DAVIS EXPEDITION FUND

REPORT ON EXPEDITION / PROJECT

Expedition/Project Title: FULL REPORT: Ant-pollinator interactions in ant-plants. Ecological costs of myrmecophily and its evolutionary consequences for host plant reproduction.

Travel Dates: 24 August 2016 – 9 January 2017

Location: Troncones, Guerrero; La Mancha, Veracruz, Mexico City, MEXICO

Group Members: Nora Villamil Buenrostro

Aims: To compare how native and introduced (*Apis mellifera*) pollinators vary in their interactions with the ant assemblage visiting the ant-plant *Turnera velutina* (Passifloraceae)

1

2016 EXPEDITION REPORT
Ant-pollinator interactions in ant-plants

_____

Ecological costs of myrmecophily and its evolutionary consequences for host plant pollination

Nora Villamil Buenrostro
The University of Edinburgh

The 2016 fieldwork expedition to Mexico was originally planned as a 10-week expedition from 20 August – 30 October 2016 at Troncones, Guerrero, and consisted of 6 weeks of fieldwork, 1 week of logistic arrangements, and 3 weeks of sample processing. Yet, due to the generous award by the Davis Expedition Fund (£4379), I was able to extend this into a 15-week expedition from 23 August 2016 – 12 January 2017, comprising:

- o 1.5 weeks of fieldwork arrangements
- o 4 weeks of fieldwork at Troncones, Guerrero
- o 2 weeks of sample processing and La Mancha fieldwork arrangements
- o 4.5 weeks of fieldwork at La Mancha, Veracruz
- o 3 weeks of sample processing in Mexico City

All the funds granted were used for the fieldwork extension and chemical analyses of floral and extrafloral nectar samples. Please see further details of predicted and actual costs provided below in Table 1.

	Item	Cost predicted (£)	Actual cost (£)	Contributions Provider (£)	Total Davis (£)
TRAVEL	**UK-Mexico return flight**	1059	1560		1560
	Transport to Troncones (Flights and taxis)	630	350		300
	Transport to La Mancha (Buses and taxis)	Additional fieldwork	200		200
				Travel Sub-Total	**2060**
SUBSISTEN	**Accommodation: Troncones**	2100	1500		720
	Food: Troncones	540	270		270

	Accommodation: La Mancha (Triple room rate)	Additional fieldwork	300			208
	Food: Troncones	Additional fieldwork	150			140
					Subsistence Sub-Total	1338
SUPPLIES	**Solvents for olfactory cues** (Dichloromethane and hexane)		120	-120	-120 (solvents were returned and money reimbursed)	0
	Nectar measuring equipment (Refractometers, callipers, microcapillaries, pipettes)		-800	-800	-800 (Nora Villamil and Stone lab)	0
	Other equipment (GPS, humidity and temperature sensors)		-250	-250	-250 (Nora Villamil and Stone lab)	0
	Disposables (Stationary, tags, jars, bags, ant excluding resin, Petri dishes)		50	50	-50 (Nora Villamil)	0
	Nectar chemical analyses (Chromatography and protein characterisation analyses)	Additional analyses	2000		-1020 (Prof. Karina Boege)	980
					Supplies Sub-Total	980
TOTAL PROJECT COSTS						4378
MONEY AWARDED						4379
REMAINING MONEY						1

Below I summarise the activities and experiments conducted, achievements and aims fulfilled, along with some key findings and observations. The work could not have been achieved without the help of five brilliant field assistants who volunteered for long hours: Rosa Isela Ríos and Alberto Bernal Herrera, both biology students, Adriana Fournier, a geography student, Andrés Iñigo, expert in Old Latin and colonial Mexican literature, and Aura Andrade Orloff, a former biology student and current philosopher and artist.

Achievements and activities

The overall aim of the expedition was achieved: I collected enough data to fully answer the main research question: How do interactions between different native pollinators and ants patrolling *Turnera velutina* vary from those observed when it is visited by an introduced and dominant pollinator- *Apis mellifera*? With this fieldwork expedition I successfully completed all fieldwork for my PhD project.

Apart from conducting fieldwork at Troncones, as mentioned in the proposal, I was also able to conduct additional fieldwork at La Mancha, Veracruz, where I had previously worked on ant-pollinator interactions in 2015. Thanks to the generous contribution of the Davis Expedition Fund I was able to collect data on ant-pollinator interactions in La Mancha where non-native European honeybees (*Apis mellifera*) compete with native species. I was also able to conduct some nectar chemistry analyses. These analyses will investigate the composition of floral and extrafloral nectar samples, particularly regarding differences in volatiles, sugars and amino acids in the nectar rewards for ants and pollinators. All these additional data increased the robustness of our findings and significantly enhanced this study.

Figure 1. Map of the locations where fieldwork was conducted. The Troncones, Guerrero community has a wide array of native pollinators including at least ten butterfly species, wasps, and native bees. In contrast, at La Mancha, Veracruz community the European honeybee (*Apis mellifera*) is the main and dominant pollinator.

In addition to planned activities, during fieldwork at La Mancha, my field assistant Rosa Isela Ríos (an undergraduate at UNAM, Mexico City) and I collected additional data on insect florivory of *T. velutina*. This exploratory work inspired Rosa's honours project, which I am jointly supervising along with Prof. Emer. Judith Márquez-Guzmán, who runs the Plant Development Lab at the Science Faculty, UNAM. Rosa's dissertation aims to understand how *Turnera velutina* defensive mechanisms and natural florivory rates change throughout floral reproductive development (*i.e.* across buds, flowers and fruits). The Davis expedition fund biography of Prof Davis says that part of his motivation for the fund was to help "broaden the experience and cultural horizons of young people". As an alumni of the Science Faculty at UNAM, a Mexican autonomous and free university, it also gave me great pleasure to help broaden the experience of a young student at UNAM. Thank you Davis Expedition Fund for helping me make this possible.

Aims and methods

Most of the planned aims were fulfilled and new ones were added, although some experimental designs had to be modified. Please find below a list of the aims proposed, achieved, or not, and additional aims. For each aim, I summarise the methods and experimental designs conducted or modified to fulfil it, or an explanation of why it was not fulfilled.

Planned and fulfilled aims:

1. Quantify pollinator visitation and fitness in plants with and without ants (i.e. with ants excluded).
   - We set up an ant exclusion experiment in plants colonised by each different ant species. Using Tanglefoot resin we excluded the ants from some branches, and marked similarly sized branches as controls. Pollinator visitation rates were assessed through direct observations of flowers in anthesis in plants colonised by each different ant species. Fruits from those flowers were collected to estimate fitness.

2. Evaluate direct ant-pollinator conflict by assessing the effect of ant patrolling on pollinator visitation and outcrossing rates.
   - We set up an ant exclusion experiment, excluding ants from the whole plant, and using similarly sized plants as control.
       i. Pollinator visitation rates were assessed through direct observations of flowers in anthesis.
       ii. Outcrossing rates were determined using different anther dyes for a central and various satellite flowers in a focal plant, surrounded by neighbouring plants with undyed anthers (naturally yellow pollen). Stigmas from the focal flowers were fixed and coloured pollen grains will be counted to estimate selfing, geitonogamy, or outcrossing rates (Fig. 2).

3. Compare whether ants have a different effect on native pollinators relative to their effect on *Apis mellifera*, an introduced honeybee that is the dominant pollinator at La Mancha population.
   - I have already conducted these experiments on the *T. velutina* population at La Mancha, Veracruz during the 2015 field season and would now be able to contrast them. Honeybees are more aggressive than butterflies and native (usually stingless) bees, therefore, we expect native pollinators to be more susceptible to aggressive ants around the flowers.

4. Test whether pollinators are deterred by the presence of ants inside the flowers.
   - Ant corpses from one of the three main most aggressive ant species were placed inside three flowers of the same plant, and a fourth flower was left as a control, with no ant corpses. Pollinator visitation in these four flower treatments was registered.

5. Determine which floral organ (corollas, stigmas or anthers) contains ant repellents and quantify their effect on each of the different ant species.
   - Plants with at least four flowers were chosen and each flower was submitted to one of the following treatments: a) anthers removed, b) pistil removed, c)

anthers+pistil removed, d) control: intact. We recorded ant patrolling inside the flowers and pollinator visitation. Any additional flowers (+4) were removed to control for floral display.

6. Cafeteria experiments involving different floral organs were conducted to determine which floral organ (corollas, stigmas or anthers) contains ant repellents and their effect on each of the different ant species.
   - This will be tested in the field by exposing each ant species to artificial nectar in plastic arenas containing either a corolla without sexual organs, stigmas, or anthers. Ant preference and behaviour in this cafeteria essays will be recorded. Artificial extrafloral nectar will be prepared to simulate 'realistic' highly concentrated extrafloral nectar, within the range of natural variation displayed by this species (Villamil unpubl. data).

Additional aims fulfilled:
7. The aggressiveness in the seven most common and abundant ant species was assessed.
   - We assessed aggressiveness by inducing damage (naturally or artificially) to apical leaves of plants guarded by each of the ant species and recorded the response time, number and ant behaviour to estimate an aggressiveness index for each ant species.
       i. Artificial damage induced using forceps and tweezers to cut and pierce leaves.
       ii. Natural damage induced by placing the natural herbivore *Euptoieta hegesia* (Lepidoptera) larvae with similar head capsule widths on apical leaves.

8. Ant and native pollinator activity patterns and peak activity hours were characterised.
   - Ten apical leaves from 90 plants bearing one bud, one flower and one fruit were surveyed every hour during 12 hours (0700-1900), recording the number of ants patrolling EFNs and ants and other floral visitors.

9. Levels of natural florivory in *Turnera velutina* were assessed in both populations.
   - We counted the number of damaged and undamaged flowers in several patches daily, during several days to obtain the mean percentage of flowers with florivory.

Planned but not fulfilled aims:
1. Test the effect of different ant species on pollen viability and seed set at Troncones.
   - Manual cross pollinations using pollen grains exposed to each of the ant species were planned to compare pollen viability and fitness with fitness values obtained from control pollen grains, not exposed to ants.
   - This aim could not be fulfilled since we found fewer reproductive plants in the Troncones population than we expected. Most of the plants available were being used in other experiments, and unfortunately we did not have enough additional plants to conduct this experiment.

2. Determine the visual or olfactory cues pollinators use to detect ants.
   - We planned to assess pollinator visitation in flowers from ant-excluded plants exposed to one of the following treatments: 1) odourless ant bodies (visual cues); 2) ant aroma extract addition (olfactory cues); 3) a frozen dead ant with its natural aroma (visual+olfactory cues); and 4) control flowers without ants or ant-aromas. Pollinator visitation in these four flower treatments would be quantified. Neither odour sequestration nor the experimental setup was successfully achieved and so this experiment could not be conducted properly. Instead, we placed frozen ant corpses from different species with different levels of aggressiveness inside flowers to test their effect on pollinator visitation (For further details see aim 4.)

Findings and observations

We found some important differences between the ecological communities in Troncones (Southern Pacific coastline) and La Mancha (Gulf of Mexico). A less diverse array of ant species patrolled *T. velutina* plants in Troncones, but this array did include the three most aggressive species. In Troncones, floral visitors comprise several guilds: nectar robbers, pollinators, and ant guards. Nectar robbers are mainly two beetle species: one Curculionidae and one from the Staphylinidae family. Only the Staphylinidae nectar robber is found at La Mancha, but is less abundant.
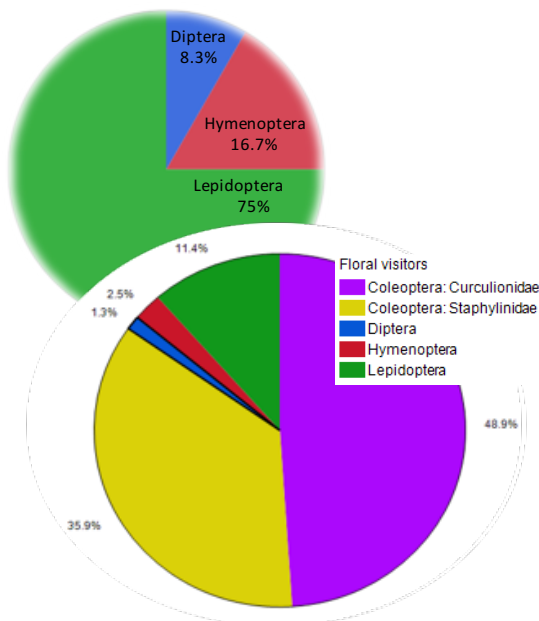

Figure 2. Composition of floral visitors (lower chart) and pollinators (top chart) found in *T. velutina* flowers at Troncones, Guerrero, Mexico.

Pollinators in Troncones include butterflies (Lepidoptera), flies (Diptera), wasps, and native bees (Hymenoptera). We recorded a high diversity of butterflies visiting the flowers in Troncones, with over 10 different morpho-species accounting for 75% of all pollinator visits. This is a striking difference with La Mancha where 94% of the floral visits are conducted by the European honeybee (*Apis mellifera*), an introduced and dominant pollinator, and where butterflies are rarely seen in the flowers and account for 1.7% of visits (Sosenski et al. 2016). Butterflies collect only floral nectar and contact both female and male sexual organs. Flies and wasps are interested in floral nectar only, whilst bees, native or honeybees, are interested in both nectar and pollen.

Although ants are also hymenopterans, ants inside the flowers are treated here as a different guild, and not regarded as pollinators because 1) ants are usually deficient pollinators (Rico-Gray and Oliveira 2007, Dutton and Frederickson 2012), and 2) ant guards inside flowers are mostly regarded as an ecological cost, since they may steal nectar and actively deter pollinators from the flowers.

In *T. velutina*, the number of overall floral visitors is not affected by the number of ants patrolling EFNs (R^2= 0.003, $F_{(1,84)}$= 0.2808, P= 0.5975, Fig. 3) and that may be because the most abundant floral visitors are nectar robbing beetles. Curculionidae and Staphylinidae beetles together account for 84.8% of floral visitors (Fig. 2) and nectar robber activity is not correlated at all with the number of ants patrolling EFNs (R^2= 0.0016, $F_{(1,84)}$= 0.1424, P= 0.706, Fig. 3). However, if we focus only on pollinators, ant patrolling EFNs in Troncones does affect the floral dynamics, since the number of ants in the extrafloral nectaries (EFN) showed a positive and significant correlation with the number of ants inside the flowers (R^2= 0.129, $F_{(1,84)}$= 12.50, P= 0.0007, Fig. 3). Furthermore, butterflies are negatively affected by the number of ants patrolling EFN (R^2= 0.054, $F_{(1,84)}$= 4.87, P= 0.029, Fig. 3), whilst dipterans and hymenopterans are not affected by ant patrolling (R^2= 0.033, $F_{(1,84)}$= 2.86, P= 0.094, Fig. 3) (see Table 1 for all statistical outputs of these correlations).

Our results show that ant guards patrolling EFNs may indeed bear an ecological cost for the native pollinators, butterflies, which account for 3/4 of pollinator visits. These results contrast with our previous findings on ant-pollinator dynamics regarding the introduced *Apis mellifera* in the western coast at La Mancha, where the number of European honeyebees visits is not affected by the number of ants patrolling EFNs. This differential response to guard ants may be related to behavioural differences in both pollinators: social bees and particularly honeybees are aggressive insects, whilst butterflies are rather shy and non-aggressive insects, and so may be more vulnerable to aggressive ant guards.
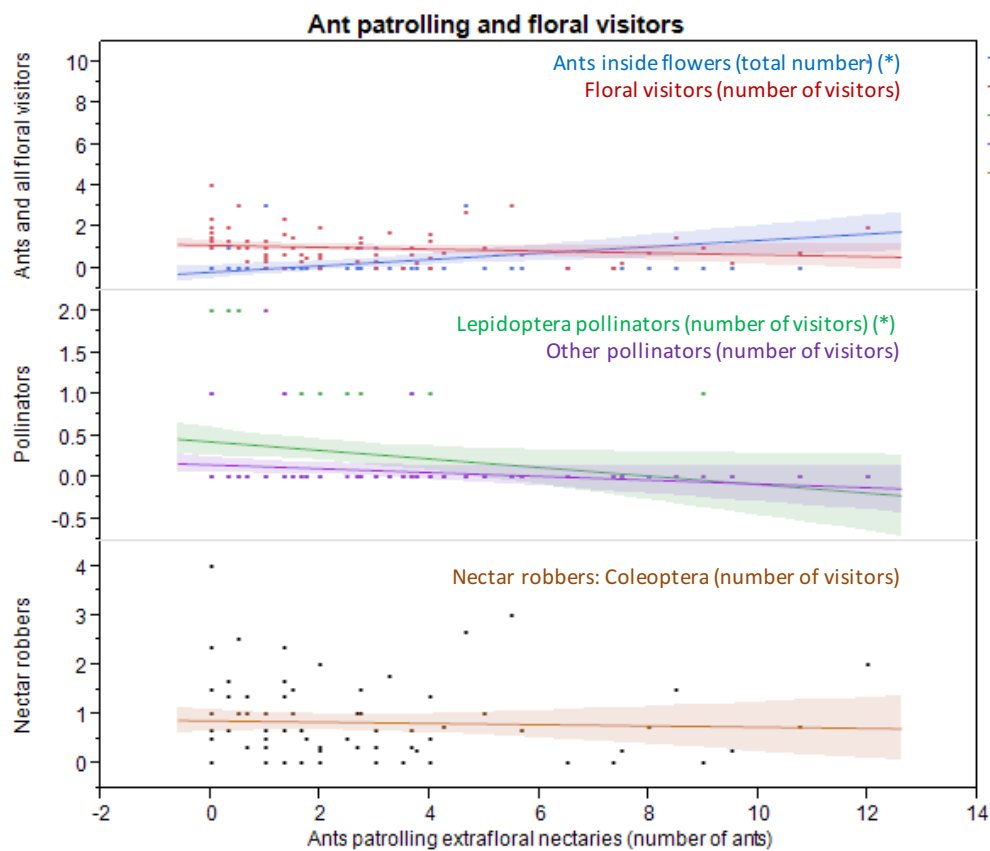

Figure 3. Correlations between the number of ants patrolling extrafloral nectaries and the number of different floral visitors (ant guards, pollinators or nectar robbers). The x and y axis depict the number of mutualists counted in each flower throughout all anthesis. Regressions lines and 95% confidence intervals (shading) are shown, and significant regressions are marked with an asterisk (*).

Interestingly, the number of ants in EFNs does not affect the number of coleopteran nectar thieves (R^2= 0.0016, $F_{(1,84)}$= 0.1424, P= 0.706, Fig. 3) and nectar thieves do not stimulate ant patrolling or recruitment to EFNs (R^2= 0.0019, $F_{(1,84)}$= 0.1654, P= 0.685, Fig. 4). However, nectar robbers do have an effect on other insect guilds visiting the flowers. Nectar robbers are negatively and significantly correlated with the number of butterflies visiting the flowers (R^2= 0.0727, $F_{(1,84)}$= 6.588, P= 0.012, Fig. 4), but not with other pollinators such as dipterans or hymenopterans (R^2= 0.0078, $F_{(1,84)}$= 0.6614, P= 0.4184, Fig. 4). Finally, the number of nectar thieves is positively correlated with the number of ants inside the flowers (R^2= 0.048, $F_{(1,84)}$= 4.23, P= 0.685, Fig. 4). Previous studies have shown that ants may reduce floral nectar robbers (O'Dowd 1979) or the number of florivorous beetles (Newman and Thomson 2005). The positive correlation we found between the number of beetle nectar thieves and the number of ants inside the flower could be explained in either of the following ways:

    I.   Ants may be recruited into the flowers to prevent robbing, defending floral nectar against beetles thieves. Previous studies have shown that ants may reduce floral nectar robbers (O'Dowd 1979) or the number of florivorous beetles (Newman and Thomson 2005).

    II.   Ants could be found in flowers with nectar robbers due to a facilitation loop between robbers and ant guards, where both act as nectar thieves hindering plant reproduction by stealing pollinators rewards. Positive feedback loops between antagonists and ants guards have been previously reported in ant-plants, and guard ants switch from a mutualistic to an antagonistic role due to the presence of a third species (Savage and Peterson, 2007).

Further experiments are required to disentangle whether this interesting observation is due to a defensive role of ants, or to a facilitative loop with antagonists.
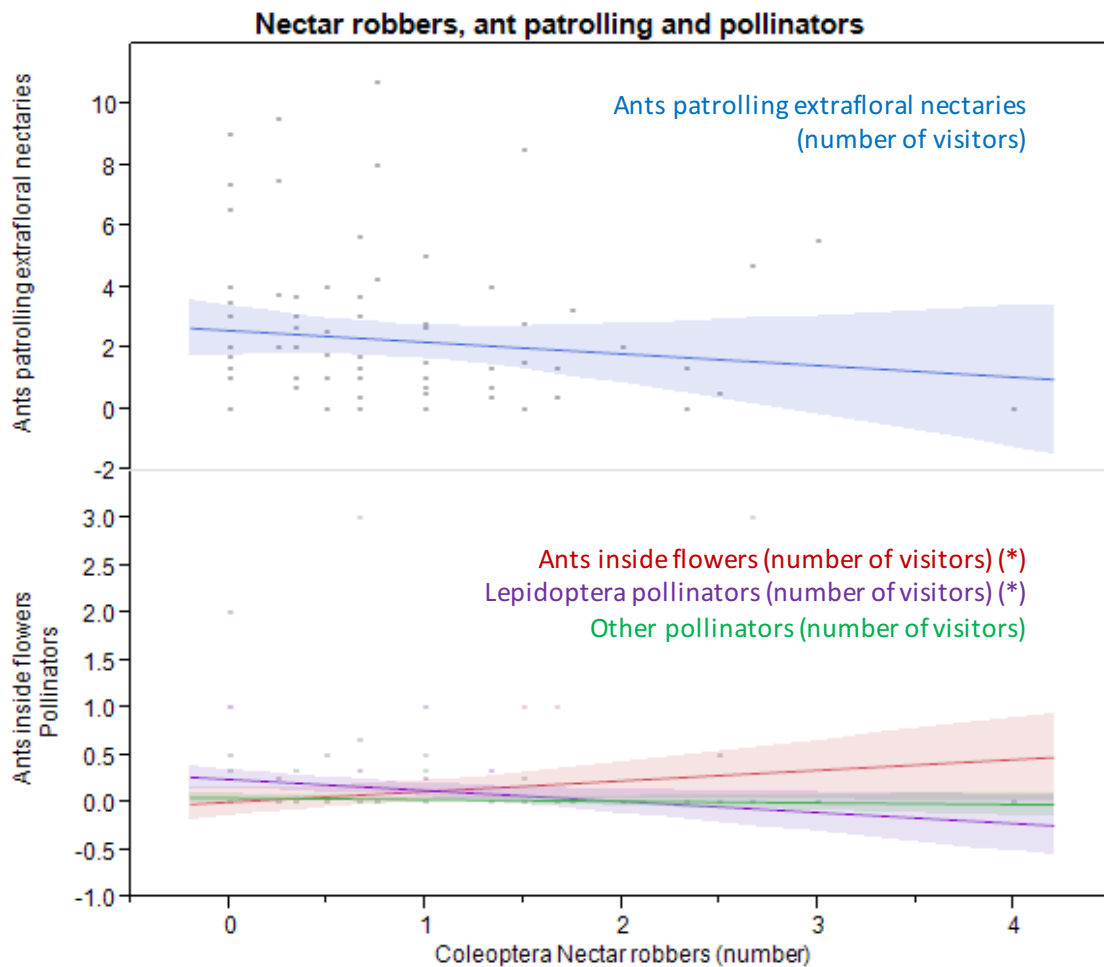
Figure 4. Correlations between the number of nectar robbers (Coleoptera: Curculionidae and Staphylinidae) and their effects on ants patrolling EFNs, ants inside flowers, and pollinator visitation of butterflies and other pollinators (dipterans and hymenopterans). The x and y axis depict the number of mutualists counted in each flower throughout all anthesis. Regressions lines and 95% confidence intervals (shading) are shown, and significant regressions are marked with an asterisk (*).

Native butterflies are the main pollinators in Troncones, but also seem to be the most sensitive visitor since butterfly visitation is negatively and significantly correlated with nectar thieves and high ant patrolling in EFNs (Figs. 3-4). Temporal segregation of pollinator and ant activities was observed in Troncones. Butterflies are active between 1000-1100 h, peaking at 1030 h, whilst almost no ants are seen patrolling until after flowers are closed (1230 h) and ant activity peaks in the afternoon between 1500-1600 h (Fig. 5). Butterfly activity peaks around 1030 am, when air temperature increases since lepidopterans activity is linked to air temperature. Butterflies usually alternate between flight and basking to thermoregulate, but spend longer periods in flight when air temperatures are higher (Shreeve 1984). Floral nectar secretion peaks around 1030 h, when butterfly visitation activity is also highest. However, nectar thieves' activity in Troncones and *Apis mellifera* visitation in La Mancha start early, as soon as flowers open, peak around 0930 am and remain active until flowers close. Butterflies, on the other hand, start their visiting later and have a clear activity peak one hour later, around 1030 am (Fig. 5).

These native butterflies seem to be more selective visitors than the generalist *Apis mellifera*. Butterflies will approach and overfly some flowers without landing on them, as if evaluating and deciding whether or nor to visit them. This contrasts with honeybees, which land on almost all the flowers they approach. If butterflies are indeed assessing floral traits before visiting them, perhaps flowers previously visited by honeybees or nectar robbers are no longer attractive to butterflies, because they bear low nectar volumes, and so butterflies are outcompeted by earlier foragers. Perhaps robbing beetles and *Apis mellifera* both outcompete butterflies using a 'first come, first served' strategy, foraging on floral nectar during the first hour of anthesis when butterflies are still inactive due to lower air temperatures.
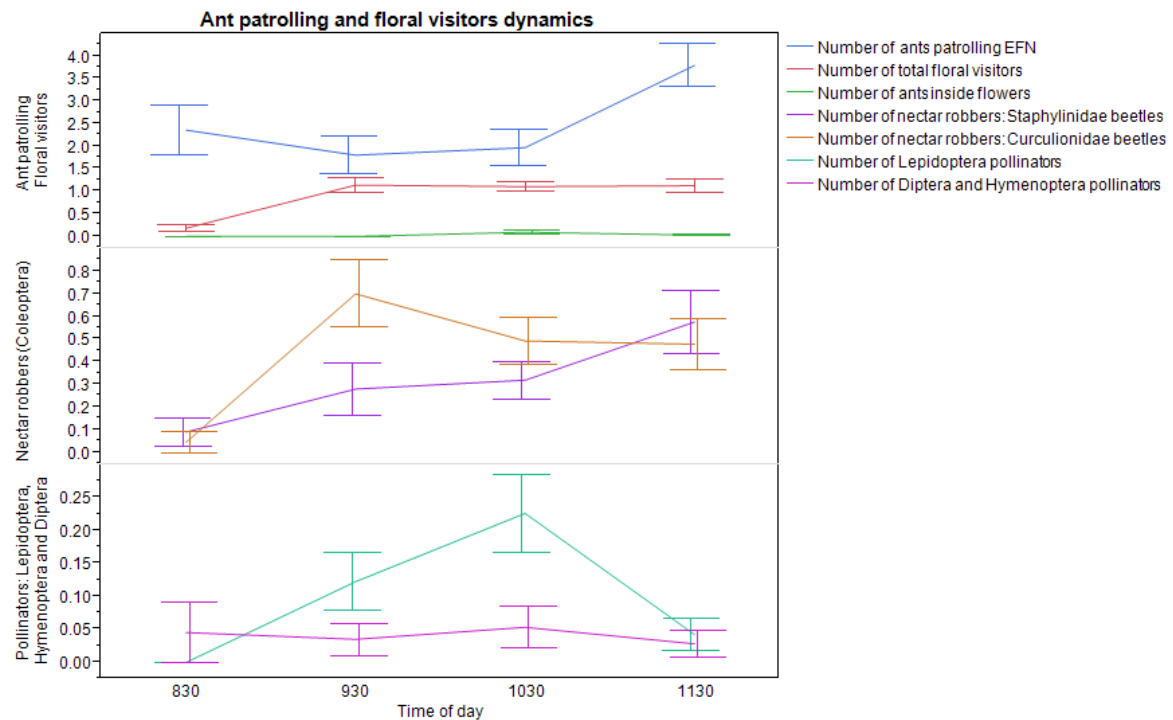

Figure 5. Ants, pollinators and nectar robber temporal dynamics and activity peaks during anthesis. The y axis shows the number of mutualists per flower in each hourly survey (mean±se).
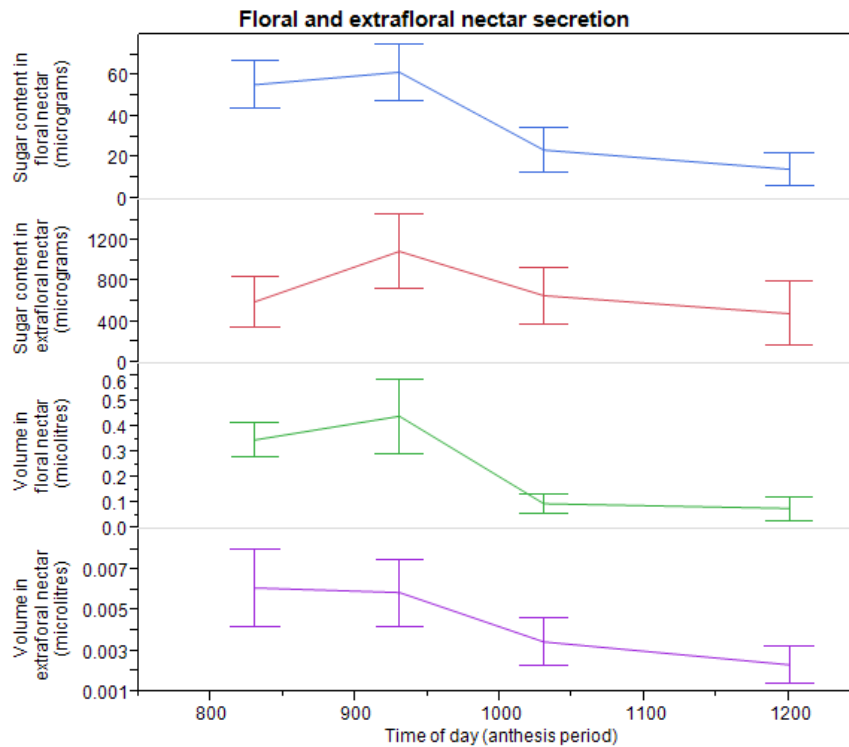
Figure 6. Floral and extrafloral nectar secretion rates per flower o per leaf in *Turnera velutina* at La Mancha, Mexico (mean±se).

Table 1. Summary of correlations between activities of the different insect guilds in *Turnera velutina.*

Response variable	R^2	F	d.f.	P	Direction
Explanatory variable: Ants patrolling in EFNs					
Ants inside flowers	0.129	12.50	1,84	0.0007	Positive (***)
All floral visitors	0.003	0.2808	1,84	0.5975	Negative
Lepidoptera pollinators	0.054	4.87	1,84	0.029	Negative (***)
Other pollinators (Diptera and Hymenoptera)	0.033	2.86	1,84	0.094	None
Nectar robbers (Coleoptera)	0.0016	0.1424	1,84	0.706	None
Explanatory variable: Nectar robbers					
Ants patrolling EFNs	0.0019	0.1654	1,84	0.685	None
Ants inside flowers	0.048	4.23	1,84	0.0426	Positive (***)
Lepidoptera pollinators	0.0727	6.588	1,84	0.0120	Negative (***)
Other pollinators (Diptera and Hymenoptera)	0.0078	0.6614	1,84	0.4184	None
Explanatory variable: Curculionidae nectar robbers					
Staphylinidae nectar robbers	0.0579	5.166	1,84	0.025	Negative (***)

Outcomes

- All fieldwork required for my PhD dissertation has been completed.
- Preliminary results were discussed at the LIPA seminars at Prof. Boege's lab seminars on plant-insect interactions, obtaining useful feedback.
- As a combined result from this and previous field seasons, the following three manuscripts are being prepared for peer-reviewed publications:
    - Patterns in ant distribution: where are the most aggressive ants?
    - Assessing the ecological costs of ant patrolling on pollination services
    - Pollinator avoidance of bodyguards and floral-ant repellence strategies

References

Dutton, E. M., and M. E. Frederickson. 2012. Why ant pollination is rare: new evidence and implications of the antibiotic hypothesis. Arthropod-Plant Interactions 6:561-569.

Newman, D., and J. Thomson. 2005. Interactions among nectar robbing, floral herbivory, and ant protection inLinaria vulgaris. Oikos 110:497506.

O'Dowd, D. J. 1979. Foliar nectar production and ant activity on a neotropical tree, Ochroma pyramidale. Oecologia 43:233-248.

Rico-Gray, V., and P. S. Oliveira. 2007. Mutualism from Antagonism: Ants and Flowers. Pages 85-98 The ecology and evolution of ant-plant interactions. University of Chicago Press.

Savage, A. M., & Peterson, M. A. (2007). Mutualism in a community context: the positive feedback between an ant–aphid mutualism and a gall-making midge. Oecologia, 151(2), 280-291.

Shreeve, T. 1984. Habitat selection, mate location, and microclimatic constraints on the activity of the speckled wood butterfly Pararge aegeria. Oikos:371-377.

Sosenski, P., S. Ramos, C. A. Domínguez, K. Boege, and J. Fornoni. 2016. Pollination biology of the hexaploid self-compatible species Turnera velutina (Passifloraceae). Plant Biology.