figure 4).

**DISCUSSION**

Our findings show that both ants and pollinators are active while flowers are open (Figure 2B), but FN and EFN are simultaneously secreted (Figure 2A), and that pollen deposition occurred when ants are actually patrolling (Figure 2C). Consequently, T. velutina guarding ants and pollinators operate in close spatial and temporal proximity, implying that both direct and indirect conflict could exist in this system. We found, however, no evidence for indirect, nest-mediated, conflict between ants and pollinators, since plants did not allocate resources towards floral nectar, even when 91% of the sugar investment during anthesis, which is in EFN, is prevented (Figure 2). We found evidence for direct conflict, as the presence of dead individuals of patrolling ant species inside flowers was associated with both higher frequency of inspection behaviors in potential pollinators, and reduced visit duration (time spent inside flowers) (Figure 3). Taken together, these effects could have increased handling time per flower and reduced pollinator foraging efficiency. Nonetheless, this could not be observed under an experimental setting using dead ants placed inside flowers. Further studies are required to test (i) the effect of having guarding ants on pollinator visitation, and (ii) the impact of any such effects on plant fitness. The latter are crucial to understanding whether ants are ongoing.

**Figures**

Figure 2: Changes in floral visitors after ants were introduced into T. velutina flowers. Data is presented as the proportion of time spent in each activity, and significance by Fisher’s exact test. A: Total visitor frequency (V), B: Pollen deposition (P), C: Durations of contact visits (D), and D: Frequency of inspector visits (F).

Figure 3: Changes in floral visitors after ants were introduced into T. velutina flowers. Data is presented as the proportion of time spent in each activity, and significance by Fisher’s exact test. A: Total visitor frequency (V), B: Pollen deposition (P), C: Durations of contact visits (D), and D: Frequency of inspector visits (F).

**Table 2**

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<td>0.03</td>
<td>Pseudomyrmicinae</td>
</tr>
<tr>
<td></td>
<td>P. flavescens var.</td>
<td>0.2</td>
<td>0.02</td>
<td>Pseudomyrmicinae</td>
</tr>
<tr>
<td></td>
<td>P. flavescens var.</td>
<td>0.1</td>
<td>0.01</td>
<td>Pseudomyrmicinae</td>
</tr>
<tr>
<td></td>
<td>P. flavescens var.</td>
<td>0.1</td>
<td>0.01</td>
<td>Pseudomyrmicinae</td>
</tr>
</tbody>
</table>
Villamil et al. Ant-Pollinator Conflict: Deterrence but no Nectar Trade-Offs

### Pollinator Deterrence?

<table>
<thead>
<tr>
<th>Response</th>
<th>Distribution</th>
<th>Fixed effects</th>
<th>H</th>
<th>UFI</th>
<th>P value</th>
<th>Random effects</th>
<th>Variance SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-recipe &amp; post-recipe</td>
<td>Gas toilet</td>
<td>Chewing treatment</td>
<td>9C</td>
<td>0.091</td>
<td>0.0007</td>
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Although some studies have documented variation among ant species in their ability to deter pollinators (Fabaceae; Nicklen and Wagner, 2006), they have not always been consistent. For example, in Fabaceae (Martínez-Bauer et al., 2015) and Malpighiaceae (Assunção et al., 2014), ant species in the genus *Turnera* induce a strong ant-repellent floral volatiles treatment (Figure 2A), while *Heteropterys* species induce a weak ant-repellent floral volatiles treatment (Figure 3A). In both cases, ants feeding on the control flowers (Figure 3B) had no effect on pollinator visitation, while ants feeding on the treatment (T) plants significantly decreased the duration of pollinator visits. This suggests that the level of ant aggressivity toward herbivores correlates positively with the ecological costs on pollination via pollinator deterrence. However, whether the level of ant aggressivity toward herbivores correlates positively with the ecological costs on pollination via pollinator deterrence remains unknown (Ness, 2006; Miller, 2007; Miller et al., 2014; Ohm and Miller, 2014), and should be tested further.

### Indirect Conflict

The effect of clogging on nectar secretion and sugar re-allocation in *Turnera velutina* flowers has been reported for facultative ants associated with many ant species simultaneously (*Stilpnoechidium* species (Nikolai and Wagner, 2006), *Amanox reepolii* (Amanox scottensis, *Rhabarbarum*; Ove; Raine et al., 2014), and *Heteropterys* species (Malpihiaceae; Assunção et al., 2014)). Our results show that *Turnera* species induce a strong ant-repellent floral volatiles treatment (Figure 2A), while *Heteropterys* species induce a weak ant-repellent floral volatiles treatment (Figure 3A). In both cases, ants feeding on the control flowers (Figure 3B) had no effect on pollinator visitation, while ants feeding on the treatment (T) plants significantly decreased the duration of pollinator visits. This suggests that the level of ant aggressivity toward herbivores correlates positively with the ecological costs on pollination via pollinator deterrence. However, whether the level of ant aggressivity toward herbivores correlates positively with the ecological costs on pollination via pollinator deterrence remains unknown (Ness, 2006; Miller, 2007; Miller et al., 2014; Ohm and Miller, 2014), and should be tested further.

We conclude that there is no indirect nectar-mediated conflict between guarding ants and pollinators in *Turnera* species, although ant-repellent floral volatiles treatment (Figure 3A) and sugar re-allocation (Table 1) were observed in *Turnera* species. Our experimental approach found no evidence for a trade-off in sugar investment in extrafloral and floral nectar in *Turnera* species. However, our results do not rule out replenishment after emptying in the morning, and from reshielding later in the day (Kearns and Inouye, 1993).

### Does Herbivore Deterrence Match Pollinator Deterrence?

Although some studies have documented variation among ant species in their ability to deter pollinators (Fabaceae; Nicklen and Wagner, 2006), they have not always been consistent. For example, in Fabaceae (Martínez-Bauer et al., 2015) and Malpighiaceae (Assunção et al., 2014), ant species in the genus *Turnera* induce a strong ant-repellent floral volatiles treatment (Figure 2A), while *Heteropterys* species induce a weak ant-repellent floral volatiles treatment (Figure 3A). In both cases, ants feeding on the control flowers (Figure 3B) had no effect on pollinator visitation, while ants feeding on the treatment (T) plants significantly decreased the duration of pollinator visits. This suggests that the level of ant aggressivity toward herbivores correlates positively with the ecological costs on pollination via pollinator deterrence. However, whether the level of ant aggressivity toward herbivores correlates positively with the ecological costs on pollination via pollinator deterrence remains unknown (Ness, 2006; Miller, 2007; Miller et al., 2014; Ohm and Miller, 2014), and should be tested further.

We found two previous studies testing indirect, nectar-mediated ant-pollinator conflict by quantifying sugary rewards...
suggests that plants may experience fewer investment trade-offs among different functional traits than previously assumed.

CONCLUSIONS

Our new knowledge about trade-offs between extrafloral and floral nectar rewards, in which traits are not independent in our models, indicates that some traits may trade off. Our finding that many traits do not trade off suggests that selection against non-selected trait combinations may be an evolutionarily relevant process. The observed trade-offs between extrafloral and floral nectar rewards may be more complex than previously thought, as it is possible that trade-offs can be difficult to detect due to the presence of trade-offs between other traits. For example, trade-offs between floral and extrafloral nectar rewards may be more complex than previously thought, as it is possible that trade-offs can be difficult to detect due to the presence of trade-offs between other traits. For example, trade-offs between floral and extrafloral nectar rewards may be more complex than previously thought, as it is possible that trade-offs can be difficult to detect due to the presence of trade-offs between other traits.

In conclusion, our findings provide new insights into the ecology and evolution of plant-ant interactions, and suggest that these relationships may be more complex than previously thought. Future studies should focus on identifying the mechanisms underlying these trade-offs, and investigating the ecological and evolutionary implications of these findings.
Villamil et al.

Ant-Pollinator Conflict: Deterrence but no Nectar Trade-Offs


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Villamil et al.

Conflict of Interest Statement. This article declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.