

UNIVERSIDADE DE SÃO PAULO  
FFCLRP - DEPARTAMENTO DE BIOLOGIA  
PROGRAMA DE PÓS-GRADUAÇÃO EM ENTOMOLOGIA

“Revelando as especificidades da interação entre formigas e plantas portadoras de nectários  
extraflorais: impactos diretos sobre plantas, herbívoros, formigas e polinizadores”

“Revealing the specificities of the interaction between ants and plants bearing extrafloral  
nectaries: direct impacts on plants, herbivores, ants and pollinators”

Eduardo Calixto Soares

Tese apresentada à Faculdade de  
Filosofia, Ciências e Letras de Ribeirão  
Preto da USP, como parte das exigências  
para a obtenção do título de Doutor em  
Ciências, Área: ENTOMOLOGIA

RIBEIRÃO PRETO – SP  
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Orientador: Kleber Del Claro

**“Versão corrigida”**

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## Resumo

A relação formiga-planta mediada por nectários extraflorais (NEFs) é um modelo clássico e bem documentado de defesa biótica de plantas, em que plantas oferecem néctar extrafloral, um recurso alimentar, e em troca as formigas defendem as plantas predando ou repelindo os herbívoros. Por outro lado, o comportamento agressivo das formigas pode ser prejudicial às plantas, uma vez que elas podem acabar repelindo ou atacando os polinizadores e, conseqüentemente, influenciando o sucesso reprodutivo da planta. Nesta perspectiva, nós avaliamos as especificidades da interação entre formiga e plantas com NEFs e os impactos que as formigas apresentam sobre os herbívoros e polinizadores. O estudo foi realizado no período de setembro de 2015 a julho de 2018, em uma reserva de cerrado stricto sensu, localizada em Uberlândia, Brasil. No Capítulo 1, nós apresentamos uma revisão de estudos científicos dos últimos 30 anos sobre a história da arte do mutualismo de proteção entre formigas e plantas com NEFs e as perspectivas dentro deste tópico. No Capítulo 2, nós mostramos que a atividade dos NEFs influencia a relação formiga-herbívoro levando a padrões de sazonalidade e sincronismo das populações de ambos, com diferentes taxas de predação de herbívoros e impactos no fitness da planta. No Capítulo 3, demonstramos os benefícios de curto e longo prazo do néctar extrafloral e de fontes de alimento artificial no fitness (número e peso de indivíduos) e sobrevivência das colônias de formigas, em que a presença de néctar extrafloral e fontes alimentares ricas em carboidratos e proteínas influenciar positivamente o crescimento e a sobrevivência da colônia. E finalmente, no Capítulo 4, nós observamos que o mutualismo de proteção entre formigas e plantas com NEFs pode influenciar negativamente o fitness da planta através da dissuasão de polinizadores. A partir dos resultados dessa tese, podemos concluir que o néctar extrafloral é um fator chave na regulação e estruturação das interações ecológicas, influenciando diretamente no crescimento e na sobrevivência de colônias de formigas, bem como na interação entre formigas e herbívoros, e formigas e polinizadores em plantas com nectários extraflorais. Nesta perspectiva, devemos estudar cada sistema ecológico detalhadamente, conhecendo todos os seres interagentes, bem como suas respectivas histórias naturais, para entender os padrões de interação e a estruturação das cadeias alimentares, os quais influenciam diretamente a dinâmica dos ecossistemas naturais.

**Palavras-chave:** Cerrado, Defesa biótica, Nectários extraflorais, Formigas, Polinizadores, Herbívoros

## Abstract

The ant-plant relationship mediated by extrafloral nectaries (EFNs) is a classic and well documented model of biotic plant defense, in which plants offer extrafloral nectar, a liquid food resource, and in return ants defend plants by preying on or repelling herbivores. On the other hand, the ant aggressive behavior might be detrimental to plants, since they can end up repelling or preying on pollinators and consequently influencing the plant's reproductive success. In this perspective, we evaluated the specificities of ant-plant interaction and the impacts of ants on herbivores and pollinators in plants bearing EFNs. The study was conducted from September 2015 to July 2018, in a reserve of cerrado stricto sensu, located in Uberlândia, Brazil. In Chapter 1, we presented a review of scientific texts of the last 30 years on the history of the art of protective mutualism between ants and plants with EFNs and the perspectives of this topic. In Chapter 2, we demonstrated the short- and long-term benefits of extrafloral nectar and artificial food source on ant colony fitness (number and weight of individuals) and survival, in which the presence of extrafloral nectar and food sources rich in carbohydrates and proteins positively influence the growth and survival of the colony. In Chapter 3, we showed that the activity of EFNs influences the ant-herbivore relationship leading to patterns of seasonality and synchronism of herbivore and ant populations, with different herbivore predation rates, and impacts on plant fitness. And finally, in Chapter 4, we observed that the protective mutualism between ants and EFN-bearing plants may negatively influence plant fitness via deterring visitation by pollinators. We showed that extrafloral nectar is a key factor in the regulation and structuring of ecological interactions, directly influencing the growth and survival of ant colonies, as well as in the interaction of ants with herbivores and pollinators in plants bearing extrafloral nectars. In this perspective, we should study each ecological system in detail, knowing all interacting beings, as well as their respective natural histories, to understand the patterns of interaction and the structuring of food chains, which directly influence the natural ecosystems dynamic.

**Key words:** Cerrado, Biotic defense, Extrafloral nectar, Predator-prey model

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# Background

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## **Introduction**

### *Trophic systems*

Insects, the most abundant organisms in terrestrial ecosystems, are impacting consumers of early trophic levels, greatly influencing the adaptive value of plants. Nevertheless, insects are also the food base for many other insects and arthropods that make up higher trophic levels, which shows a bottom-up and top-down effects on the trophic chains (Del-Claro and Torenzan-Silingardi 2012).

Food chain studies have challenged researchers from different fields for many years (May 1973, Post 2002, Dyer 2011) and the degree of omnivory or specialization in these systems has received much attention since the last century (Reagan and Waide 1996, Blüthgen et al. 2003). For example, the predator-prey model developed by Lotka (1925) and Volterra (1926) is a classic example of a model that helps understanding the interaction between two trophic levels, more specifically the predator-prey relationship.

Thus, insect-plant interactions, which are extremely abundant in terrestrial environments in both temperate and tropical climates (Price et al. 2011), may be an excellent tool for studying these trophic chains and multitrophic systems. These two taxa have a close relationship, in which different types of interactions have emerged, such as mutualism of pollination, protection mutualism, plant tissue feeding (herbivory), and seed dispersal (Stotz et al. 1999).

### *Ant-herbivore-plant bearing extrafloral nectary interaction*

The ant-plant mutualism is a classic and well documented model of biotic defense, including plants with extrafloral nectaries (EFNs) in the Brazilian Cerrado (Del-Claro 2004, Rico-Gray and Oliveira 2007, Calixto et al. 2018a). EFNs are glands that produce a sugar-rich liquid, but can also present different diluted compounds (e.g. phenols, alkaloids, amino acids, lipids, among others) (Baker 1977, Koptur 1994a, González-Teuber and Heil 2009), mainly

attracting predatory arthropods such as spiders, wasps, and especially ants (Nahas et al. 2012, Del-Claro et al. 2016, Calixto et al. 2018b).

Extrafloral nectar is a key resource for associations between plants and their mutualistic predators, and ants are considered the main biotic agents of defense against herbivory (Rosumek et al. 2009, Trager et al. 2010, Calixto et al. 2018a). Ants that feed on EFNs may increase their individual colony survival and even show a significant increase in body size when compared to ants that do not feed on this resource (Byk and Del-Claro 2011). In response to this food source, ants exhibit aggressive patrolling behaviors, which may decrease the rate of herbivory in these plants, thus representing an efficient biotic defense of the plant (e.g. Lange and Del-Claro 2014, Calixto et al. 2015). This kind of defense can be an effective plant protection strategy, since herbivorous insects usually have ways of resisting physical and chemical defenses (see “arms race theory”), but rarely have anti-defense adaptations performed by ants, except for the action of jumping from plants (Coley and Kursar 1996, Dejean et al. 2006).

It is known that in addition to extrafloral nectar, ants can feed on several other types of resource, for example, hemiptera exudates (honeydew), captured or dead arthropods, carrion, among others (Blüthgen and Feldhaar 2010, Cerdá and Dejean 2011), and that some of these resources (other trapped arthropods) present a large amount of nitrogen, an essential element for living beings (Hölldobler and Wilson 1990, Tobin 1995), fundamental in structuring diverse structures, such as nucleic acids and proteins, the two groups of substances which can be considered elementary to life (Tobin 1995). According to Davidson (1997), the main sources of nitrogen for arboreal ants are animals, that is, prey and carrion. However, the prey availability on plants is unpredictable, as these animals may exhibit different “escape” systems against predators (Dejean et al. 2006). Then, many arboreal ants have developed numerous morphological, behavioral and physiological adaptations to facilitate the collection,

monopolization and/or digestion of specific food resources (Blüthgen and Feldhaar 2010). For example, there are ants that have specific foraging and feeding behavior, but in certain periods, when the availability of the food resource is changed, they can change the foraging behavior and the food resource sought. This fact was shown by Anjos et al. (2019), who highlighted the role of *Pogonomyrmex naegelii* ants as seasonal invertebrate carrion removers. Whitford and Jackson (2007), studying the seed collecting ant, *Pogonomyrmex rugosus*, showed that the colonies of this ant increase the foraging rate in the moments of low quantity of available seeds and high concentrations of prey, being these alive or dead (also see Belchior et al. 2012). This “pulse” of predation on these insects demonstrates the importance of foods with high concentrations of essential compounds for ants, as is the case of nitrogen.

*Ant-pollinator-plant bearing extrafloral nectary interaction*

Uncountable studies have shown the impact that ants have on EFN-bearing plants acting as biotic defense, mainly related to herbivory rates and plant reproductive success (see review in Rosumek et al. 2009, Trager et al. 2010). However, this interaction does not always represent a benefit to plants (O’Dowd and Catchpole 1983, Ness 2006, Byk and Del-Claro 2010, Lange and Del-Claro 2014, Villamil et al. 2018), demonstrating the existence of conditionalities (Bronstein 1994). An example of this conditionality is the negative influence that ants cause on the pollination of EFN-bearing plants, scaring or preying their pollinators (Assunção et al. 2014). Another example, evidenced by Pires and Del-Claro (2014a), is that ants act as vectors of endophytic fungi, which diminish the photosynthetic plant leaf area, interfering with their fitness.

Regardless of the outcome of ant-plant interactions for both partners, predatory ants interfere in some way with the plant visitor community, be they herbivores, predators or pollinators. According to Rico-Gray and Oliveira (2007), predatory ants can significantly affect prey behavior and decrease the potential size of herbivore populations considered as pests. In

addition, the literature shows that some dominant ant groups (*Oecophylla*, *Dolichoderus*, *Anoplolepis*, *Wasmannia*, *Azteca*, in the tropics, *Solenopsis*, in the tropics and subtropics, and *Formica* in temperate environments) are beneficial or potentially beneficial, acting as predatory ants (Tschinkel 2006).

In this perspective, predator-prey interaction involving ant-herbivore and ant-pollinator represents an excellent tool for applying dynamic systems theory in ecology. From these models, we can better understand the dynamics of populations within communities (Goel et al. 1971), and to better understand the functioning of multitrophic systems. Therefore, this thesis sought to verify in an insect-plant system the predator-prey relationship using the ant-herbivore and ant-pollinator models in plants bearing EFNs. To evaluate this central objective, and supported by the hypothesis that both models (ant-herbivore and ant-pollinator) could fit the predator-prey relationship, we outlined this study in four main objectives:

1. present a review of scientific studies of the last 30 years on the history of the art of protective mutualism between ants and plants with EFNs and the perspectives of this topic;
2. investigate whether the activity of EFNs influences the ant-herbivore relationship leading to patterns of seasonality and synchronism of herbivore and ant populations, with different herbivore predation rates, and impacts on plant fitness.
3. evaluate the short- and long-term benefits of extrafloral nectar and artificial food sources on ant colony fitness (number and weight of individuals) and survival;
4. test whether the protective mutualism between ants and EFN-bearing plants negatively influences plant fitness by dissuading the visitation by pollinators.

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# **Chapter 1**

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**Protection mutualism: an overview of ant-plant interactions  
mediated by extrafloral nectaries**

**Protection mutualism: an overview of ant-plant interactions mediated by extrafloral nectaries**

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## **Abstract**

Ants and plants bearing extrafloral nectaries (EFNs) are among the most abundant interacting organisms in the Neotropics, being considered excellent models for studies of ecological interactions. These mutualisms have been studied for more than 150 years. The first studies on this subject addressed the indirect benefit of the presence of ants on plants, reducing the foliar herbivory in most cases. Recently, the direct and indirect benefits of these interactions for ants and for EFNs-bearing plants survivorship, growth and reproduction, have shown conditionality to spatial and temporal variations. Here, we reviewed how the topic “protection mutualism in ant-plant interactions mediated by EFNs” has been approached more recently. A great number of papers dealing with this theme have been published in the last 30 years and new perspectives have emerged in the last decade. We showed how scientific and academic areas are working to improve the knowledge on protection mutualisms considering ant-plant ecological networks and how they can shape communities. Furthermore, we discuss some aspects related with the EFNs evolutionary hypotheses, the existence of conditionalities in ant-plant protection mutualism mediated by EFNs, and we provide some perspectives to inspire new studies that will help in the understanding of these fascinating ecological interactions.

**Keywords:** Biotic defense; Ecological interactions; Herbivory; Myrmecophilous plants; Predators.

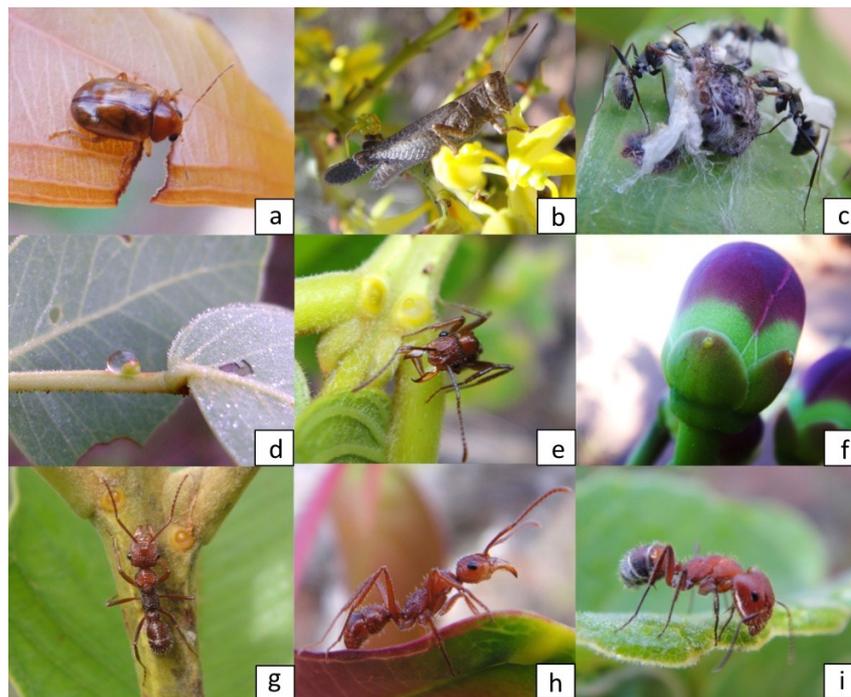
## Introduction

Insects, the most abundant organism in terrestrial ecosystems, correspond to more than 50% (about 1 million species) of the total species of living beings described so far (Grimaldi & Engel 2005, but also see May 1988, Stork 1988, Stork *et al.* 2015). They have different life histories, most of which affecting consumers of first trophic levels, but also greatly influencing the adaptive value of plants (Del-Claro & Torezan-Silingardi 2012). Mainly first level consumers, they are also the food base for the higher trophic levels. As predators, there are thousands of insect species, feeding on innumerable other insects (see Stork 1988). Thus, this group of organisms acts both as bottom-up and top-down forces of the food webs, being essential for the maintenance of the most diverse ecosystems (Price *et al.* 2011). On the other hand, there are 350,000 vascular plant species (<http://www.theplantlist.org>), of which about 305,000 are angiosperms. Insects and plants together correspond to most of living macroscopic organisms on Earth.

The origin of these two groups has been studied extensively and evidence indicates that their success is related to their mutual interactions (Torezan-Silingardi 2012). Although the first plants appeared in a period prior to the first insects (Misof *et al.* 2014), the largest group of extant plants, the angiosperms, arose in the Cretaceous period, when the insects were abundantly present (Labandeira & Sepkoski 1993, Grimaldi & Engel 2005, Schoonhoven *et al.* 2005). The period of greater diversification of the insects is superimposed with that of angiosperms', showing how this interaction is ancient and interrelated (Kukalová-Peck 1991, Labandeira 1998, Del-Claro 2012).

The earliest interactions between plants and insects recorded so far occurred at the beginning of the Devonian, about 400 million years ago (Labandeira 1998, Schoonhoven *et al.* 2005, Misof *et al.* 2014). Some fossils indicate that the first interaction between these two groups was antagonistic (herbivory) (Figures 1a and 1b), in which the insects fed on plant

spores or had a perforation and suction habit of other plant tissues (Labandeira & Sepkoski 1993, Labandeira 1998, Schoonhoven *et al.* 2005). Records of potentially mutually beneficial relationships between insects and plants emerged in the Permian, 290 million years ago (Labandeira 1998, Grimaldi & Engel 2005), and apparently, spores, “pre-pollen”, and pollen were important components of the diets of insects (Labandeira 1998). This feeding strategy is an important precursor to the Paleozoic pollinating mutualisms (Labandeira 1997).



**Figure 1.** Insect-plant interactions: (a-b) herbivores feeding on vegetative and reproductive tissues of plants; (c) *Camponotus* sp. Mayr, 1861 (Hymenoptera, Formicidae) ants capturing and preying spider; (d) extrafloral nectar drop in *Stryphnodendron adstringens* (Fabaceae); (e) *Ectatomma tuberculatum* (Olivier, 1792) (Hymenoptera, Formicidae) with mandibles full of nectar; (f) extrafloral nectary on the sepals of *Caryocar brasiliense* (Caryocaraceae); (g-h) *E. tuberculatum* on extrafloral nectary of *Qualea multiflora* (Vochysiaceae) and *Lafoensia pacari* (Lythraceae); and (i) *Camponotus leydigi* Forel, 1886 foraging in *C. brasiliense*. Photos by Denise Lange.

Associations involving insects and plants (whether antagonistic or mutual) correspond to most of the existing ecological interactions, considered as the structural basis of natural ecosystems (Torezan-Silingardi 2012). However, it was only at the end of the 19<sup>th</sup> century that studies addressing interactions between insects and plants emerged (*e.g.* Packard 1890, Riley 1892), becoming numerous in the last 30 years. From the 1960s until the early 1980s, several

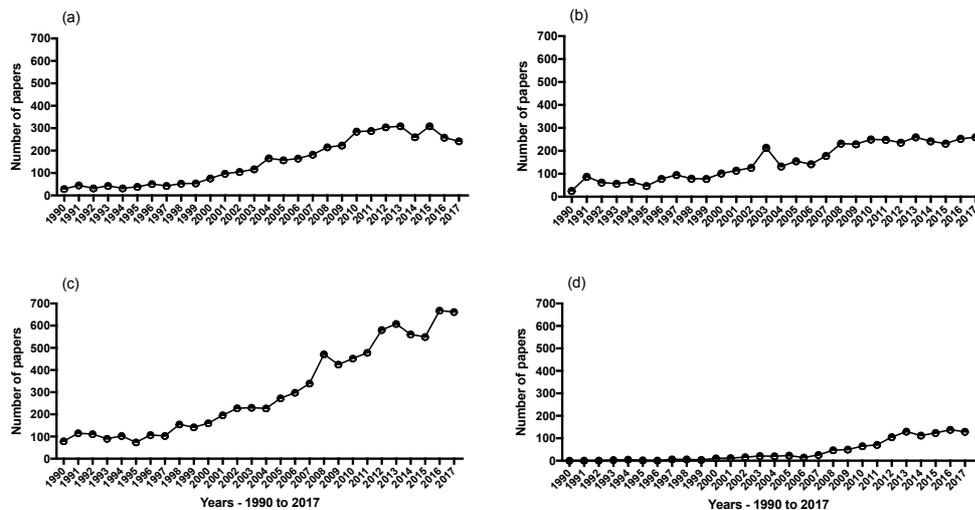
studies were carried out, proposing new mechanisms to explain the pattern of interaction observed between vascular plants and insects, strengthening the multidisciplinary character of the theme (e.g. Ehrlich & Raven 1964, Berenbaum 1983). In the last two decades (see Figure 2), a greater interest in the mechanisms that generate the biodiversity has appeared, focusing on complex associations between plants and insects (Thompson 1994, 2014, Del-Claro 2004), as well as studies including ecological communities (Hunter *et al.* 1988), cladistic classifications (Vane-Wright *et al.* 1991), hierarchical composition of different organization levels (Noss 1990), and complex network analysis (e.g. Bascompte & Jordano 2007, Dáttilo *et al.* 2016).

Therefore, the purpose of this review is to present how the scientific and academic areas are working to provide and improve the knowledge about insect-plant relationships, particularly mutualism between ants and plants, and how these interactions can shape the ecosystem. In other words, our review aims to work specifically with ant-plant mutualism mediated by extrafloral nectaries (EFNs) and try to show the plants/ants-eye view, how this theme has been approached in recent years and to provide future perspectives, going beyond the general ant-plant interaction themes presented in other recent revisions. We hope that our review can provide new perspectives to research in this area and to inspire new studies that will help in the understanding of these fascinating ecological interactions.

### **Material and methods**

For acquisition and choice of articles in this review, we initially used Google Scholar (<https://scholar.google.com>) and complemented with Web of Science ISI database set (<https://apps.webofknowledge.com/>) and JStor (<http://www.jstor.org>). To make the Figure 2, we used the total number of articles per year from Google Scholar and we refined the search with the following terms: “ant-plant mutualism” OR “ant-plant mutualisms” (Figure 2a), “ant-plant interaction” OR “ant-plant interactions” (Figure 2b), “extrafloral nectaries” OR

“extrafloral nectar” OR “extrafloral nectary” (Figure 2c), and “biotic defense” OR “biotic defence” (Figure 2d). We used this methodology to reach the largest quantity of articles available with these terms. All available and relevant papers until 2017 were used. In addition, some references within articles chosen by database systems have been acquired. Our search for these topics showed a large number of works with the term “extrafloral nectaries” OR “extrafloral nectar” OR “extrafloral nectary” (Fig. 2c) and a small number of published works with “ant-plant mutualism” OR “ant-plant mutualisms” (Fig. 2a), “ant-plant interaction” OR “ant-plant interactions” (Fig. 2b) and “biotic defense” OR “biotic defence” (Fig. 2d). These data showed us a great amount of papers dealing with protection mutualism and helped us to better understand how this topic has been approached over the years considering certain characteristics.



**Figure 2.** Number of published papers per year from 1990 to 2017 found in the Google Scholar database. The search was refined with the following words (a) “ant-plant mutualism” OR “ant-plant mutualisms”, (b) “ant-plant interaction” OR “ant-plant interactions”, (c) “extrafloral nectaries” OR “extrafloral nectar” OR “extrafloral nectary”, and (d) “biotic defense” OR “biotic defence”.

### Ant-plant interaction

Ants are dominant insects in most terrestrial environments, with key roles in energy and nutrients flow within ecosystems. Currently, 15,933 valid ant species are known (Ant Web,

2018), although it is estimated that there are about 21,800 species in the world (Agosti & Johnson 2003). In many habitats, especially in tropical regions, ants comprise most of the arthropod fauna found on vegetation (Oliveira & Freitas 2004). Several ant species have established interactions with plants using plants' surfaces as a foraging substrate to search for live (Figure 1c) or dead prey, nectar (Figures 1d and 1e), exudates from herbivorous insects or from the plants themselves, as well as sites to build their nests. As a result, ants form facultative or obligate associations with plants and contribute to the decrease of herbivore population, mediating interactions between herbivores and plants (Del-Claro *et al.* 2016).

Some species of plants, known as myrmecophytes, have adequate and specific structures for the colonization and nesting of ant colonies (Rico-Gray & Oliveira 2007). These structures can originate from modifications of several plants parts, such as leaves, trunk, bulbs or even roots, called domatia (the plural of *domatium*, meaning 'home'). The diversity of myrmecophyte plants and ants associated with them is quite high in several regions, with approximately 250 species of myrmecophytes in the Neotropics, distributed in 14 families (Benson 1985). The association between swollen-thorn *Acacia cornigera* (Fabaceae) and *Pseudomyrmex ferrugineus* (Pseudomyrmecinae) is the best-known example of ant-plant mutualism; in this association plants offer several different resources to ants, including domatia, extrafloral nectar, and Beltian food bodies (Janzen 1966). The case of *Tococa guianensis* Aublet (Melastomataceae) in the Brazilian Cerrado is also a typical example of this type of interaction, in which individuals of this shrubby plant have colonies of ants in their hollow thorns (Michelangeli 2005).

There are also myrmecophilous plants (plant species associated with ants, but not specialized) (Rico-Gray & Oliveira 2007). These species can provide food through food bodies and extrafloral nectaries (EFNs) (Figures 1d-1h). Food bodies are structures rich in lipids, carbohydrates, proteins and amino acids and may be present at the base of leaf petiole, as occurs

in species of the genus *Cecropia* (Urticaceae). The EFNs are secretory glands that are not involved with pollination (Koptur 1992, Del-Claro *et al.* 2016), but produce an aqueous liquid rich in sugars and several other diluted compounds, such as amino acids, lipids, phenols, alkaloids and volatile organic compounds (Baker & Baker 1983, Koptur 1994, Wäckers 2001, González-Teuber & Heil 2009). These structures are extremely variable in structure and morphology (Díaz-Castelazo *et al.* 2005, Machado *et al.* 2008, Aguirre *et al.* 2013), and can occur in practically all plant organs.

Among the resources provided by plants for insects, extrafloral nectar is the main classical example (see Bentley 1977, Beattie 1985, Rico-Gray & Oliveira 2007, Heil 2015), attracting a great diversity of predatory arthropods, such as wasps (Cuautle & Rico-Gray 2003, Eubanks & Styrsky 2005, Wäckers *et al.* 2005), spiders (Ruhren & Handel 1999, Whitney 2004, Nahas *et al.* 2012), and mainly, ants (Rosumek *et al.* 2009, Marazzi *et al.* 2013, Del-Claro *et al.* 2016) (Figure 1i). The EFNs can be found in 3,941 species belonging to 108 families of vascular plants (see the world list of extrafloral nectaries <http://www.extrafloralnectaries.org>, Weber & Keeler 2013). Many examples of EFNs are found in Brazilian savannas, occurring in 8 to 31% of the plant individuals and in 15 to 26% of the tree species in these regions (see Oliveira & Leitão-Filho 1987, Oliveira & Pie 1998), including the most abundant trees (Lange & Del-Claro 2014).

Protective mutualism involving ants and plants mediated by EFNs is characterized by foraging of predatory ants on plants (myrmecophilous or myrmecophytes), resulting in benefits to plants (herbivory decrease and/or fitness enhancement). In exchange, ants receive direct or indirect food from plants that increase colony growth and survivorship (Byk & Del-Claro 2011). The first study evaluating the interaction between ants and tropical plants was proposed by Belt in 1874. Subsequently, the classic work developed by Von Wettstein in 1889 with Asteraceae species, *Jurinea mollis* and *Serratula lycopifolia*, demonstrated for the first time

that plants benefit with the interaction, a decrease in leaf area loss. Oliveira *et al.* (1987) were the first to present experimental evidence in EFNs-bearing plants in the Brazilian savanna (*cerrado*). Since then, interactions involving ants and plants, especially in tropical regions, have increasingly drawn the attention of biologists to the importance of the various processes involved and the factors that govern their establishment (Del-Claro 2004, Rico-Gray & Oliveira 2007, Oliveira *et al.* 2012, Del-Claro *et al.* 2016). Recently, studies have been directed to specific questions about these interactions, looking at conditional features of systems (biotic and abiotic) that influence the outcomes.

### **“Plants-eye” view**

Herbivores exert high evolutionary pressure on plants (Marquis 2012, Thompson 2013) that responded and developed different anti-herbivore mechanisms, such as direct chemical and physical defenses (see Crawley 1983, Coley & Barone 1996, Fürstenberg-Hägg *et al.* 2013, Calixto *et al.* 2015). Besides chemical and physical defenses, plants have other defensive strategies, such as biotic defense, a kind of indirect defense mainly promoted by EFNs (Beattie 1985, Rico-Gray & Oliveira 2007).

This defensive plant system is dynamic, where plants can synchronize their defenses over time to have a better performance in protection or can produce different amounts and qualities of extrafloral nectar influencing the foraging of mutualistic ants (Lange *et al.* 2017). Plants can replace defensive strategies over their phenological development, as in *Qualea multiflora* (Vochysiaceae) that presents different defenses during foliar development (trichomes, toughness and EFNs), where each defense is expressed and presents peaks of effectiveness according to leaf stage (Calixto *et al.* 2015). This study corroborates the Optimal Defense Theory (McKey 1974, 1979, Rhoades 1979), where plants seek to minimize costs of producing defenses and maximize herbivore resistance. According to this theory, plants allocate their defenses to structures according to their value (plant-related tissues) and the

probability of attack. In this way, it is predicted that constitutive defenses (see next paragraph) should be used in plants parts of high value and/or probability of attack, whereas induced defenses, *e.g.* EFNs, should be used in parts of lower value and/or probability of attack.

From another perspective, plants may exhibit defenses that are either constitutive, defenses that are always expressed, or induced, defenses that are expressed after damage or a risk of damage, or both (Zangerl & Rutledge 1996, Karban & Baldwin 1997). Some studies have shown that after herbivorous attack, plants increase the levels of biotic defenses (*e.g.* Ness 2003, Heil & Silva-Bueno 2007, Heil 2008, Pulice & Packer 2008, Bixenmann *et al.* 2011), which may negatively affect herbivorous insects (Karbon 1993, Stout & Duffey 1996, Marquis 2012). Genetic and molecular manipulations have provided evidence of various biochemical mechanisms and signaling pathways in the induction process (*e.g.* Baldwin & Preston 1999, Paschold *et al.* 2007, Heil & Land 2014, Duran-Flores & Heil 2016). Induced responses of plants to herbivory are analogous to immune responses of animals and aim to reduce the performance and/or preference of herbivores by changes in their chemical composition and/or the quality of their tissues (Baldwin & Schultz 1983, Karban & Myers 1989, Karban & Baldwin 1997, Agrawal 1998, Korndörfer & Del-Claro 2006). Other factors should be considered within the induction system of extrafloral nectar by herbivorous (see Heil *et al.* 2000), as the induction trigger (natural or artificial damage) (*e.g.* Heil *et al.* 2000), the stage of plant development or the region that suffered the damage (Jones & Koptur 2015, Holland *et al.* 2009) and the nature of the attacker (Carrillo *et al.* 2012).

The relationship between induced defense and biotic defense mediated by EFNs has been demonstrated in many systems. There is an increase in number or productivity of EFNs located on leaves and buds after being damaged by herbivores (Agrawal & Rutter 1998, Heil *et al.* 2000, Pulice & Packer 2008, Jones & Koptur 2015). In addition to these examples, studies have also shown the functioning of EFNs located in flowers, inflorescences and/or fruits (Rico-

Gray 1989, Díaz-Castelazo *et al.* 2005, Falcão *et al.* 2014) to promote both the protection of vulnerable parts and seed dispersal. These EFNs on reproductive parts also respond to simulated herbivory, providing more examples of induced defense (Zangerl & Rutledge 1996, Wäckers & Bonifay 2004, Holland *et al.* 2009). Other works related to induced defense have shown that some plants can develop systems of damage recognition through certain substances or molecules present in insect saliva or eggs (Arimura *et al.* 2005, 2011, Carrillo *et al.* 2012), showing that this defensive strategy is complex and deserves more attention.

### **Ants-eye view**

For ants, plants may be a source of food and place for nesting. Several authors have shown that ants prefer to forage on plants with EFNs than in other plants (Rico-Gray & Oliveira 2007). In plants with EFNs, at times when there is more nectar production, there is also more ant species foraging on plants, decreasing competition and increasing coexistence (Lange *et al.* 2013, Belchior *et al.* 2016). In addition, plants that produce nectar in higher quantities and richer in calories are more visited by ants (Fagundes *et al.* 2017, Lange *et al.* 2017) and this supply of nectar varies during the day (Lange *et al.* 2017). Therefore, the nectar produced in these structures is a key resource for the ant-plant interaction.

Byk & Del-Claro (2011) experimentally showed that extrafloral nectar consumption from *Chamaecrista desvauxii* (Caesalpinaceae) has a positive effect on the colonies of *Cephalotes pusillus* (Myrmicinae), regarding the number of individuals per colony, body weight and number of eggs. On the other hand, in addition to extrafloral nectar, ants can feed on various other types of resource, for example, Hemiptera exudates (“honeydew”) and sources rich in nitrogen, such as captured or dead arthropods and carrion (Hölldobler & Wilson 1990, Blüthgen & Feldhaar 2010, Cerdá & Dejean 2011). According to Davidson (1997), the main sources of nitrogen for arboreal ants are animals, both prey and carrion. Thus, ants find in

EFNs-bearing plants a perfect place to supplement their diets with nitrogen, sugars, amino acids, lipids, and water (Ness *et al.* 2009).

Several ant species forage on EFNs-bearing plants around the world, mainly in the tropics. Of the 17 Formicidae subfamilies, five have common representatives foraging on plants: Pseudomyrmecinae, Dolichoderinae, Ponerinae, Formicinae and Myrmicinae. In addition to the species diversity, there is behavioral diversity, ranging from opportunistic to extremely aggressive species, and from generalists, who nest on the ground and occasionally forage on plants, to specialists, who nest and feed exclusively on EFNs (Hölldobler & Wilson 1990, Silvestre *et al.* 2003). Many species can forage on the same plant, coexisting, depending on the aggressiveness of each species, while in some cases, the aggressiveness of ant species inhibits the presence of other species (Davidson *et al.* 1989, Heinze *et al.* 1994).

Niche partitioning is also present in ant communities associated with EFNs. Some forage exclusively at night, others only during the day, patrolling extrafloral nectar (Dáttilo *et al.* 2014, Lange *et al.* 2017). All these aspects demonstrate that ants that forage on plants do not have this resource always available and free from competitors. They must deal with biotic and abiotic factors to obtain the resource.

### **Context-dependence in ant-plant interaction**

Many studies have shown the effects that ants have on EFN-bearing plants acting as biotic defenses, mainly related to herbivory and the reproductive success of plants (see Rico-Gray & Oliveira 2007, Rosumek *et al.* 2009, Zhang *et al.* 2015). However, this interaction does not always represent a benefit to the plant (*e.g.* O'Dowd & Catchpole 1983, Rashbrook *et al.* 1992, Rosumek *et al.* 2009, Byk & Del-Claro 2010, Lange & Del-Claro 2014), revealing the existence of conditionalities (Bronstein 1994). The variation in protective mutualism involving ants and plants is dependent on associated ant species (Floren *et al.* 2002, Del-Claro & Marquis 2015, Anjos *et al.* 2017), ant density (O'Dowd & Catchpole 1983, Rashbrook *et al.* 1992), ant

colony nutritional demand (Wilder & Eubanks 2010, Pohl *et al.* 2016), ant size (Rico-Gray & Thien 1989), herbivore defense strategy (Floren *et al.* 2002, Alves-Silva & Del-Claro 2014, Alves-Silva *et al.* 2015), associated plant species (Blüthgen *et al.* 2000, Lange & Del-Claro 2014, Koptur *et al.* 2015), and plant phenological stage (Lange *et al.* 2013, Vilela *et al.* 2014, Belchior *et al.* 2016). In addition, ants can scare away pollinators of EFN-bearing plants, or prey on them, decreasing the fruit set (see Holland *et al.* 2011, Assunção *et al.* 2014). In this context, may plants produce more extrafloral nectar to distract ants from flowers (Chamberlain & Holland 2008; see next section “Evolutionary aspects”)? Ants can also act as vectors of endophytic fungi, which diminish the photosynthetic plant leaf area, interfering with their fitness (Pires & Del-Claro 2014). These conditionalities point out to the complexity of ant-plant interactions. In addition, Baker-Meio & Marquis (2011) showed that the outcomes from interactions of co-occurring varieties of *Chamaecrista desvauxii* with ants are context dependent within and among taxa. They observed that the effectiveness of ants against herbivory depends on the variety of *C. desvauxii* considered and on the presence of seed predators, as well as the EFNs size and the amount of nectar produced.

Bronstein (1998) argues that the presence of another trophic level, such as herbivores, alters the mutualistic interactions between plants and ants due to variations in behavior and feeding modes among different species of herbivores, in addition to variations in herbivore abundance and richness over time. Furthermore, morphological and behavioral characteristics of ants often have an impact on the density, spatial distribution and diversity of herbivorous assemblages (*e.g.* Oliveira & Del-Claro 2005). On the other hand, the ant community structure has been studied in a variety of habitats and it is clear that ant assemblages are dynamic, with spatial and temporal variation that characterizes these communities (see review in Rico-Gray & Oliveira 2007). Thus, plants with EFNs usually associate with guilds of omnivorous ant

species that change in composition and abundance over time and space, which may compromise the benefit magnitude received by the mutual partner (*e.g.* Blüthgen *et al.* 2000).

Although biotic factors are often presented as the main reasons for spatiotemporal variations within ant-plant systems (Marquis & Braker 1994, Romero & Vasconcellos-Neto 2004), abiotic factors have relevant impact as determinants of the outcomes of mutualistic interactions (Bronstein 1994, 1998, Kersch & Fonseca 2005, Vilela *et al.* 2014). Some studies that recorded spatiotemporal variations in the ant-plant mutualistic interactions attributed the observed patterns to the hypothesis of climatic conditions variation (Rico-Gray *et al.* 2012, Dáttilo *et al.* 2013, Leal & Peixoto 2016) and environmental disturbances, *e.g.* fire (Del-Claro & Marquis 2015, Fagundes *et al.* 2015). Leal & Peixoto (2016) demonstrated that the ant effect on performance of EFN-plants increased as mean annual precipitation decreased, suggesting that ants provide greater benefits to plants in these environments to compensate the higher costs for production and maintenance of the EFNs. They also found that the frequency of dominant ants on EFNs-bearing plants increased in drier areas. Del-Claro & Marquis (2015) found that fire modified the impact of ants on the leaf area consumed by insect herbivores, but the ant-plant protective mutualism remained efficient after the fire. These results confirm that abiotic factors may increase service costs and/or benefits of rewards offered by mutual partners.

Despite the existence of conditionalities in ant-plant mutualism mediated by EFNs, Chamberlain & Holland (2009) in their meta-analysis showed that ant effects on plants are not generally context dependent, but, instead, are routinely positive and rarely neutral. These data suggest that the costs and benefits of a mutualism may change, as well as its result. However, the benefit will occur most of the time proving that this interaction has a mutualistic character. On the other hand, when mutual species are inserted within a network of multitrophic interactions, it becomes hard to predict the ecological dynamics of the interaction (McCann

2000). Then, long-term studies can help us understand the maintenance and diversity of multitrophic systems.

### **Evolutionary aspects**

In spite of the amplitude and general occurrence of interactions, especially the factors related to plant resistance through ants attracted by EFNs, studies testing the proposed evolutionary theories are abundant in some aspects and rare in others. These evolutionary hypotheses become even more complex when we evaluate the evolutionary aspects of ant-plant interactions together with other trophic levels, such as herbivores (Ohgushi 2016), essential in the evolutionary direction of ant-plant with EFNs protection mutualism. According to Bittleston *et al.* (2016), morphology and/or structures help and can demonstrate a particular type of ant-plant interaction, and if a new plant species, with certain attractive structures such as domatia, food bodies and EFNs, was found, it is very likely that it would be an ancient and long-term association with ants, since all these structures are related to the attraction of predators, such as ants. However, despite the understanding that species evolution and interactions are intrinsically linked, it remains challenging to study ecological and evolutionary aspects at the same time over longer time scales (Weber *et al.* 2017).

The fact that ant-plant interactions may have conditionalities, and EFNs may not always be linked to plant protection, has led to questions about possible other functions. Some alternative hypotheses related to the evolution and the functioning of EFNs have been raised (see Marazzi *et al.* 2013, Heil 2015, Del-Claro *et al.* 2016): (i) EFNs may act as a distraction, preventing the foraging of ants on flowers (see Becerra & Venable 1991), which may interfere with the plant's reproductive success, (ii) EFNs can be a food source, which induces the establishment of ant nests closer to plants and, consequently, can improve the plant nutrition (Wagner & Nicklen 2010), (iii) plants secrete extrafloral nectar to eliminate excess sugar (see Bentley 1977, Baker *et al.* 1978, Koptur 2005), (iv) EFNs have a defensive/protective function

(see review in Heil 2015, Del-Claro *et al.* 2016), and (v) EFNs can distract tending ants from myrmecophilous hemipterans (Becerra & Venable 1989).

### **Perspectives**

In our current perspective, biodiversity should be viewed and evaluated in a way that seeks to understand the ecological interactions, including aspects such as: (i) life history, biology, and behavior of related species (see Oliveira & Del-Claro 2005, Del-Claro *et al.* 2013), (ii) observations of conditionalities within each interaction (*e.g.* Byk & Del-Claro 2010, Holland *et al.* 2011, Assunção *et al.* 2014), (iii) sophisticated tools for analyzing certain parameters, such as those derived from graph theory, which can provide better conclusions of these interactions (see Bascompte & Jordano 2013, Lange *et al.* 2013, Dáttilo *et al.* 2015), (iv) studies about several ecosystem levels (see Lange *et al.* 2017), and (v) research related to the genetics and physiology of defense-related traits (Heil 2015). We suggest that it is also important to take into account the anthropic effects and climate change, evaluating how these factors are influencing ant-plant interactions (see Leal & Peixoto 2016, Vilela *et al.* 2017). It is important to concentrate studies in places with strong influence of climatic seasonality, as in the Americas (see Rico-Gray & Oliveira 2007), or even to evaluate direct impacts on interactions and ecosystem, such as fire events in very dry and/or propitious areas (see Del-Claro & Marquis 2015, Fagundes *et al.* 2015). From these perspectives, biodiversity should be re-named “Interaction Biodiversity” (*sensu* Thompson 1996, 2005).

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## **Chapter 2**

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**Ant-herbivore interaction influenced by plant-based resource:  
impact of extrafloral nectar on predator-prey relationship**

**Ant-herbivore interaction influenced by plant-based resource: impact of extrafloral nectar on predator-prey relationship**

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## **Abstract**

Predator-prey relationships are excellent models for understanding ecosystem dynamics, mainly due the fact that the outcomes of these interactions are conditional to predators and/or prey's presence/absence, or behavior and natural history. In this perspective, ant-herbivore interaction mediated by extrafloral nectaries (EFNs) activity represents an excellent experimental model to understand predator-prey relationships. We tested whether extrafloral nectar supply indirectly influences the ant-herbivore relationship, eliciting: (i) a seasonality and synchronism in ant and herbivore population throughout the year; (ii) a higher predation rate at times of greater herbivore abundance; and (iii) a positive impact of ants on plant fitness (fruit set production). For that, we conducted manipulative experimental field surveys in EFN-bearing plants over one year. Our results showed that there was a greater abundance of plants with active EFNs, herbivores and ants in the rainy season (October to March). There was a great temporal overlap especially for herbivore and ant abundance over the year, which the period of the greatest EFNs activity increased the likelihood of herbivore predation. Our data also showed that more aggressive herbivores were less preyed upon by ants, while a larger number of ants resulted in a higher probability of predation, and longer path length of ants to plants resulted in higher herbivory rates and lower fruit set production. Additionally, plants with ants showed less herbivory rates. We concluded that in the Brazilian tropical savanna the ant-herbivore relationship is affected by a number of factors, especially by EFNs activity, which influences both ants and herbivores, and result in positive or negative impacts on the fitness of plants that host these interactions.

**Key words:** Seasonality, Synchronism, Temporal overlap, Biotic defense, Extrafloral nectary, Cerrado.

## Introduction

The study of trophic interactions is a key element in understanding the ecosystem dynamics (May 1973, Post 2002, Dyer 2011), where the level of specialization or generalization of species, considering eating habits, make up these trophic systems. This issue has been treated since the last century and with different methodological approaches (Simpson and Raubenheimer 1993, Reagan and Waide 1996, Blüthgen et al. 2003). For example, the predator-prey relationship is a classic example of a mathematical model that helps to understand patterns within the interaction between two trophic levels. From a cyclical fluctuation perspective, predators may decrease their populations in the absence of prey, and in contrast prey may increase their population in the absence of predators (Lotka 1925, Volterra 1926). In a perspective of synchronicity, there seems to be a synchronism between predator and prey population (Lindén 1988).

Despite ants being recognized as predators, they have developed specific eating behaviors and habits, being considered more correctly as omnivores, combining predation, scavenging dead animals and feces, and consumption of plant-based resources (Hölldobler and Wilson 1990). Variations in eating habits may also be present during the ant ontogeny, or even according to the individual caste. For instance, proteins and lipids are generally required for larval growth, ovary development and egg production in queens, while carbohydrates are required for metabolism in adults, sperm production, and like basic energy source (Nation 2015).

Regarding plant-based resources, which mediate the ant-plant-herbivore interaction, extrafloral nectar is the main resource provided (Del-Claro et al., 2016; Koptur, 1992), being fundamental for ant colony growth and survival (Byk and Del-Claro 2011). In this mutualistic interaction, plants provide food resources and ants offer protection against herbivores (Calixto et al., 2018), consequently decreasing herbivory rate and increasing fruit set production

(Rosumek et al. 2009, Nascimento and Del-Claro 2010, Trager et al. 2010). Extrafloral nectar is considered a low nitrogen resource (see Koptur 1994a, Blüthgen et al. 2004, González-Teuber and Heil 2009) and foliage-dwelling ants have access to a diet composed basically of plant-based resources (Blüthgen et al. 2003, Davidson et al. 2003), then workers need to seek other sources of nitrogen for colony nutrition (Blüthgen and Feldhaar 2010). According to Davidson (1997), prey and carrion are the main nitrogen sources for these foliage-dwelling ants; directly contribution to the ant defensive role on plant tissues (Del-Claro et al. 2016).

A large number of studies have been shown that the presence of extrafloral nectaries (glands which produce nectar; EFNs) affects the foraging activity of ants (e.g. Lange et al. 2017), and that the nutritional composition of nectar (especially carbohydrates and amino acids) is also an important factor in aggressiveness, foraging (Blüthgen and Fiedler 2004, Heil 2011) and prey choice by ants (Wilder and Eubanks 2010). Pacelhe et al. (2019) showed that more nutritious artificial extrafloral nectar (based on sugar and amino acids) induce a greater rate of ant recruitment, ant aggressiveness, and efficiency in the predation of herbivores, consequently increasing plant defensive protection (see also Flores-Flores et al. 2018).

In this perspective, changes and behavioral adaptations are present in ant colonies to regulate their nutrient intake, such as balancing the carbohydrate-protein ratio. For instance, Lange et al. (2013) showed that the phenology of EFNs affects the ecological network properties between plants bearing EFNs and ants through its influence on resource availability and competition among ants. Anjos et al. (2019) showed that *Pogonomyrmex naegelii* ants may present changes in dietary habits, showing preferences for specific food types in different moments of the year (Belchior et al. 2012).

Considering that foliage-dwelling worker ants can change their feeding habits and prey herbivores to balance colony needs, in this study we investigated whether extrafloral nectar activity indirectly influences the ant-herbivore relationship. To reach this goal, we sought to

answer the following questions: (1) is there seasonality and synchronism of EFNs activity with ant and herbivore abundance? (2) May different factors related to ants (e.g. distance between ant nest and plant; or time to find the herbivore), herbivores (e.g. size; or more or less aggressive), and EFN-bearing plants (e.g. EFNs activity; or shape) influence the herbivore predation rate by ants? (3) Do nest distance and the path length of ants interfere in the decrease of herbivory rate and increase of fruit production? We hypothesized: (H1) seasonality and synchronism in EFNs activity, ant and herbivore abundance throughout the year; (H2) a higher predation rate at times of greater herbivore abundance and of less aggressive herbivores; and (H3) a positive impact of ants on plant fitness, influenced by the presence of extrafloral nectar.

## **Material and methods**

### *Study area*

The fieldwork was conducted in the Ecological Reserve of the Clube de Caça e Pesca Itororó de Uberlândia (CCPIU: 48°17'W; 18°58'S), in Uberlândia, Brazil. The reserve located in the Cerrado Biome (Oliveira and Marquis 2002) has about 230 ha of cerrado sensu stricto. The reserve contains palm swamp areas, open areas with shrubs and small trees, and more enclosed areas with trees reaching up to 15 meters in height. From October to March, we can observe a marked rainy season, while from April to September, we can observe a marked dry season. The mean annual temperature varies from 18 to 28° C and the rainfall from 800 to 2,000 mm (Ferreira and Torezan-Silingardi 2013).

### *Data collection*

*H1 – there is a seasonality and synchronism in EFNs activity, ant and herbivore abundance throughout the year*

To verify if there is seasonality and synchronism of the greater abundance of ants, herbivores, and plants with active EFNs, we used ten transects of 50 meters (length) each and

at least 50 meters apart. Transects were made inside the reserve and perpendicular to a trail of 2.5 meters wide that crosses the reserve (Bächtold et al. 2016). To decrease edge effects, we started each transect 10 meters away from the trail. All transects were made in the same side of the way, and presented similar conditions of soil, light and humidity.

After that, we selected 10 individuals of the 20 most common plant species with EFNs at the reserve: *Bionia coriacea* (Fabaceae), *Banisteriopsis malifolia* (Malpighiaceae), *Bauhinia rufa* (Fabaceae), *Banisteriopsis stellaris* (Malpighiaceae), *Caryocar brasiliense* (Caryocaraceae), *Eriotheca gracilipes* (Malvaceae), *Heteropterys pteropetala* (Malpighiaceae), *Lafoensia pacari* (Lythraceae), *Manihot caerulescens* (Euphorbiaceae), *Ouratea hexasperma* (Ochnaceae), *Ouratea spectabilis* (Ochnaceae), *Palicourea rigida* (Rubiaceae), *Qualea grandiflora* (Vochysiaceae), *Qualea multiflora* (Vochysiaceae), *Qualea parviflora* (Vochysiaceae), *Stryphnodendron polyphyllum* (Fabaceae), *Senna rugosa* (Fabaceae), *Senna velutina* (Fabaceae), *Smilax polyantha* (Smilacaceae), *Tocoyena Formosa* (Rubiaceae). These plants were divided in 20 individuals (one per species) per transect (n = 200 plant individuals; n=10 transects). Plants of the same species were selected with similar characteristics of height and architecture and distant up to five meters from transects. All plants were monitored monthly over one year, between March 2017 and February 2018, to know the herbivores and ants present in each individual, as well as the phenology variation of EFNs over time. We visited the transects always on two consecutive days, where we evaluated the first five transects on the first day, and the remaining five on the second day. The observations were divided into two shifts: daytime (8:00 a.m. to 12:00 p.m.) and nighttime (7:00 p.m. to 11:00 p.m.). All herbivores and ants were quantified and qualified and, whenever possible, a specimen of each ant and herbivore species was collected for later identification in the Laboratório de Ecologia Comportamental e de Interações (LECI). The activity of EFNs was analyzed monthly as a dummy variable, that is, if plant individuals presented (value = 1) or not

(value = 0) active EFNs. Active EFNs are light in color, bright and are generally producing; non-active EFNs are dark with necrosis aspects and do not produce nectar (Calixto et al. 2015).

*H2 – there is a higher predation rate at times of greater herbivore abundance and of less aggressive herbivores*

To verify which factors influence the predation of herbivores by ants, we conducted a termite predation experiment. This surrogate herbivore was used because (1) they are commonly used to test ant aggressiveness (e.g. Fagundes et al. 2017), (2) they are abundant and easier to manipulate than natural herbivores, (3) it is a form of standardization of the experiment, and (4) we can test the impact of herbivore aggressiveness using termite soldiers and workers. We selected 20 individuals (not related to the previous selected plants) of five plant species bearing EFNs (n = 100): *Banisteriopsis malifolia* Nees & Mart. (Malpighiaceae), *Lafoensia pacari* (A. St.-Hil.) (Lythraceae), *Ouratea spectabilis* (Mart.) Engl. (Ochnaceae), *Qualea multiflora* (Mart.) (Vochysiaceae) e *Stryphnodendron polyphyllum* (Mart.) (Fabaceae). Each of these species has a type and location of EFN (Weber et al. 2015), which allows us to test whether predation of herbivores can be influenced by these factors. Each plant was at least 10 meters apart, and plants from the same species had similar height, and number of branches and leaves.

This analysis was conducted in the dry (lower EFN activity) and rainy (greater EFN activity) seasons (Lange et al. 2013), between 09:00 am and 11:00: am, and with the same plant individuals. In each plant individual we selected and tagged two similar apical leaves without any apparent damage. Each leaf received a worker or a soldier, both alive, of *Nasutitermes* sp. (Isoptera: Termitidae). We only added the termites if all ants on the plant were at least 30 cm away from the selected leaves. After releasing each termite, we started the observations. Termites remained on plants for a maximum time of 15 minutes, where we evaluated: the ant

abundance and species, the time to find the termite, termite predation or not, the path length of ants until the nest after preying the termite, and the straight distance from the nest to the plant (henceforth “straight distance”). To evaluate the path length of ants, we carefully followed each ant, and with the aid of a sewing line, we re-make the path. If the termite left the leaf, or fell from the plant, we discarded the experiment and began again.

*H3 – there is a positive impact of ants on plant fitness, influenced by the presence of extrafloral nectar*

In order to analyze if the straight distance of the nest and the path length of ants is an important factor in the protection and final fruit production of plants, in the beginning of October 2017 we selected 20 similar individuals of *Q. multiflora*, which already had branches with sprouting leaves, some active EFNs, and ants. We only selected plants that contained the ant *Camponotus crassus* ant to standardize the protective effect of the ants along the plants, since different species of ants present different levels of protection (see Del-Claro and Marquis 2015, Fagundes et al. 2017). We checked plants biweekly to assure that ant species had not changed.

In each plant three branches were selected, in which the herbivory rate of all leaves was measured after the necrosis of all EFNs (when the leaves become developed and tough) of the selected branches (see Calixto et al. 2015). When most of leaves of the selected branches were in the intermediate phase of development and with active EFNs (for more details see Calixto et al. 2015), we placed a worker of *Nasutitermes* sp. (Termitidae) in the first bifurcation of each plant, awaited some predation event by ants, and then we followed the ant to the nest (e.g. Yamamoto and Del-Claro 2008). At this stage, we evaluated the straight distance from the plant to the nest, and the path length of ants. Similar to hypothesis *H2*, we used a sewing line to re-make the path length of ants.

During the plant reproductive stage, we first counted the total number of inflorescences, and then the total number of fruits produced in each selected plant. In this way, we were able to evaluate whether the straight distance from the nest to the plant, the path length of ants, or the herbivory rate influence the production of fruits per inflorescence per plant.

Finally, to analyze if the herbivory rate is influenced by the presence of ants, 20 different individuals of *Q. multiflora* presenting branches with sprouting leaves, active EFNs, and *C. crassus* ants were selected, and each individual was divided into two similar parts. We prevented the ant access in one part using a non-toxic resin (Tanglefoot) around the base of stem insertion; and in the other part, we added the same resin, but only in a half part of the stem, allowing ant access. Three branches of each part ( $n = 6$  branches per plant) were selected and the herbivory rate of these branches was quantified when leaves became full expanded and EFNs were no longer active. Herbivory rate was measured according to Calixto et al. (2015), using photos and the program ImageJ 1.47.

#### *Data analysis*

All statistical analyses and graphs were performed using RStudio 3.5.1 at 5% of probability. In all models, we checked the residuals to verify the suitability of the model and overdispersion whenever applicable.

*H1 – there is a seasonality and synchronism in EFNs activity, ant and herbivore abundance throughout the year*

We used circular statistical analyses to evaluate whether there is a seasonality of plants bearing active EFNs, ants, and herbivores. Then, months were converted into angles, and the abundance of plants with active EFNs, and the abundance of ants and herbivores were used to calculate the mean vector ( $\mu$ ), mean vector length ( $r$ ), median, standard deviation, Rayleigh test  $Z$ , and Rayleigh test  $p$ . Rayleigh test  $p$  with  $p$ -value  $< 0.05$  and mean vector length ( $r$ ) close to 1 indicate seasonality of the data, that is, phenological activity is concentrated around one

single date or mean angle. Circular analyses were conducted with the package “circular” (Agostinelli and Lund 2017).

To verify the synchronism patterns over the year, we constructed three temporal abundance matrices, based on the collection months and on the evaluated groups (abundance of plants with active EFNs; abundance of ants; and abundance of herbivores), in order to conduct niche overlap analysis using the Pianka (1973) and Czechanowski (1981) indices, and the coefficient of overlapping presented by Ridout and Linkie (2009). Pianka and Czechanowski indices ranges between 0 to 1, where 0 means that a species pair never co-occurs in time, and 1 means complete temporal overlap. The coefficient of overlapping is fitted by Kernel density functions, and represents a quantitative measure of overlap (0 – no overlap, 1 – identical activity patterns). We used the estimator Dhat4, since samples are larger than 75 (Ridout and Linkie 2009). In addition, we also analyzed our temporal abundance matrix with a niche overlap null model using RA3 and RA4 algorithms from package “EcoSimR” (Gotelli et al. 2015). In brief, RA3 reshuffles the row values, and RA4 reshuffles the non-zero row values; however, both retain the observed “niche breadth” of each species. We simulated 1000 null assemblages, designed the histogram of simulated against observed niche overlap value, and we estimated the one-tailed probability of observed data.

*H2 – there is a higher predation rate at times of greater herbivore abundance and of less aggressive herbivores*

We conducted a survival analyzes to evaluate whether the predatory ant activity is influenced by EFNs activity and termite caste (our proxy for less and more aggressive herbivores), implementing the G-rho family of Harrington and Fleming (1982) using the function *survdiff* in the package “survival” (Therneau 2015) followed by pairwise comparisons using Log-Rank test and p-value correction of Benjamini and Hochberg (Benjamini and

Hochberg 1995) in the package “survminer” (Kassambara and Kosinski 2018). For that, we considered all termite observations (200 during dry season, 100 for termite workers and 100 for soldiers; and 200 during rainy season, 100 for workers and 100 for soldiers). Termite predation and time to find the termite were considered as response variables, and season and termite caste as explanatory factor: Survival (Termite predation + Time)~Season + Termite caste. We used season as proxy of EFNs activity, since our results of EFN seasonality showed that EFNs activity is concentrated in the rainy season (also see Lange et al. 2013). Termite caste as predictor shows us whether ant behavior is changed according to herbivore aggressiveness/defense. Finally, termite predation was fitted as dummy variable: 1 = removed and 0 = not removed.

To test which other variables are the best to explain termite predation, we used only data from rainy season, because there were a small number of predation events ( $n = 7$ ) during dry season. First, we conducted a model selection, where our complete model was represented by termite caste, abundance of ants, plant species, and ant species as fixed factors, and termite nested within plant individual was fit as random factor to control spatial (‘plant individuals/termite) repeated measure. We used the package ‘MuMIn’ (Barto’n 2018) to extract the AICc (lowest value), delta ( $< 2$ ), and weight (highest value), and select the best model (AICc = 108.6, delta = 0, weight = 0.639), which presented termite caste and abundance of ants as fixed factor and termite nested within plant individual as random factor. After that, we conducted a GLMM with binomial error distribution followed by Wald chi-square test, using the package “lme4” (Bates et al. 2015) and “car” (Fox and Weisberg 2011), respectively.

To test if the time to find the termites is influenced by the ant nest distance and ant abundance, we used only data from rainy season, and where there were predation events, since only in that moment we were able to measure the nest distance. Then, we ran a LMM followed by Wald chi-square test using the package “lme4” and “car” respectively. Ant nest distance

and ant abundance were fitted as fixed effect and plant individual was considered a random factor. Since we did not observe any recruitment event, we considered the time to find a termite worker or soldier in the same plant individual as independent measures.

To test if there is difference in the average speed of ants carrying termites between the two termite castes, we ran a LMM followed by Wald chi-square test. Termite caste was considered as fixed effect and plant individual was considered as random factor. Average speed was calculated dividing the ant nest distance (straight distance) by the time carrying the termite. Termite worker and soldier were considered as independent measures, and we used the data only from *Camponotus crassus* (n = 68), since it was the ant with the highest number of predation events.

*H3 – there is a positive impact of ants on plant fitness, influenced by the presence of extrafloral nectar*

To test whether the straight distance of the nest and path length of ants influence the plant protection, we ran a GLM with gaussian error distribution. We used the herbivory average of the three branches per plant as dependent variable.

To test whether the path length of ants and the herbivory rate influence the number of fruits produced, we ran a GLM with gaussian error distribution, since we quantified the total number of fruits produced divided by the total number of inflorescence per plant (number of fruits/inflorescence/plant). In addition, as we observed that path length of ants significantly affected the herbivory rate, and herbivory rate significantly affected the number of fruits (see results), we ran a path analysis to show the effects of the direct and indirect relations of the predictive variables on the fruits produced. With this analysis we can observe the linear and causal relationship among the predictor variables, and through the *coefficient of*

*nondetermination* ( $1-R^2$ ) we can estimate the fraction of the variance that is not explained by the model.

To compare the herbivory rate between control (with ants) and treatment (without ants) branches, we used the average herbivory percentage of each branch as response variable. As we analyzed the two treatments in the same plant individual and we tagged three branches for each treatment, we used branches nested within treatment nested within plant individual (hierarchical design) to control spatial repeated measure. Then, we ran a GLMM with beta error distribution and “logit” link followed by Wald chi-square test using the package “glmmTMB” (Brooks et al. 2017).

## Results

*H1 – there is a seasonality and synchronism in EFNs activity, ant and herbivore abundance throughout the year*

Our field observations revealed a total of 5326 ant individuals found in the 200 individuals belonging to the 20 EFN-bearing plant species during the day and night periods over the year (Appendix 1). Of this total, we found 40 species of ants distributed in six subfamilies. The most representative subfamily was Formicinae, with 16 species, followed by Myrmicinae with 13. The ant species most present and abundant was the diurnal *Camponotus crassus*, which visited all plant species and represented almost 15% of the total ant individuals sampled. In addition, we observed strictly diurnal or nocturnal species, and species that foraged in both shifts.

Regarding herbivores, we found a total of 1620 herbivores, distributed in seven orders (Appendix 2). Hemiptera and Coleoptera were the most abundant orders presenting 45.0% and 34.3%, respectively, of all herbivores sampled. The most abundant herbivorous families were Chrysomelidae and Cicadellidae, which had species present in 17 plant species.

Circular analyses showed that EFNs activity is concentrated around December (Fig. 1a), and ants (Fig. 1b) and herbivores (Fig. 1c) around November, and that the mean angle of EFNs, ant and herbivore abundance were seasonally significant (Table 1). Despite of significant, the mean vector length ( $r$ ) for ants and herbivores was 0.31 and 0.12, respectively, which indicates that their abundance is concentrated at a specific time of the year, but they also may be present throughout the year (Fig. 1).

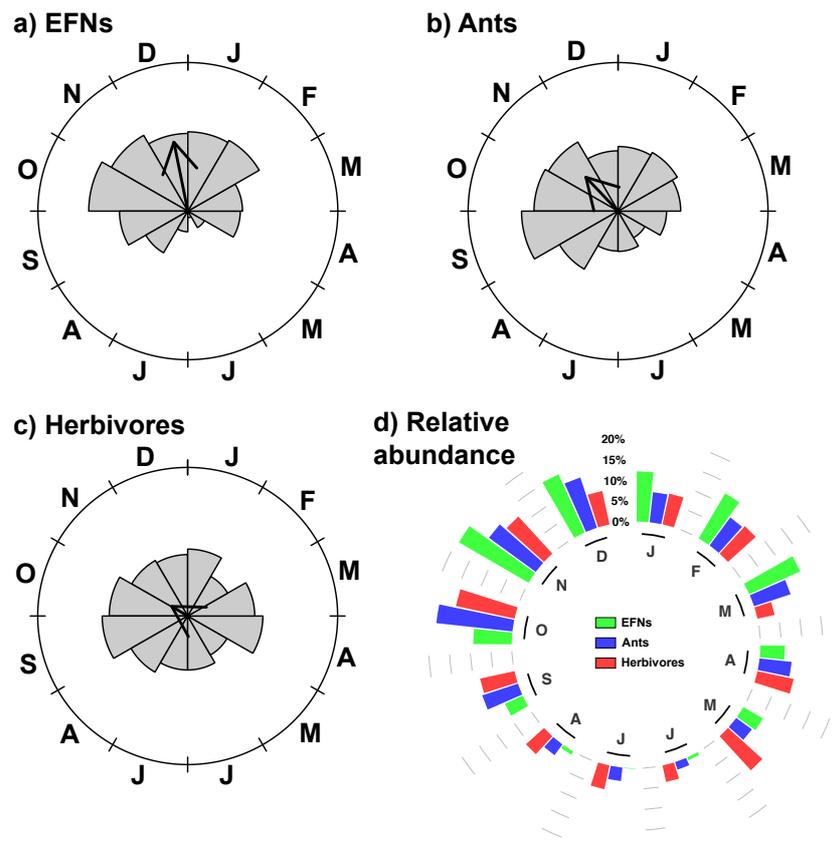


Figure 1. Annual activity of (a) plants bearing extrafloral nectaries (EFNs), (b) abundance of ants, (c) abundance of herbivores, and (d) relative abundance of plants with active EFNs, ants and herbivores in a cerrado reserve, Brazil. Arrow position represents the mean angle (mean month) and arrow length the length of mean vector ( $r$ ) in a), b), and c). Circular analysis result. detailed in Table 1.

Table 1 – Circular statistical analysis testing for seasonality patterns of extrafloral nectaries, ants and herbivores abundance in twenty plants bearing extrafloral nectaries in a cerrado reserve, in Uberlândia, Brazil, between March 2017 and February 2018. EFNs – extrafloral nectaries.

	<b>Mean vector (<math>\mu</math>)</b>	<b>Month</b>	<b>Length of mean vector (r)</b>	<b>Median</b>	<b>Circular standard deviation</b>	<b>Rayleigh test (Z)</b>	<b>Rayleigh test (p)</b>
<b>EFNs</b>	348.656	December	0.47	360.000	289.917	0.473	0.000
<b>Ants</b>	316.740	November	0.31	330.033	272.655	0.312	0.000
<b>Herbivores</b>	301.270	November	0.12	300.010	242.996	0.124	0.000

From September, we can observe an increase of the relative abundance of EFNs activity, ants and herbivores. This growth is maintained until December, where we can clearly see a decrease in the abundance of ants and herbivores (Fig. 1d). In the dry season (April to September), there is a low EFNs activity and a low abundance of ants and herbivores. However, at that time it seems to be the only time where the relative abundance of herbivores remains higher than that of ants, indicating a negative effect of the presence of ants on herbivores.

Niche overlap indices together with niche overlap null models showed significant overlap when implementing pairwise comparisons between EFNs x ants, and ants x herbivores (Table 2, Fig. 2). The comparison between EFNs x herbivores was not significant. All analyzed factors were relatively more abundant in the rainy season, and at least for EFNs x ants, and ants x herbivores, we visualized an evident overlap for these two pairs of groups.

Table 2 – Niche overlap indices and one-tailed  $p$ -value based on 1000 randomizations for pairwise comparisons among extrafloral nectaries (EFNs), ants and herbivores. Values of niche overlap indices close to 1 mean complete temporal overlap.

	Niche overlap indices			$p$ -value	
	Piank a	Czechanow ski	Coefficient overlap	Null model (RA3)	Null model (RA4)
<b>EFNs x Ants</b>	0.909	0.778	0.789	<b>0.002</b>	<b>0.003</b>
<b>EFNs x Herbivores</b>	0.839	0.681	0.680	0.092	0.090
<b>Ants x Herbivores</b>	0.939	0.840	0.815	<b>&lt;0.001</b>	<b>&lt;0.001</b>

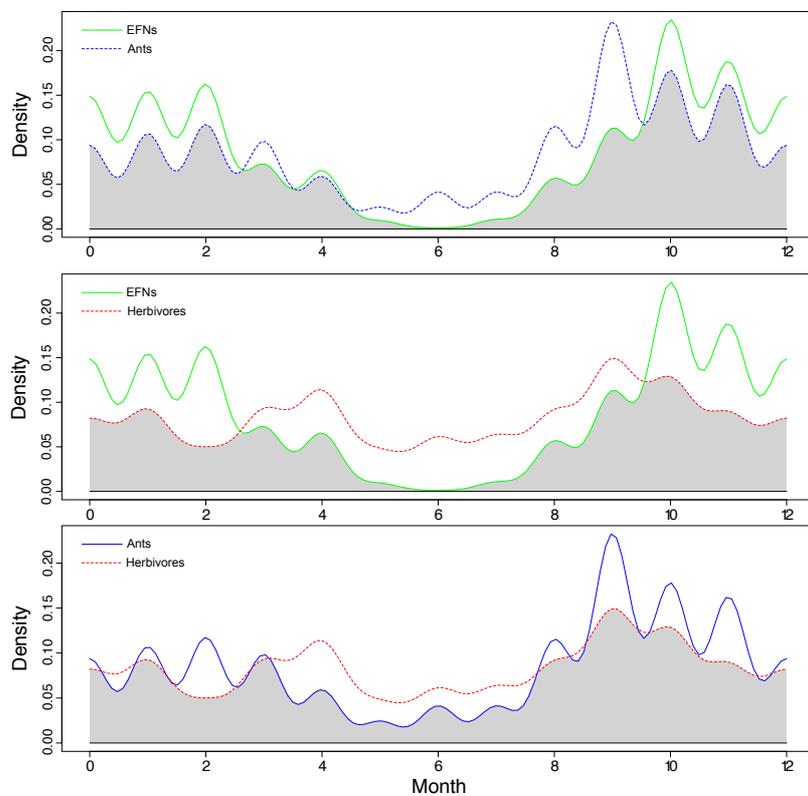


Figure 2 – Kernel density functions (based on the abundance of each factor analyzed) of pairwise comparisons among extrafloral nectaries (EFNs), ants and herbivores throughout the year. Shading area correspond to the coefficient of overlap (see Table 2). Number ranges at the bottom of each graph indicates months of the year starting in January (0 to 1) and finishing in December (11 to 12).

*H2 – there is a higher predation rate at times of greater herbivore abundance and of less aggressive herbivores*

We found that the ant predatory activity is influenced by EFN activity and termite caste ( $\chi^2 = 114.0$ ,  $df = 3$ ,  $p < 0.001$ ; Fig. 3). From a total of 400 termites used in the experiment (200 in the rainy season and 200 in dry season), we observed 92 (46%) ant predation events during the period of greatest activity of EFNs (rainy season) and only seven (4%) during the lowest moment (dry season). Of the 200 termites used in the lowest period of EFNs activity, we observed two soldier predations, and five worker predations. Of the other 200 termites used in the greatest period of EFNs activity, we observed 37 predation events of soldiers and 55 of workers. Our pairwise comparisons showed a significant difference in termite predation between the greatest and lowest period of EFNs activity, and between worker and soldier predation in the greatest period of EFNs activity (Fig. 3).

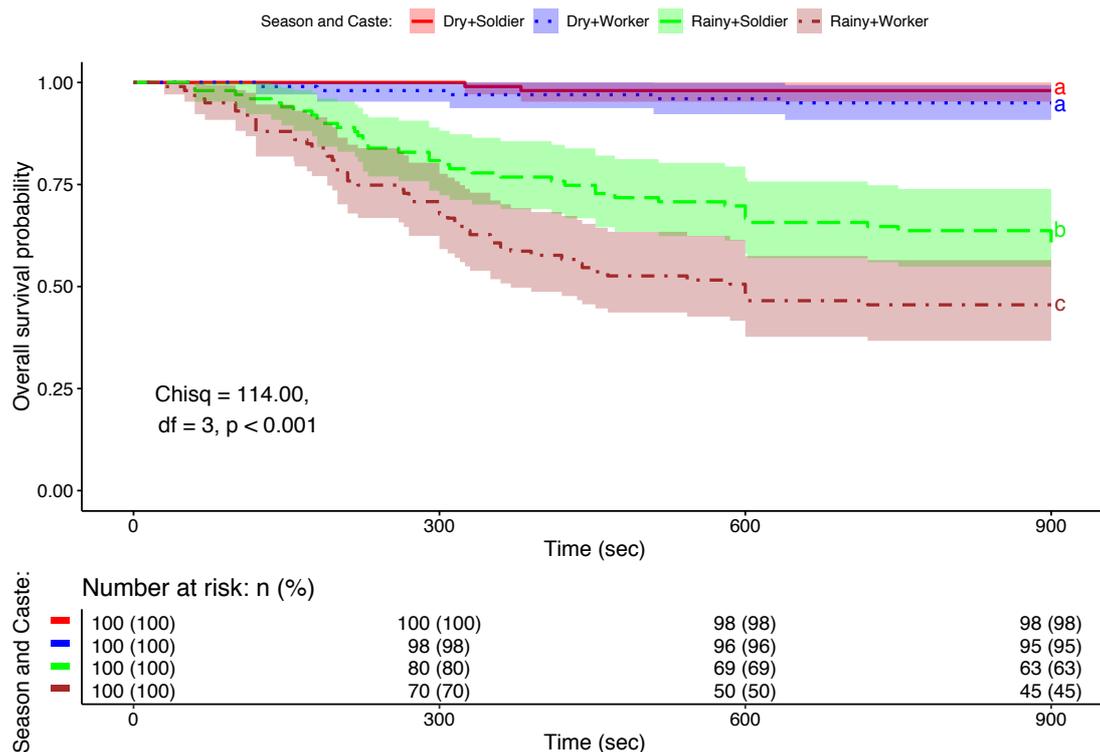


Figure 3 – Time spent by ants to remove live termites (workers and soldiers) from plants during the period of the lowest (dry season) and greatest activity (rainy season) of extrafloral nectaries (EFNs) in five EFN-bearing plants species. Different letters at the end of each curve differ from each other by pairwise comparisons using Log-Rank test and p-value correction of Benjamini and Hochberg (1995). Number at risk table means absolute number and percentage of subjects at risk by time, respectively.

We found that surrogate herbivore predation is influenced by both herbivore aggressiveness and abundance of ants, where the abundance of ants is the most explanatory factor within the model (Termite caste – GLMM:  $\chi^2 = 6.237$ ,  $p < 0.05$ ; Abundance of ants – GLMM:  $\chi^2 = 38.625$ ,  $p < 0.001$ ; Fig. 4). We observed that a smaller abundance of ants is necessary for the removal of workers and that the probability of removing a worker is greater than that of a soldier.

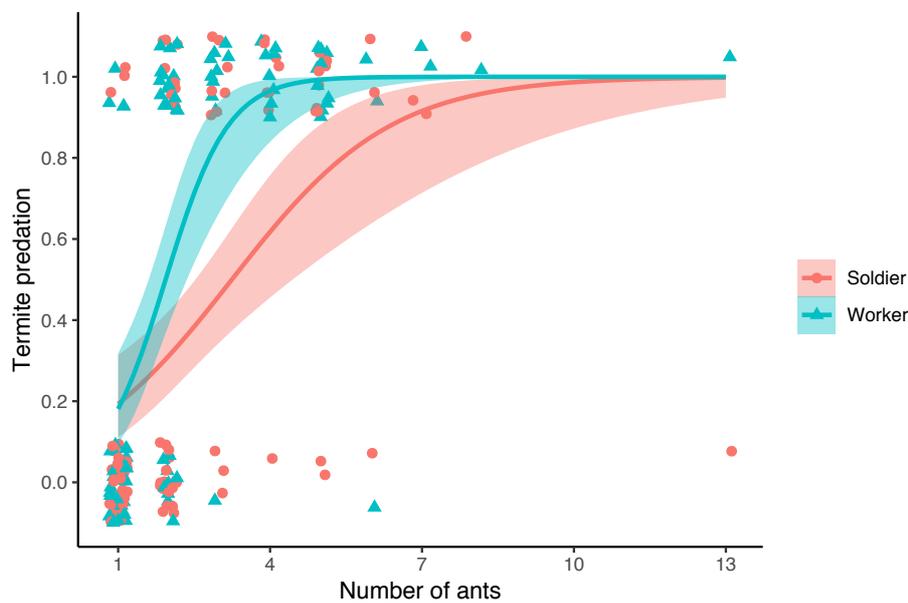


Figure 4 – Logistic regression plot with binomial fitted curves of the termite predation probability in function of ant abundance. Each curve was produced with a binomial smooth function. Jitter function (width=0.18, height=0.1) was used to add a random noise to the plot, decreasing dots overlap and making the plot easier to read.

The time spent to find a termite was influenced negatively by the abundance of ants (LMM:  $\chi^2 = 8.788$ ,  $p < 0.01$ ; Fig. 5), but not for ant nest distance (LMM:  $\chi^2 = 0.138$ ,  $p = 0.709$ ). The average speed of *C. crassus* carrying termites did not differ between the two termite castes (LMM:  $\chi^2 = 1.936$ ,  $p = 0.164$ ). However, we observed that ants carrying workers were slightly faster ( $0.75 \pm 0.55$  cm/s; mean  $\pm$  SD) than those carrying soldiers ( $0.59 \pm 0.34$  cm/s).

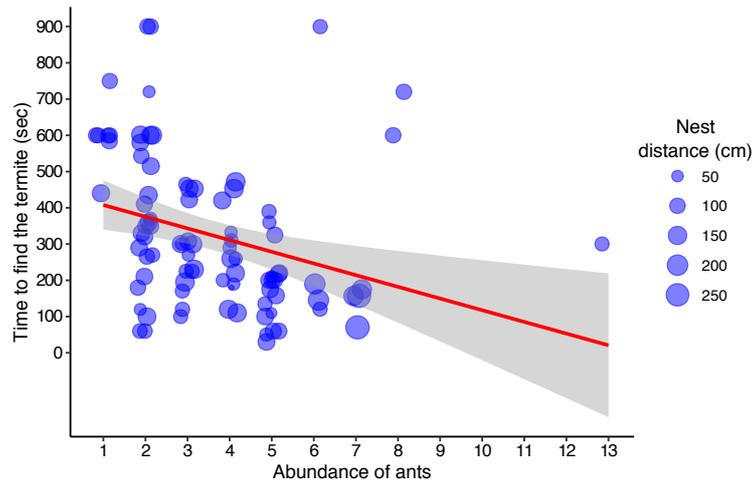


Figure 5 – Time to find the surrogate herbivore (termite) influenced by ant abundance (LMM:  $\chi^2 = 8.788$ ,  $p < 0.01$ ) in five abundant extrafloral nectaried plants in the Brazilian savannah. Regression line is based on the linear model between time to find the termite and abundance of ants ( $y = 439.96 - 32.24x$ ). Despite not significant (LMM:  $\chi^2 = 0.138$ ,  $p = 0.709$ ), we plotted nest distance based on circles. Jitter function (width=0.18) was used to add a random noise to the plot, decreasing dots overlap and making the plot easier to read.

*H3 – there is a positive impact of ants on plant fitness, influenced by the presence of extrafloral nectar*

The herbivory rate increased as the path length of ants increased (GLM:  $\chi^2 = 13.450$ ,  $p < 0.001$ ; Fig. 6), while the straight distance of the nest did not influence the rate of herbivory (GLM:  $\chi^2 = 1.046$ ,  $p = 0.306$ ). The number of fruits/inflorescence/plant was negatively influenced by the herbivory rate (GLM:  $\chi^2 = 8.191$ ,  $p < 0.01$ ; Fig. 7a). However, the path analysis showed that there is a direct influence of the herbivory rate and an indirect influence of the path length of ants via herbivory rate on the number of fruits produced (Fig. 7b), even though there has not been a direct effect of path length of ants on fruit set (GLM:  $\chi^2 = 0.011$ ,  $p = 0.915$ ). Furthermore, 52% of the variance of the herbivory rate and 50% of the variance of the number of fruits are explained by the causal relationship stated in our model (Fig. 7b). Finally, our ant removal experiment showed that plants without ants had an herbivory rate three times higher ( $8.16 \pm 4.08$  %; mean  $\pm$  SD) than plants with ants ( $2.64 \pm 1.90$  %) (GLMM:  $\chi^2 = 59.483$ ,  $p < 0.001$ ; Fig. 8).

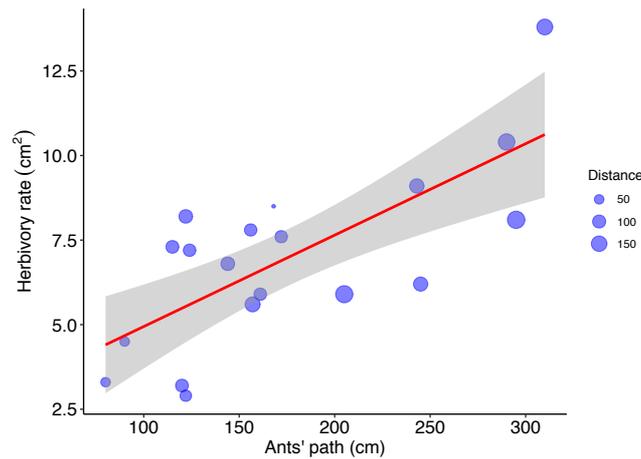


Figure 6 – Influence of straight distance of the ant nest and path length of ants in the herbivory rate of *Qualea multiflora*. Straight distance of the nest – GLM:  $\chi^2 = 1.046$ ,  $p = 0.306$ ; Path length of ants – GLM:  $\chi^2 = 13.450$ ,  $p < 0.001$ . Regression line is based on the linear model between herbivore rate and path length of ants ( $y = 2.24 + 0.02x$ ;  $R^2 = 0.53$ ). Despite not significant (GLM:  $\chi^2 = 1.046$ ,  $p = 0.306$ ), we plotted nest distance based on circles size.

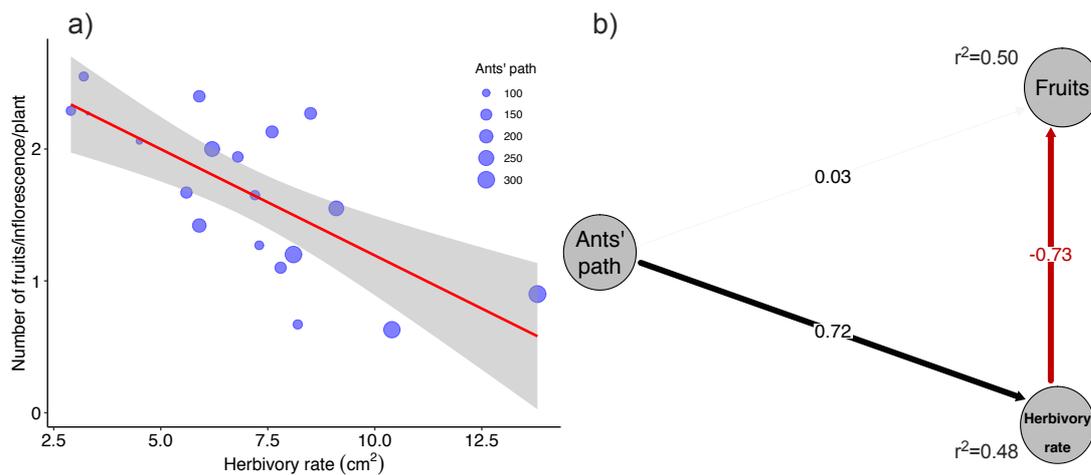


Figure 7 – (a) Influence of herbivory rate and path length of ants in the number of fruits produced per inflorescence per plant in *Qualea multiflora*. Herbivory rate – GLM:  $\chi^2 = 8.191$ ,  $p < 0.01$ ; Path length of ants – GLM:  $\chi^2 = 0.011$ ,  $p = 0.915$ . Regression line is based on the linear model between number of fruits produced and herbivore rate ( $y = 2.80 - 0.16x$ ;  $R^2 = 0.50$ ). (b) Path diagram showing the causal relationships between herbivory rate and path length of ants on the number of fruits produced. The  $r^2$  represents the *coefficient of nondetermination* ( $1-R^2$ ), which estimates the fraction of the variance that is not explained by the model.

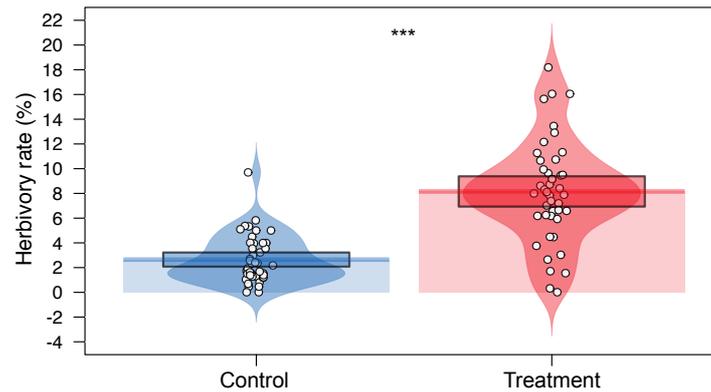


Figure 8 – Foliar herbivory rate in *Qualea multiflora* in the control (with ants) and treatment (without ants) groups. \*\*\*(GLMM:  $\chi^2 = 59.483$ ,  $p < 0.001$ ). Figures show raw data (points), descriptive statistics (line and density), and inferential statistics (Confidence interval – 95%).

## Discussion

Our results are consistent with the hypothesis that in extrafloral nectaried plants, the nectar supply directly influences the predator-prey relationship represented by the ant-herbivore interaction. The outcomes of these interactions are directly related to seasonality and synchronism of herbivore and ant populations (higher predation rate at times of greater herbivore abundance), and positive impact of ants on plant fitness (higher fruit set production and lower leaf area loss). Specifically, our results indicate that (i) there is a seasonality of EFN activity and herbivore and ant abundances, and a great temporal overlap especially for herbivore and ant abundance over the year; (ii) EFN activity, herbivore features and ant abundance are fundamental factors in predation of herbivores by ants; and (iii) the herbivory and fruit production are respectively influenced directly, and indirectly via herbivory rate, and also by path length of ants.

Ants have developed a series of morphological and behavioral adaptations to meet the needs of the colony as well as their own metabolic needs (Blüthgen and Feldhaar 2010). Even presenting a diet composed of plant-based resources (Blüthgen et al. 2003, Davidson et al. 2003), foliage-dwelling ants seek different nitrogen-rich resources, mainly obtained from preys

and carrion (Davidson 1997). Thus, from our results of seasonality and synchronism, foliage-dwelling ants present higher rates of abundance of their populations at times of greater abundance of herbivores, which is closely related to the activity of EFNs. In the Cerrado, EFNs are more active in the rainy season and are directly related to the production of new leaves (Lange et al. 2013) or flower buds and flowers (Vilela et al. 2014), which are highly consumed by herbivorous insects (Coley and Barone 1996, Ferreira and Torezan-Silingardi 2013). For instance, Vilela et al. (2014) showed that there was synchronism among the flowering period, EFNs activity, and presence of ants and herbivores in four species of Cerrado Malpighiaceae. From this perspective, we believe that EFN activity and the presence of young leaves and flowers attract ants and herbivores, influencing the predator-prey relationship represented by the ant-herbivore interaction.

In addition, ants may exhibit seasonal changes associated with foraging behavior by altering the amount of food collected, as well as changes in food preferences (Cook et al. 2011). Regarding the switching food preferences, ants and other animals may shift their food preferences due to the change in specific food availability, or to regulate nutrient intake (Whitford and Jackson 2007, Belchior et al. 2012, Anjos et al. 2019). For instance, Judd (2006) showed that *Pheidole ceres* colonies can switch their food preference in order to regulate nutrient intake, despite of relative abundance of available foods. In this way, many insects regulate their nutrient intake when there is opportunity; and when exposed to an unbalanced diet they seek to regulate the intake of foods that are in excess or in deficit (Behmer 2009).

The time to find the surrogate herbivore was influenced by the abundance of ants, but not by the straight nest distance, while the predation rate of the surrogate herbivore was influenced by the EFN activity, herbivore features and ant abundance. Plant protection by ants is influenced by several factors such as the quality of the resource (Bixenmann et al. 2011, Fagundes et al. 2017, Flores-Flores et al. 2018, Pacelhe et al. 2019), ant species and

aggressiveness (Cuautle et al. 2005, Byk and Del-Claro 2010, Fagundes et al. 2017), and the type of herbivore (Heads and Lawton 1985, Alves-Silva and Del-Claro 2016). Wagner (1997) showed that plants with basal nests presented much more foraging ants than plants without these nests, which positively influenced the number of seeds produced (see also Lanan and Bronstein 2013). In addition, EFNs activity influenced the predation rate of herbivores, since a greater abundance of ants was present in the period of greater activity of EFNs (Fig. 1), which increases the likelihood of encounter between ants and herbivores. As EFNs are highly carbohydrate-rich, ants that feed on this resource may exhibit an imbalance in the carbohydrate: protein ratio, which may influence the demand for nitrogen-rich resources as a way of balancing the nutrient deficiency, consequently increasing predation rate, and decreasing herbivory (see Behmer 2009).

Different surrogate herbivores (termite castes) presented different predation rates, where small and less aggressive herbivores (termite workers) were more preyed upon. Fewer herbivores have developed counter-adaptations against ant attack (Coley and Kursar 1996, Heil and McKey 2003, Dejean et al. 2006, Sousa-Lopes et al. 2019). However, there are some behavioral and morphological adaptations to avoid enemy attack, namely, flying, jumping to another place, jumping from the plant, secreting chemicals that hinder mobility or repel an enemy, curling up, among other types of defenses (see Heads and Lawton 1985). For instance, Alves-Silva and Del-Claro (2016) showed that the number of thrips and percentage of herbivory were higher in branches with the presence of ants than in branches without ants, since thrips could use the small spaces between the flower buds to hide from ants (thigmotaxis behavior). Another reason for the low predation rate of less aggressive herbivores is that we used termite soldiers as aggressive herbivores. As ants are major predators of termites (Traniello 1981) and there are records of this interaction more than 100 million years ago

(Engel et al. 2016), many species of termites have developed different defenses, which are especially employed against ants (Prestwich 1984).

Herbivory and fruit production are respectively influenced directly and indirectly via herbivory rate, by path length of ants. Our results showed that smaller path length of ants decreased the herbivory rate. Mutualistic ant nests closer to plants do not mean greater protection of leaves and/or fruit production, since the path length of ants is an important component for ant-plant interactions (see Dansa and Rocha 1992). Some ants tend to nest near food resources (see Wagner 1997, Wagner and Nicklen 2010) and then present a larger number of workers seeking for these resources. In this context, this greater number of workers can influence negatively the frequency of attacks of herbivores, decreasing herbivory rate (González-Teuber et al. 2012), which consequently, resulted in a higher fruit production. This result is consistent to Mothershead and Marquis (2000), who showed that individual plants of *Oenothera macrocarpa* (Onagraceae) with lower herbivory rates produced higher numbers of flowers, fruits and seeds. Another reason for lower fruit production in plants with higher herbivory rate is that plants with more leaf damage presented a higher loss of photosynthetic area, which results in a lower potential for production of photosynthates that would be allocated to fruit production (Taiz and Zeiger 2009). Finally, ant-free plants showed a higher herbivory rate and lower fruit production than plants with ants, which is confirmed by several studies (see review in Rosumek et al. 2009, Trager et al. 2010). These results together show that the nest distance and the presence of ants on plants may influence the herbivory rate, which can influence the fruit production, as a cascade effect.

In summary the extrafloral nectar supply is essential for predator-prey relationship represented by ant-herbivore interaction in plants bearing EFNs. These very conditional outcomes can fit the Lotka-Volterra model (predator-prey model) and can lead (i) to patterns of seasonality and synchronism of herbivore and ant populations, (ii) to higher predation rates

at times of greater herbivore abundance, and (iii) to impacts on plant fitness. Our study shows that ant-herbivore relationship is affected by a number of factors, which influence both interactors, and result in positive and/or negative impacts on the fitness of plants that host these interactions. We proposed a new approach on ant-herbivore interaction in EFN-bearing plants that could help in a better understanding of the patterns and dynamics of predator-prey relationships.

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Appendix 1 – Absolute and relative abundance of ant species observed during the day and at night in twenty species of plants bearing extrafloral nectaries from March 2017 to February 2018 in a cerrado reserve, Brazil. A. Ab (R. Ab%) – Absolute (Relative abundance%); D – Day time, N – Night time; DN – Day and Night time. Bc – *Bionia coriacea*, Bm – *Banisteriopsis malifolia*, Br – *Bauhinia rufa*, Bs – *Banisteriopsis stellaris*, Cb – *Caryocar brasiliense*, Eg – *Eriotheca gracilipes*, Hp – *Heteropterys pteropetala*, Lp – *Lafoensia pacari*, Mc – *Manihot caerulescens*, Oh – *Ouratea hexasperma*, Os – *Ouratea spectabilis*, Pr – *Palicourea rigida*, Qg – *Qualea grandiflora*, Qm – *Qualea multiflora*, Qp – *Qualea parviflora*, Sp – *Stryphnodendron polyphyllum*, Sr – *Senna rugosa*, Sv – *Senna velutina*, Sxp – *Smilax polyantha*, Tf – *Tocoyena formosa*.

Subfamily/species	A. Ab (R. Ab%)	Activity period	Plant species associated
<b>Dolichoderinae</b>	<b>521(9.78)</b>		
<i>Azteca</i> sp.1	509(9.55)	DN	Br, Cb, Oh, Qp, Sp, Sr, Tf
<i>Dolichoderus lutosus</i> (Smith, 1858)	11(0.20)	N	Cb, Sr, Sv
<i>Dolichoderus</i> sp.	1(0.01)	N	Sp
<b>Ectatomminae</b>	<b>337(6.32)</b>		
<i>Ectatomma brunneum</i> Smith, 1858	9(0.16)	DN	Bm
<i>Ectatomma tuberculatum</i> (Olivier, 1792)	323(6.06)	DN	Bc, Bm, Br, Bs, Cb, Eg, Hp, Mc, Oh, Pr, Qg, Qm, Qp, Sp, Sr, Sv, Sxp, Tf
<i>Gnamptogenys semiferox</i> Brown, 1958	5(0.09)	DN	Mc, Oh, Sp, Sv
<b>Formicinae</b>	<b>2744(51.5)</b>		
<i>Brachymyrmex</i> sp.1	644(12.0)	DN	Bc, Bm, Br, Bs, Eg, Hp, Lp, Mc, Oh, Os, Pr, Qg, Qm, Qp, Sp, Sr, Sv, Sxp, Tf
<i>Brachymyrmex</i> sp.2	397(7.45)	DN	Br, Bs, Hp, Lp, Mc, Oh, Os, Qg, Qm, Qp, Sp, Sr, Sv, Sxp, Tf
<i>Camponotus ager</i> (Smith, 1858)	4(0.07)	N	Eg, Oh, Qm
<i>Camponotus atriceps</i> (Smith, 1858)	264(4.95)	N	Bc, Bm, Br, Bs, Cb, Eg, Hp, Lp, Mc, Oh, Os, Qg, Qm, Qp, Sp, Sr, Sxp, Tf
<i>Camponotus blandus</i> (Smith, 1858)	123(2.30)	D	Bm, Eg, Hp, Mc, Os, Qm, Sp, Sv, Tf
<i>Camponotus crassus</i> Mayr, 1862	790(14.8)	DN	Bc, Bm, Br, Bs, Cb, Eg, Hp, Lp, Mc, Oh, Os, Pr, Qg, Qm, Qp, Sp, Sr, Sv, Sxp, Tf
<i>Camponotus lespesii</i> Forel, 1886	9(0.16)	N	Eg, Oh, Qg, Qm
<i>Camponotus leydigii</i> Forel, 1886	7(0.13)	DN	Bm, Qp
<i>Camponotus renggeri</i> Emery, 1894	374(7.02)	DN	Bc, Bm, Br, Bs, Cb, Eg, Hp, Lp, Oh, Os, Pr, Qg, Qm, Qp, Sp, Sr, Sv, Sxp, Tf
<i>Camponotus rufipes</i> (Fabricius, 1775)	15(0.28)	N	Oh, Pr, Qg, Sxp, Tf
<i>Camponotus</i> sp.1	24(0.45)	N	Bm, Cb, Eg, Lp, Sp, Sxp
<i>Camponotus</i> sp.2	15(0.28)	DN	Eg, Oh
<i>Camponotus</i> sp.3	9(0.16)	N	Oh
<i>Camponotus</i> sp.4	7(0.13)	D	Qg, Qm
<i>Camponotus</i> sp.5	5(0.09)	N	Br
<i>Camponotus substitutus</i> Emery, 1894	57(1.07)	N	Bc, Bm, Br, Bs, Cb, Eg, Hp, Oh, Qg, Qp, Sp, Sr, Sxp
<b>Myrmicinae</b>	<b>1668(31.3)</b>		
<i>Cephalotes pusillus</i> (Klug, 1824)	226(4.24)	DN	Bm, Br, Cb, Eg, Hp, Lp, Mc, Oh, Os, Qg, Qm, Qp, Sp, Sr, Sv, Sxp, Tf
<i>Cephalotes</i> sp.1	1(0.01)	D	Sxp
<i>Cephalotes</i> sp.2	1(0.01)	N	Oh

## Appendix 1. Continued

Subfamily/species	A. Ab (R. Ab%)	Activity period	Plant species associated
<i>Crematogaster</i> cf. <i>victima</i>	6(0.11)	D	Sp
<i>Crematogaster erecta</i> Mayr, 1866	296(5.55)	DN	Bm, Bs, Eg, Hp, Os, Pr, Tf
<i>Crematogaster</i> sp.1	557(10.4)	DN	Bc, Bm, Br, Cb, Eg, Hp, Lp, Oh, Os, Qm, Qp, Sp, Sr, Sxp, Tf
<i>Crematogaster</i> sp.2	170(3.19)	DN	Oh, Os, Sr
<i>Pheidole</i> cf. <i>oxyops</i>	16(0.30)	N	Bm, Oh, Os
<i>Pheidole</i> sp.1	5(0.09)	DN	Bs, Sp
<i>Pheidole</i> sp.2	14(0.26)	DN	Pr
<i>Solenopsis</i> sp.1	111(2.08)	DN	Bm, Br, Lp, Mc, Oh, Pr, Qg, Qm, Sxp
<i>Solenopsis</i> sp.2	137(2.57)	DN	Bs, Cb, Os, Pr, Qg, Qm, Sp, Sr
<i>Wasmannia</i> sp.	128(2.40)	DN	Eg, Mc, Qg, Qp, Sp, Sxp
<b>Ponerinae</b>	<b>17(0.31)</b>		
<i>Neoponera rostrata</i> (Emery, 1890)	5(0.09)	N	Eg, Os, Qp
<i>Neoponera villosa</i> (Fabricius, 1804)	12(0.22)	DN	Bm, Br, Eg, Qg, Sv, Sxp
<b>Pseudomyrmecinae</b>	<b>39(0.73)</b>		
<i>Pseudomyrmex</i> cf. <i>pallidus</i>	8(0.15)	DN	Bm, Eg, Hp, Qp, Sp
<i>Pseudomyrmex flavidulus</i> (Smith, 1858)	7(0.13)	D	Bm, Lp, Os, Qm, Qp
<i>Pseudomyrmex gracilis</i> (Fabricius, 1804)	24(0.45)	D	Bs, Eg, Hp, Lp, Mc, Oh, Os, Pr, Qg, Qm, Qp, Sp, Sr, Tf
<b>Total</b>	<b>5326(100)</b>		

Appendix 2 – Absolute and relative abundance of herbivore species observed during the day and night in twenty species of plants bearing extrafloral nectaries from March 2017 to February 2018 in a cerrado reserve, Brazil. A. Ab (R. Ab%) – Absolute (Relative abundance%); D – Day time, N – Night time; DN – Day and Night time. Bc – *Bionia coriacea*, Bm – *Banisteriopsis malifolia*, Br – *Bauhinia rufa*, Bs – *Banisteriopsis stellaris*, Cb – *Caryocar brasiliense*, Eg – *Eriotheca gracilipes*, Hp – *Heteropterys pteropetala*, Lp – *Lafoensia pacari*, Mc – *Manihot caerulescens*, Oh – *Ouratea hexasperma*, Os – *Ouratea spectabilis*, Pr – *Palicourea rigida*, Qg – *Qualea grandiflora*, Qm – *Qualea multiflora*, Qp – *Qualea parviflora*, Sp – *Stryphnodendron polyphyllum*, Sr – *Senna rugosa*, Sv – *Senna velutina*, Sxp - *Smilax polyantha*, Tf – *Tocoyena formosa*.

Order/Family/species	A. Ab (R. Ab%)	Activity period	Plant species associated
<b>Coleoptera</b>	<b>557(34,3)</b>	<b>DN</b>	
Buprestidae	3(0,18)	N	
Buprestidae sp.	3(0,18)	N	Os, Qm, Qp
Cerambycidae	1(0,06)	N	
Lamiinae sp.	1(0,06)	N	Qg
<b>Chrysomelidae</b>	<b>312(19,2)</b>	<b>DN</b>	
Cassidinae sp.	3(0,18)	DN	Br, Hp
Chalepini sp.	3(0,18)	D	Br
Chrysomelidae sp.1	79(4,87)	DN	Bm, Bs, Cb, Eg, Hp, Lp, Mc, Oh, Os, Pr, Qg, Qm, Qp, Sp, Sr, Sxp, Tf
Chrysomelidae sp.2	98(6,04)	DN	Bc, Bm, Br, Bs, Cb, Eg, Hp, Lp, Oh, Os, Qg, Qm, Qp, Sp, Sr, Sxp
Chrysomelidae sp.3	51(3,14)	DN	Bc, Bm, Br, Bs, Cb, Eg, Lp, Mc, Oh, Os, Qg, Qm, Qp, Sp, Sr, Sv, Tf
Chrysomelidae sp.4	1(0,06)	N	Sp
Chrysomelidae sp.5	1(0,06)	N	Oh
Chrysomelidae sp.6	21(1,29)	DN	Br, Bs, Hp, Lp, Oh, Qm, Qp, Sp, Sr
Chrysomelidae sp.7	12(0,74)	DN	Bs, Cb, Lp, Sr
Chrysomelidae sp.8	41(2,53)	DN	Bm, Br, Bs, Cb, Eg, Hp, Mc, Qg, Sr, Sxp
Chrysomelidae sp.9	1(0,06)	N	Oh
<i>Disonycha</i> sp.	1(0,06)	N	Sr
<b>Curculionidae</b>	<b>135(8,33)</b>	<b>DN</b>	
Curculionidae sp.1	76(4,69)	DN	Bc, Bm, Hp, Lp, Oh, Os, Qg, Qm, Sp, Sr, Tf
Curculionidae sp.2	1(0,06)	N	Os
Curculionidae sp.3	1(0,06)	D	Sr
Curculionidae sp.4	4(0,24)	DN	Sp
Curculionidae sp.5	3(0,18)	N	Lp, Os
Curculionidae sp.6	12(0,74)	DN	Lp, Oh, Qm, Qp
Curculionidae sp.7	4(0,24)	N	Os
Curculionidae sp.8	2(0,12)	D	Qg, Tf
Curculionidae sp.9	1(0,06)	N	Mc, Qp
Curculionidae sp.10	3(0,18)	DN	Cb
Curculionidae sp.11	20(1,23)	DN	Bm
Curculionidae sp.12	1(0,06)	N	Tf

## Appendix 2. Continued

<b>Order/Family/species</b>	<b>A. Ab (R. Ab%)</b>	<b>Activity period</b>	<b>Plant species associated</b>
Curculionidae sp.13	3(0,18)	DN	Bs
Curculionidae sp.14	3(0,18)	N	Bm, Eg, Hp, Sp, Tf
Entiminae sp.	1(0,06)	N	Qm
<b>Elateridae</b>	<b>7(0,43)</b>	<b>DN</b>	
Agrypninae sp.	2(0,12)	N	Qp
Elateridae sp.1	4(0,24)	DN	Bm, Qm, Sp, Sv
Elateridae sp.2	1(0,06)	N	Lp
<b>Phengodidae</b>	<b>1(0,06)</b>	<b>N</b>	
Phengodidae sp.	1(0,06)	N	Tf
<b>Scarabaeidae</b>	<b>26(1,60)</b>	<b>DN</b>	
<i>Catharsius molossus</i>	1(0,06)	N	Cb
Cyclocephala sp.1	22(1,35)	DN	Cb, Hp, Qg, Qp
Cyclocephala sp.2	2(0,12)	N	Hp
Scarabaeidae sp.2	1(0,06)	D	Qm
<b>Tenebrionidae</b>	<b>48(2,96)</b>	<b>DN</b>	
Allecula sp.1	10(0,61)	DN	Eg, Hp, Lp, Os, Qm, Qp, Sp
Allecula sp.2	20(1,23)	DN	Br, Cb, Lp, Qg, Qp
Pimeliinae sp.1	1(0,06)	D	Tf
Pimeliinae sp.2	3(0,18)	DN	Eg, Qm, Sp, Sv
Tenebrionidae sp.1	9(0,55)	DN	Bm, Hp, Sp
Tenebrionidae sp.2	4(0,24)	N	Tf
Tenebrionidae sp.3	1(0,06)	D	Sr
<b>Unidentified</b>	<b>24(1,48)</b>	<b>DN</b>	
Coleoptera sp.1	1(0,06)	D	Hp
Coleoptera sp.2	12(0,74)	DN	Lp, Qg, Qp
Coleoptera sp.3	1(0,06)	N	Bm
Coleoptera sp.4	4(0,24)	DN	Eg, Sp
Coleoptera sp.5	3(0,18)	N	Qm, Tf
Coleoptera sp.6	2(0,12)	DN	Cb
Coleoptera sp.7	1(0,06)	D	Sv
<b>Hemiptera</b>	<b>729(45,0)</b>	<b>DN</b>	
<b>Acanaloniidae</b>	<b>1(0,06)</b>	<b>N</b>	
Acanaloniidae sp.1	1(0,06)	N	Eg
<b>Aleyrodidae</b>	<b>20(1,23)</b>	<b>DN</b>	
Aleyrodidae sp.	20(1,23)	DN	Qm, Qp, Sr
<b>Alydidae</b>	<b>59(3,64)</b>	<b>DN</b>	
Alydidae sp.1	6(0,37)	DN	Br, Oh, Qp, Sv
<i>Alydus</i> sp.	1(0,06)	D	Oh
<i>Hyalymenus</i> sp.1	33(2,03)	DN	Bc, Br, Lp, Oh, Os, Qg, Qm, Sr, Sv
<i>Hyalymenus</i> sp.2	19(1,17)	DN	Bc, Hp, Os, Qg, Sr, Sv

## Appendix 2. Continued

Order/Family/species	A. Ab (R. Ab%)	Activity period	Plant species associated
Aphididae	212(13,0)	DN	
Aphididae sp.1	187(11,5)	DN	Bm, Eg
Aphididae sp.2	13(0,80)	DN	Eg, Oh, Os
Aphididae sp.3	11(0,67)	D	Sr, Sv
Aphididae sp.4	1(0,06)	N	Os
Cercopidae	8(0,49)	DN	
Cercopidae sp.1	4(0,24)	DN	Oh, Os, Sxp
Cercopidae sp.2	4(0,24)	N	Bs, Qp
Cicadellidae	245(15,1)	DN	
Cicadellidae sp.1	5(0,30)	DN	Sv, Sxp, Tf
Cicadellidae sp.2	8(0,49)	DN	Bm, Br, Oh, Os
Cicadellidae sp.3	2(0,12)	N	Os
Cicadellidae sp.5	1(0,06)	N	Bm, Eg, Lp, Qm, Sr
Cicadellidae sp.6	1(0,06)	N	Lp, Qg, Qm, Sp
Cicadellidae sp.7	2(0,12)	DN	Qm
Cicadellidae sp.8	3(0,18)	DN	Br, Cb, Hp, Lp, Oh, Qm, Sp, Sr
Cicadellidae sp.9	33(2,03)	DN	Oh, Qg, Qm, Sv, Sxp
Cicadellidae sp.10	6(0,37)	DN	Cb, Lp, Os
Cicadellidae sp.11	1(0,06)	N	Br, Tf
Cicadellidae sp.12	5(0,30)	DN	Qg
Cicadellidae sp.13	11(0,67)	N	Qm
Cicadellidae sp.14	2(0,12)	DN	Qg, Qp
Cicadellidae sp.15	12(0,74)	N	Br, Lp, Sp
Cicadellidae sp.16	9(0,55)	DN	Bm, Br, Bs, Cb, Eg, Hp, Lp, Oh, Os, Qm, Qp, Sp, Sv, Sxp, Tf
Coronigonalia sp.	3(0,18)	N	Br, Qg
<i>Empoasca kraemeri</i>	140(8,64)	DN	Bc, Bm, Br, Bs, Cb, Eg, Hp, Lp, Oh, Os, Pr, Qg, Qm, Qp, Sv, Sxp, Tf
<i>Macugonalia cavifrons</i>	1(0,06)	D	Qg
Coccidae	29(1,79)	DN	
Coccidae sp.1	9(0,55)	D	Lp, Sr
Coccidae sp.2	20(1,23)	DN	Bm, Qm
Coreidae	63(3,88)	DN	
<i>Amorbini</i> sp.	1(0,06)	N	Os
<i>Cebrenis cauta</i>	60(3,70)	DN	Bm, Br, Bs, Hp, Lp, Qg, Qm, Qp, Sp, Sv, Tf
Coreidae sp.1	1(0,06)	D	Bc
Coreidae sp.2	1(0,06)	D	Lp
Fulgoridae	3(0,18)	N	
Fulgoridae sp.1	3(0,18)	N	Os
Membracidae	7(0,43)	DN	
Membracidae sp.1	3(0,18)	D	Sr

## Appendix 2. Continued

<b>Order/Family/species</b>	<b>A. Ab (R. Ab%)</b>	<b>Activity period</b>	<b>Plant species associated</b>
<i>Membracis trimaculata</i>	3(0,18)	D	Bs
<i>Oeda (Oedacanthus) informis</i>	1(0,06)	N	Qg
<b>Miridae</b>	<b>5(0,30)</b>	<b>N</b>	
<i>Lygus</i> sp.	5(0,30)	N	Bm, Oh, Sv
<b>Ortheziidae</b>	<b>5(0,30)</b>	<b>DN</b>	
Ortheziidae sp.	5(0,30)	DN	Oh, Qm
<b>Pentatomidae</b>	<b>15(0,92)</b>	<b>DN</b>	
<i>Dichelops</i> sp.	1(0,06)	D	Os
Discocephalinae sp.	2(0,12)	N	Bc, Mc
Edessinae sp.	1(0,06)	N	Hp
Pentatomidae sp.1	5(0,30)	DN	Sp, Sv
Pentatomidae sp.2	1(0,06)	N	Sv
Pentatomidae sp.3	4(0,24)	DN	Hp, Lp, Tf
<i>Proxys albopunctulatus</i>	1(0,06)	N	Tf
<b>Pyrhocoridae</b>	<b>4(0,24)</b>	<b>D</b>	
Pyrhocoridae sp.1	1(0,06)	D	Hp
Pyrhocoridae sp.2	3(0,18)	D	Lp, Sv
<b>Rhopalidae</b>	<b>2(0,12)</b>	<b>D</b>	
Rhopalidae sp.1	2(0,12)	D	Mc
<b>Scutelleridae</b>	<b>1(0,06)</b>	<b>N</b>	
Pachycorinae sp.1	1(0,06)	N	Bm
<b>Unidentified</b>	<b>50(3,08)</b>	<b>DN</b>	
Fulgoroidea sp.1	11(0,67)	DN	Bm, Br, Eg, Qg, Sxp, Tf
Fulgoroidea sp.2	8(0,49)	DN	Cb, Hp, Oh, Os, Qp
Fulgoroidea sp.3	14(0,86)	DN	Br, Sxp
Lygaeoidea sp.1	14(0,86)	DN	Br, Eg, Hp, Oh, Os, Qg
Lygaeoidea sp.2	3(0,18)	N	Hp, Oh, Os
<b>Hymenoptera</b>	<b>4(0,24)</b>	<b>D</b>	
<b>Unidentified</b>	<b>4(0,24)</b>	<b>D</b>	
Tenthredinoidea sp.1	4(0,24)	D	Eg
<b>Lepidoptera</b>	<b>64(3,95)</b>	<b>DN</b>	
<b>Arctiidae</b>	<b>9(0,55)</b>	<b>DN</b>	
Arctiinae sp.1	2(0,12)	DN	Os
Arctiinae sp.2	2(0,12)	DN	Eg, Os
Arctiinae sp.3	2(0,12)	DN	Os
Arctiinae sp.4	1(0,06)	N	Qm
Arctiinae sp.5	2(0,12)	DN	Oh
<b>Elachistidae</b>	<b>1(0,06)</b>	<b>D</b>	
<i>Chlamydistis</i> sp.	1(0,06)	D	Sxp
<b>Erebidae</b>	<b>2(0,12)</b>	<b>DN</b>	

## Appendix 2. Continued

<b>Order/Family/species</b>	<b>A. Ab (R. Ab%)</b>	<b>Activity period</b>	<b>Plant species associated</b>
Erebidae sp.1	1(0,06)	D	Sp
Erebidae sp.2	1(0,06)	N	Bs
<b>Geometridae</b>	<b>17(1,04)</b>	<b>DN</b>	
Geometridae sp.1	1(0,06)	D	Bm
Geometridae sp.2	3(0,18)	N	Lp
Geometridae sp.3	2(0,12)	D	Lp
Geometridae sp.4	3(0,18)	N	Cb
Geometridae sp.5	2(0,12)	N	Hp, Sv
Geometridae sp.6	1(0,06)	D	Bs
<i>Pseudasellodes</i> sp.	5(0,30)	DN	Oh, Tf
<b>Limacodidae</b>	<b>4(0,24)</b>	<b>DN</b>	
Limacodidae sp.1	2(0,12)	N	Bs
Limacodidae sp.2	1(0,06)	N	Sr
<i>Phobetron</i> sp.	1(0,06)	D	Os
<b>Lycaenidae</b>	<b>2(0,12)</b>	<b>D</b>	
Lycaenidae sp.1	1(0,06)	D	Bm
<i>Tmolus</i> sp.	1(0,06)	D	Bm
<b>Notodontidae</b>	<b>3(0,18)</b>	<b>DN</b>	
Notodontidae sp.1	3(0,18)	DN	Cb
<b>Oecophoridae</b>	<b>11(0,67)</b>	<b>DN</b>	
Oecophoridae sp.1	1(0,06)	D	Lp
Oecophoridae sp.2	1(0,06)	D	Bm
Oecophoridae sp.3	1(0,06)	D	Bm
Oecophoridae sp.4	6(0,37)	DN	Sp
Oecophoridae sp.5	2(0,12)	D	Os
<b>Oxytenidae</b>	<b>5(0,30)</b>	<b>DN</b>	
Oxyteninae sp.1	5(0,30)	DN	Tf
<b>Psychidae</b>	<b>3(0,18)</b>	<b>DN</b>	
Psychidae sp.	3(0,18)	DN	Sxp
<b>Saturniidae</b>	<b>1(0,06)</b>	<b>N</b>	
<i>Citheronia laocoon</i>	1(0,06)	N	Tf
<b>Unidentified</b>	<b>6(0,37)</b>	<b>DN</b>	
Lepidoptera sp.1	1(0,06)	D	Sp
Lepidoptera sp.2	1(0,06)	D	Qm
Lepidoptera sp.3	1(0,06)	N	Pr
Lepidoptera sp.4	1(0,06)	N	Sp
Lepidoptera sp.5	1(0,06)	D	Sp
Lepidoptera sp.6	1(0,06)	D	Oh
<b>Orthoptera</b>	<b>167(10,3)</b>	<b>DN</b>	
Acrididae	31(1,91)	DN	

## Appendix 2. Continued

Order/Family/species	A. Ab (R. Ab%)	Activity period	Plant species associated
Acrididae sp.1	10(0,61)	DN	Br, Cb, Eg, Os, Qg, Qm, Tf
Acrididae sp.2	9(0,55)	DN	Bc, Oh, Qp, Sr, Tf
Acrididae sp.3	3(0,18)	N	Hp
Acrididae sp.4	1(0,06)	D	Hp
Acrididae sp.5	4(0,24)	DN	Bc, Qm, Sr
Acrididae sp.6	1(0,06)	N	Br
Acrididae sp.7	3(0,18)	DN	Bm, Bs, Qg
<b>Eumastacidae</b>	<b>23(1,41)</b>	<b>DN</b>	
Eumastacidae sp.1	3(0,18)	DN	Oh, Sp
Eumastacidae sp.2	10(0,61)	DN	Bm, Br, Cb, Qg, Sp
Eumastacidae sp.3	8(0,49)	DN	Bs, Cb, Qg, Qp
Eumastacidae sp.4	2(0,12)	D	Cb, Sp
<b>Gryllidae</b>	<b>35(2,16)</b>	<b>DN</b>	
Gryllidae sp.1	10(0,61)	DN	Br, Oh, Qm, Qp, Sp
Gryllidae sp.2	14(0,86)	N	Br, Bs, Hp, Os, Qm, Sr, Sv, Sxp
Gryllidae sp.3	3(0,18)	N	Sr
Gryllidae sp.4	4(0,24)	DN	Lp, Sr
Gryllidae sp.5	4(0,24)	N	Sxp, Tf
<b>Proscopiidae</b>	<b>1(0,06)</b>	<b>N</b>	
Proscopiidae sp.	1(0,06)	N	Sxp
<b>Romaleidae</b>	<b>5(0,30)</b>	<b>N</b>	
Romaleidae sp.1	1(0,06)	N	Os
Romaleidae sp.2	4(0,24)	N	Cb, Oh, Qg
<b>Tettigoniidae</b>	<b>72(4,44)</b>	<b>DN</b>	
Conocephalinae sp.1	1(0,06)	N	Bm
Conocephalinae sp.2	1(0,06)	N	Lp
<i>Microcentrum</i> sp.	2(0,12)	N	Qp
Neoconocephalus sp.1	1(0,06)	N	Qm
Neoconocephalus sp.2	1(0,06)	N	Tf
Neoconocephalus sp.3	1(0,06)	N	Cb
Phaneropterinae sp.1	11(0,67)	DN	Bm, Bs, Hp, Oh, Sp, Sxp
Phaneropterinae sp.2	1(0,06)	N	Oh
Phaneropterinae sp.3	1(0,06)	D	Qg
Pseudophilinae sp.4	1(0,06)	N	Os
Pseudophyllinae sp.1	1(0,06)	D	Eg
Pseudophyllinae sp.2	3(0,18)	N	Oh, Qp
Pseudophyllinae sp.3	1(0,06)	N	Oh
Pycnopalpini sp.	2(0,12)	N	Oh
<i>Scudderia</i> sp.1	2(0,12)	D	Bc, Oh
<i>Scudderia</i> sp.2	2(0,12)	D	Bm

## Appendix 2. Continued

<b>Order/Family/species</b>	<b>A. Ab (R. Ab%)</b>	<b>Activity period</b>	<b>Plant species associated</b>
<i>Scudderia</i> sp.3	2(0,12)	DN	Oh
Tettigoniidae sp.1	7(0,43)	DN	Oh, Qg, Qm, Tf
Tettigoniidae sp.2	7(0,43)	DN	Bs, Cb, Eg, Oh, Sr
Tettigoniidae sp.3	9(0,55)	DN	Br, Bs, Eg, Oh, Os, Qg, Sp
Tettigoniidae sp.4	2(0,12)	N	Oh
Tettigoniidae sp.5	9(0,55)	DN	Eg, Mc
Tettigoniidae sp.6	2(0,12)	N	Sxp
Tettigoniidae sp.7	1(0,06)	N	Lp
<i>Topana</i> sp.	1(0,06)	N	Eg
<b>Psocoptera</b>	<b>32(1,97)</b>	<b>DN</b>	
Unidentified	32(1,97)	DN	
Psocoptera sp.1	18(1,11)	DN	Br, Cb, Eg, Mc, Os, Sp, Sxp
Psocoptera sp.2	14(0,86)	DN	Qg, Qm, Qp
<b>Thysanoptera</b>	<b>67(4,13)</b>	<b>DN</b>	
Heterothripidae	48(2,96)	DN	
Heterothrips sp.1	48(2,96)	DN	Bm, Bs, Cb
Unidentified	19(1,17)	DN	
Thysanoptera sp.1	3(0,18)	D	Bs
Thysanoptera sp.2	9(0,55)	DN	Bm
Thysanoptera sp.3	7(0,43)	DN	Bm, Qm
<b>Total</b>	<b>1620(100)</b>		

## **Chapter 3**

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**Balanced carbohydrate-protein diet and extrafloral nectar  
increase ant colony fitness and survival over short- and long-term**

**Balanced carbohydrate-protein diet and extrafloral nectar increase ant colony fitness and survival over short- and long-term**

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## Abstract

Ants forage on plants mainly for sugar and proteins, and extrafloral nectar (a carbohydrate-rich liquid with small amounts of amino acids) is the main plant-based resource offered in exchange for ant protection. In this perspective, we investigated the benefits of extrafloral nectar and liquids based on different carbohydrate-protein balance on the ant colony fitness (number and weight of individuals) and survival. We conducted two sets of experiments: in the field (1) we attached artificial ant nests to plants with clogged and unclogged EFNs; and at the laboratory (2) we offered artificial liquid food resources with different carbohydrate-protein balance ratio, in order to evaluate colony fitness and survival in short and long-term. Our results showed that the availability and short-term provision of extrafloral nectar had a positive influence on ant colony fitness. Additionally, regardless of time the supply of more concentrated carbohydrate and nitrogen food increased the colony fitness and survival corroborating our main hypotheses. We provided evidence that a sugar-rich liquid source like extrafloral nectar significantly benefits ant colonies, which is a basic step in the establishment of most ant-plant mutualisms.

**Key words:** *Camponotus crassus*, Extrafloral nectaries, Mutualism, Biotic defense.

## Introduction

Ants are extremely abundant and diverse in the terrestrial environment (Hölldobler and Wilson 1990, Agosti and Johnson 2003). Due to their high representativeness and diversity, several distinct behavioral and eating habits have evolved. Thus, based on food sources and feeding modes (Carroll and Janzen 1973), ants can be classified into four groups: detritivores, herbivores, omnivores and carnivores (Hölldobler and Wilson 1990). Among these groups, we can highlight the omnivorous ants that feed on resources found or provided by plants, mainly the extrafloral nectar.

The system in which arboreal ants feed on extrafloral nectar is common in terrestrial environments and very studied (Belt 1874, Zimmermann 1932, Rico-Gray and Oliveira 2007, Calixto et al. 2018a). Plants bearing EFNs provide nectar as a food resource in exchange to ants' protection against herbivores (Rico-Gray and Oliveira 2007, Heil 2015, Del-Claro et al. 2016, Calixto et al. 2018a). Besides this defensive role, ants can still increase plant's fitness, reducing leaf are loss and increasing seed set production (Nascimento and Del-Claro 2010). On the other hand, extrafloral nectar is a key resource for growth and development of colonies. By feeding on EFNs, ants can increase the colony survival and even show a significant increase in the individual weight of queen and workers (Byk and Del-Claro 2011). Lach et al. (2009) showed that both native and invasive ant species can benefit from extrafloral nectar, where omnivorous ants had a survival rate of up to seven times greater than ants that did not feed on EFNs.

Indeed, the extrafloral nectar is expected to influence the fitness and survival of arboreal ant colonies, since this food resource is rich in carbohydrates (Nicolson and Thornburg 2007, González-Teuber and Heil 2009), which has been suggested to be a fundamental resource for foliage-dwelling ants (Davidson et al. 2003). In addition to carbohydrates, nitrogen which is the basis of nucleic acids and proteins, is essential for ant larvae development (Blüthgen and

Feldhaar 2010) influencing directly the colony fitness. Due to low nitrogen concentration in EFNs (Koptur 1994b, González-Teuber and Heil 2009), foraging ants must harvest large volumes of nectar in order to sustain protein demands of growing larvae (Blüthgen and Feldhaar 2010) or search for preys and carrion on hosted plants (Davidson 1997).

In this context, it is fundamental to understand how different types of food resources, mainly carbohydrate- and nitrogen-based resources, influence the ant colony fitness and survival. Thus, our aim was to evaluate the short- and long-term benefits of extrafloral nectar and artificial liquid food source on the ant colony fitness (number and weight of individuals) and survival. We sought to answer two main questions: (i) may the presence and the short-term supply of extrafloral nectar affect the fitness and survival of ants?; and (ii) will the short- and long-term supply of more concentrated carbohydrate and nitrogen food resources increase colony fitness and survival of individuals compared to nutrient-poor resources? We believed that (1) the availability and short-term supply of extrafloral nectar would increase the colony fitness and survival, in contrast to colonies that do not feed on this resource; and that (2) carbohydrate- and nitrogen-rich food resources would have a positive impact on ant regardless of exposure time.

## **Materials and Methods**

### **Study area, plant species and ant species**

The fieldwork was conducted in the Ecological Reserve of the Clube de Caça e Pesca Itororó de Uberlândia (CCPIU: 48°17'W; 18°58'S), in Uberlândia, Brazil. The ~200 ha reserve is located in the Cerrado biome, which presents a gradient of open areas with sparse cover of shrubs and small trees to more enclosed areas with trees reaching from 12 to 15 meters in height. The region is characterized by rainy summer (from October to March) and dry winter

(from April to September); the mean annual temperature varies from 18 to 28° C and the rainfall from 800 to 2,000 mm (Ferreira and Torezan-Silingardi 2013, Vilela et al. 2014).

The extrafloral nectaried plant used was *Banisteriopsis malifolia* Nees & Mart. (Malpighiaceae), which is a shrub, reaching up to 3 meters tall (Alves-Silva 2011, Alves-Silva et al. 2013, Ferreira and Torezan-Silingardi 2013). The leaves have a pair of EFNs at the base, near the petiole, one on each side of the leaf central vein, which remain active during flowering. This plant species is abundant in the study area and is visited for a large number of ant species that feed on EFNs (Vilela et al. 2014).

The ant species used in our study was *Camponotus crassus* (Formicinae). The genus *Camponotus* is often found in plants bearing EFNs, and especially *C. crassus* seems to be one of the main ants that interact with this plant in the Cerrado (Lange and Del-Claro 2014, Anjos et al. 2017, Lange et al. 2017). It has a diurnal habit, nests in the soil and is an aggressive and dominant ant on hosted plants (Anjos et al. 2017, Fagundes et al. 2017).

## **Data collection**

### *Extrafloral nectar feeding experiment*

During the morning (~11:00 am) in a single day in the rainy season at the beginning of January 2018, we observed ant queens walking freely on the surface of the ground at the edge of a trail inside the ecological reserve. We collected 18 queens of *C. crassus*, and separated each fertilized queen (wingless) into a 50 ml falcon tube, which contained small hydrophilic cotton moistened with water.

In the laboratory, we weighed and put each queen into a squared plastic container 12x12x6 cm (hereafter “artificial nest”), which had an empty plastic microtube eppendorf (hereafter “eppendorf”) of 2 ml wrapped in red transparent cellophane paper and a 20 ml test tube filled with water concealed by hydrophilic cotton. The eppendorf and cellophane paper were used to simulate an ant queen chamber, and the test tube was used to allow water

evaporation and humidification of the container. Artificial nests were kept in the laboratory under natural conditions and fed every four days with 2 ml of a sugar diet composed of 10  $\mu\text{g}\cdot\mu\text{L}^{-1}$  of sugar and 1  $\mu\text{g}\cdot\mu\text{L}^{-1}$  of cysteine. This solution is similar to extrafloral nectar composition (see González-Teuber and Heil 2009, Lange et al. 2017) and consisted of a sugar ratio of 1 mg sucrose/(0.5 mg fructose + 0.5 mg glucose) (adapted from Bixenmann et al. 2011).

At the same study area and day, we selected 18 *B. malifolia* individuals with similar characteristics (similar height, number of branches, number of inflorescences, and habitat). All plants contained active EFNs during all the experiment, and were isolated from other ants through a non-toxic resin (Tanglefoot) applied around the stem base 30 cm from the ground. All stems that could be used as bridges to ants climb onto the plants were manually removed. Then, we randomly divided the plants into two groups: “With EFNs” - plants had active and available EFNs for ants; and “Without EFNs” - where all EFNs were clogged with colorless and non-toxic nail polish (Risqué brand). In With EFNs treatment, we also added a similar amount of nail polish next to each EFN without blocking the production of nectar. The nail polish was replaced every week.

Randomly, each artificial nest was allocated to an individual plant. We took care to place the nest horizontally and always in similar height on all plants (1.0-1.5 m from the ground). At the beginning of February, all nests were placed between 6 am and 9 am, when *C. crassus* individuals are still with low foraging activity, and therefore the nest manipulation should have caused low stress on the colonies (Lange et al. 2019). Small holes were made in the lid to allow ventilation, and three holes (larger than the workers and smaller than the queen) were made laterally in the containers allowing the foraging of the worker ants on the plant. Before placing the artificial nests on plants, we made sure that each colony had only one queen, three adult workers and five eggs, to standardize our “initial colony” (following Byk and Del-Claro 2011) and to have a better control of colony development. Weekly and for two months

we placed 2 ml of the sugar diet used previously inside the artificial nests of the Without EFNs treatment, and 1.5 ml of the same solution inside the nests placed in the plants of With EFNs treatment. This difference in the volume of sugar diet was balanced by the production of extrafloral nectar. We also checked and removed weekly honeydew-producing hemipterans and/or caterpillars (Lycaenidae/Riodinidae) that produce sugar-rich secretions (Bächtold et al. 2016, Alves-Silva et al. 2018).

Finally, after two months in the field, we collected all nests and their members, and took them to the laboratory for counting and weighting the individuals. We removed the artificial nest in two months, because we intended to evaluate the short-term benefits of extrafloral nectar, and because the plants no longer had active EFNs (ESC pers. obs.), which could influence the colony growth rate (e.g. Korndörfer and Del-Claro 2006). We counted the total number of workers, pupae, larvae, and eggs, and evaluated the weight of all individuals of these stages, as well as the queen. As each colony presented more than one individual per stage of development, we used the mean weight of each stage per colony in the analyses.

#### *Food resource experiment*

In the same morning that we caught the 18 queens in the previous experiment, we collected other 32 queens of the same ant species and at the same study site, inserted them separately into falcon tubes, and then we transferred all individuals to containers with the eppendorf, the test tube filled with water, and the sugar diet, similar to the previous experiment. We kept the artificial nests in the laboratory under controlled conditions and fed every four days with 2 ml of the sugar diet.

When every four colonies had five adult workers, we removed the sugar diet, as well as all pupae, larvae and eggs, and we randomly selected one type of artificial liquid food treatment for each colony (Table 1). The liquid solution consisted of a sugar ratio of 1 mg sucrose/(0.5 mg fructose + 0.5 mg glucose) (adapted from Bixenmann et al. 2011). Sugar

concentration is based on the average of extrafloral nectar produced by plants in the study area (Lange et al. 2017). We used cysteine because some studies have shown a high and common presence of this amino acid in the extrafloral nectar (Koptur 1979, 1994b, Dress et al. 1997). The colonies were fed every 4 days with 2 ml of their respective food solution. In the case of the High sugar + insect treatment, we also provided one final larval instar of *Tenebrio* sp. per feeding moment.

Table 1 – Artificial liquid food treatment with different carbohydrate-protein balance ratio.

	Sugar concentration (µg/µL)	Cysteine concentration (µg/µL)	Final larval instar ( <i>Tenebrio</i> sp.)
Low sugar	5	0	No
Low sugar + aa	5	1	No
High sugar + aa	50	1	No
High sugar + insect	50	0	Yes

After established the experiment (eight colony per treatment), we monthly counted the total number of individuals at each stage (egg, larva, pupa and adult worker) after the beginning of the experiment until the 60<sup>th</sup> day, when some colonies of Low sugar treatment began to die. Thus, we used the data from the 30<sup>th</sup> and 60<sup>th</sup> day after the beginning of the experiment to analyze the abundance of colony individuals (short-term analysis). As other colonies remained alive, we evaluated the survival of these colonies until 210 days after starting the experiment (long-term analysis).

### Data analysis

All statistical analyses and graphs were performed using RStudio 3.5.1 at 5% of probability. In all models, we checked the residuals to verify the suitability of the model and overdispersion.

### *Extrafloral nectar feeding experiment*

To compare if the number of adult workers, pupae, larvae and eggs differed between the two treatments (With and Without EFNs), we ran a GLM with Poisson error distribution followed by Anova from the package “car” (Fox and Weisberg 2011). We used the final number (after two months) of each individual stage as the response variable, while treatments were fitted as predictor variable.

Difference in the weight of the queens, adult workers, pupae, larvae, and eggs per colony between the two treatments (with and without EFNs) was checked by running a GLM with Gaussian error distribution followed by Anova. Except for queens, we used the average weight per colony of all individuals as our response variable. For queens, we used the difference between the final and initial weight evaluation after two months.

To evaluate if there is variation in the colony fitness as a whole, we summed the number and total weight of all individuals (queen, adult workers, pupae, larvae, and eggs) per colony. We used the variation of the queen’s weight for the analysis of total weight. Then, we fitted a GLM with Gaussian error distribution followed by Anova, in which the total number and weight were considered as response variable and treatments as predictor variable.

#### *Food resource experiment*

To test short-term benefits and whether the number of adult workers, pupae, larvae, and eggs per colony differs among the four treatments, we conducted two analyses, one in the 30<sup>th</sup> day and one in the 60<sup>th</sup>. We did not compare the other future days, because colonies of some treatments began to die (see results). We fitted a GLM with Poisson error distribution followed by Anova from package “car” for all variables. Pairwise comparisons were performed using Estimated Marginal Means (EMMs) with “emmeans” package (Lenth 2018) whenever significant.

To test long-term benefits, we compared ant colony survival between the different treatments. For that, we conducted a survival analysis implementing the G-rho family of

Harrington and Fleming (1982) using the function *survdif* in the package “survival” (Therneau 2015). Pairwise comparisons were made using Log-Rank test and p-value correction of Benjamini and Hochberg (Benjamini and Hochberg 1995) in the package “survminer” (Kassambara and Kosinski 2018). Colony survival (dummy variable: 1 = survived and 0 = not survived) and time of survival (continuous variable measured in days) were considered as response variables, and the different treatments of food resource as explanatory variable: Survival (Colony survival + Time)~Group. We considered in our model only the seven first months (210 days) of colony survival.

## Results

### *Extrafloral nectar feeding experiment*

Ant colonies with access to EFN-bearing plants had a significant increase in the number of adult workers (GLM:  $\chi^2 = 9.923$ , d.f. = 1,  $p < 0.01$ ), pupae (GLM:  $\chi^2 = 4.968$ , d.f. = 1,  $p < 0.05$ ), larvae (GLM:  $\chi^2 = 5.609$ , d.f. = 1,  $p < 0.05$ ), and eggs (GLM:  $\chi^2 = 15.835$ , d.f. = 1,  $p < 0.001$ ) (Fig.1). These colonies with access to EFNs showed in average 1.6 times more adult workers, 1.8 times more pupae, 1.35 more larvae, and 1.7 times more eggs than the other colonies (Without EFNs – adult workers:  $3.0 \pm 1.1$ ; pupae:  $2.3 \pm 0.8$ ; larvae:  $11.5 \pm 2.6$ ; eggs:  $9.4 \pm 2.1$ ; mean  $\pm$  sd).

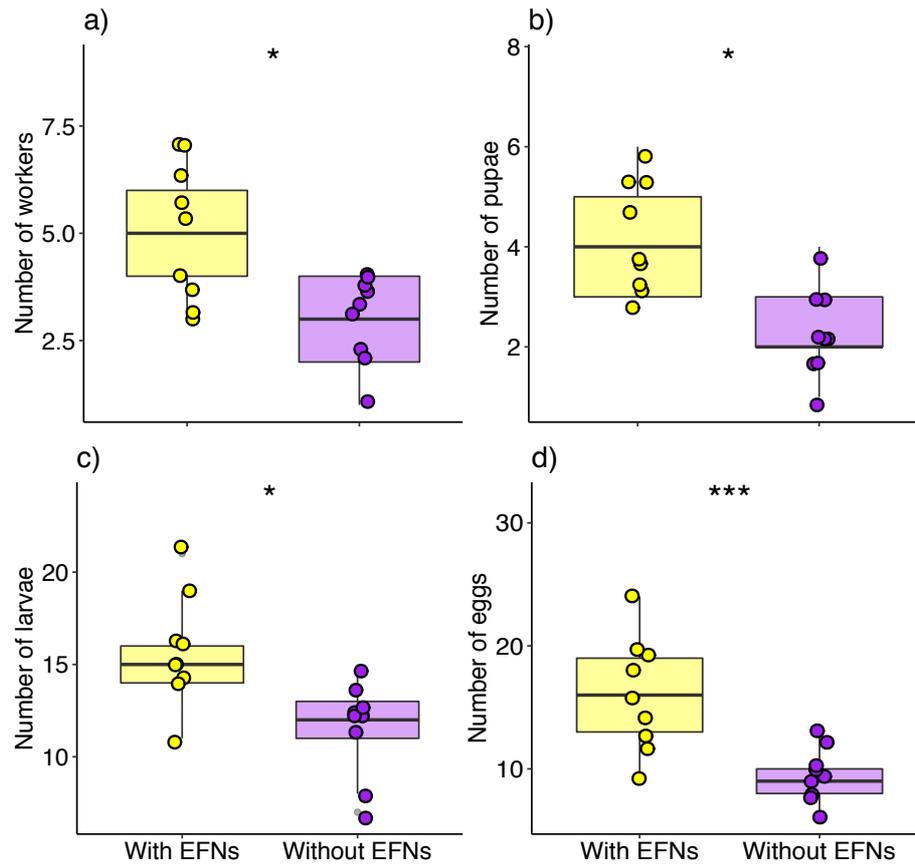


Fig. 1. Number of adult workers (a), pupae (b), larvae (c) and eggs (d) per colony of *Camponotus crassus* ants suggested to the treatments: With extrafloral nectaries (EFNs) and Without EFNs. Figures show median with maximums and minimums. GLM: \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

Colonies fed with extrafloral nectar had heavier queens (GLM:  $\chi^2 = 25.617$ , d.f. = 1,  $p < 0.001$ ), pupae (GLM:  $\chi^2 = 6.754$ , d.f. = 1,  $p < 0.01$ ), larvae (GLM:  $\chi^2 = 9.660$ , d.f. = 1,  $p < 0.01$ ) and eggs (GLM:  $\chi^2 = 9.635$ , d.f. = 1,  $p < 0.01$ ) (Fig. 2). Adult workers' weight showed no significant difference between the two treatments (GLM:  $\chi^2 = 1.257$ , d.f. = 1,  $p = 0.262$ ; Fig. 2). Heavier queens, pupae, larvae and eggs were observed in colonies that had access to plants with active EFNs.

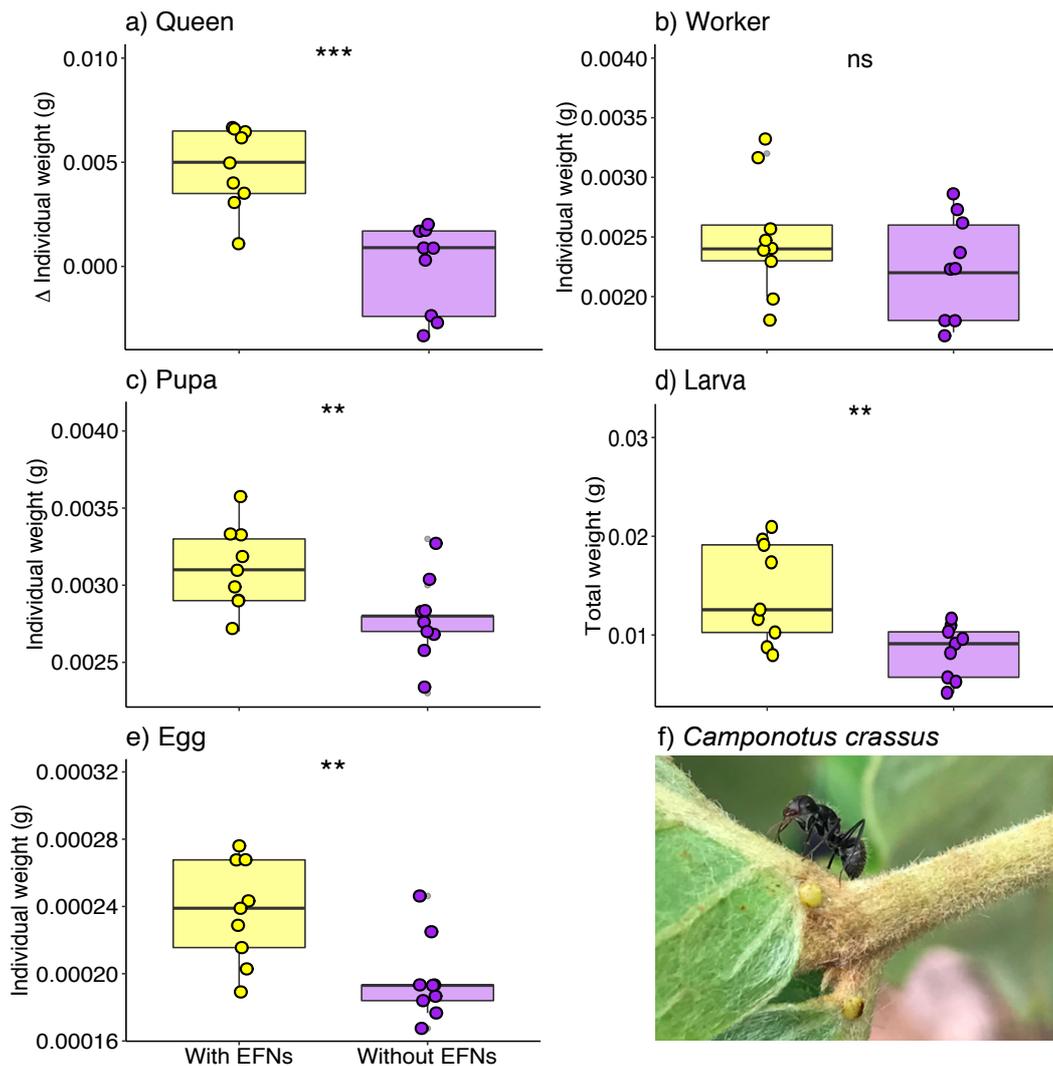


Fig. 2. Mean weight variation for queens (a), adult workers (b), pupae (c), larvae (d), and eggs (e) per colony of *Camponotus crassus* ants (f) suggested to the treatments: With extrafloral nectaries (EFNs) and Without EFNs. For queens (a), we used the difference between final and initial weight, and for larvae (d), we used the total weight per colony. Figures show median.

Both the total number and weight of individuals per colony differed between the two treatments (Number: GLM:  $\chi^2 = 28.984$ , d.f. = 1,  $p < 0.001$ ; Weight:  $\chi^2 = 36.73$ , d.f. = 1,  $p < 0.001$ ) (Fig. 3). Colonies that had access to EFNs had 1.5 more individuals and, were 2.07 times heavier than the colonies that did not had access.

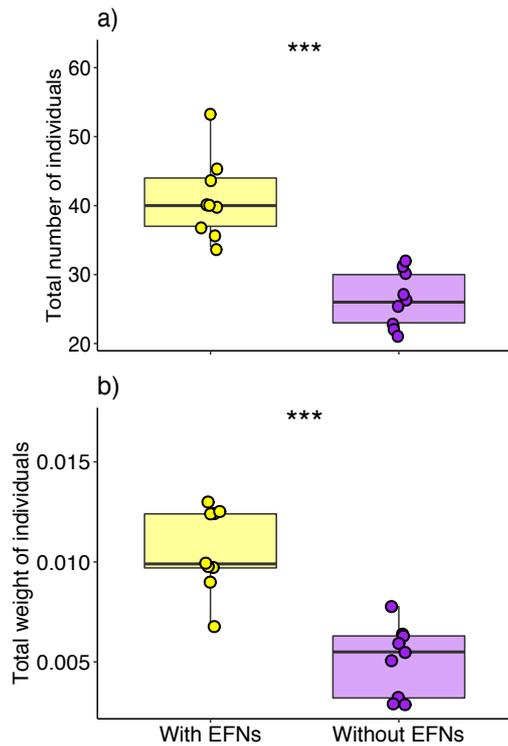


Fig. 3. Total number (a) and weight (b) of individuals (queen, adult workers, pupae, larvae, and eggs) per colony of *Camponotus crassus* ants suggested to the treatments: With extrafloral nectaries (EFNs) and Without EFNs. Figures show median with maximums and minimums. GLM: \*\*\*  $p < 0.001$ .

#### *Food resource experiment*

In the 30<sup>th</sup> day of analyses, we observed that there was no significant difference in the abundance of adult workers (GLM:  $\chi^2 = 0.942$ , d.f. = 3,  $p = 0.815$ ) and pupae (GLM:  $\chi^2 = 0.591$ , d.f. = 3,  $p = 0.898$ ) (Fig. 4). However, the abundance of larvae (GLM:  $\chi^2 = 21.489$ , d.f. = 3,  $p < 0.001$ ) and eggs (GLM:  $\chi^2 = 11.009$ , d.f. = 3,  $p < 0.05$ ) differed among the treatments (Fig. 4). The High sugar + insect treatment showed the greatest number of all colony parameters analyzed (number of adult workers, pupae, larvae, and eggs) followed by High sugar + aa, Low sugar + aa, and Low sugar treatments, respectively (Table 2).

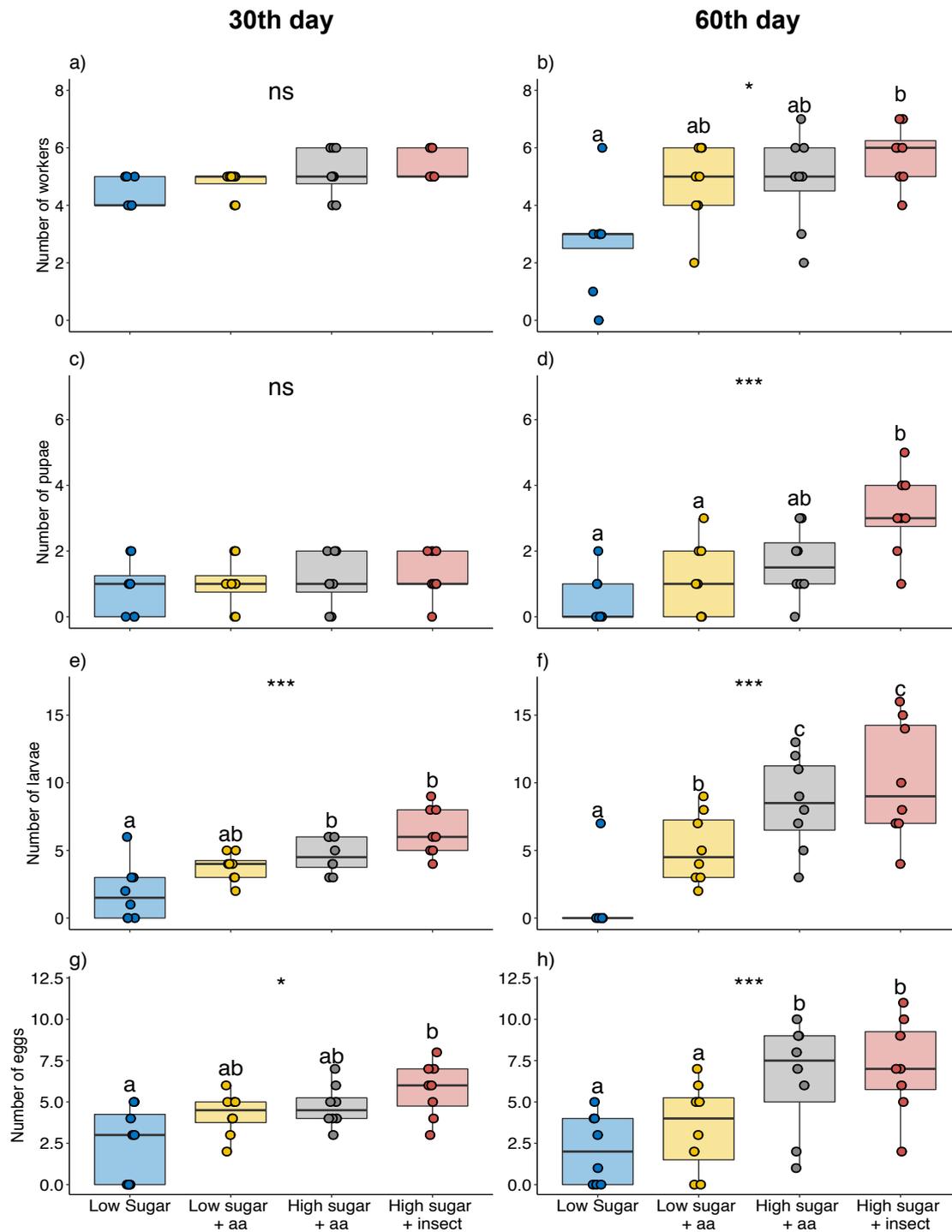


Figure 4. Number of adult workers, pupae, larvae, and eggs per colony of *Camponotus crassus* ants suggested to the four treatments of food resource in the 30<sup>th</sup> (a, c, e, g) and 60<sup>th</sup> (b, d, f, h) day. GLM: \*  $p < 0.05$ , \*\*\*  $p < 0.001$ , ns – non-significant. Letters represent statistic difference among treatments by Estimated Marginal Means (EMMs). Figures show median with maximums and minimums.

We observed a significant difference in the number of adult workers (GLM:  $\chi^2 = 9.227$ , d.f. = 3,  $p < 0.05$ ), pupae (GLM:  $\chi^2 = 18.629$ , d.f. = 3,  $p < 0.001$ ), larvae (GLM:  $\chi^2 = 82.127$ ,

d.f. = 3,  $p < 0.001$ ), and eggs (GLM:  $\chi^2 = 30.367$ , d.f. = 3,  $p < 0.001$ ) per colony among treatments in the 60<sup>th</sup> day (Fig. 4). Similarly, the High sugar + insect treatment showed the greatest number of all colony parameters (number of adult workers, pupae, larvae, and eggs) analyzed in the 60<sup>th</sup> day followed by High sugar + aa, Low sugar + aa, and Low sugar treatments, respectively (Table 2).

Table 2 – Mean ( $\pm$  sd) number of adult workers, pupae, larvae, and eggs per colony of *Camponotus crassus* ants suggested to the four treatments (eight colonies per treatment) of food resource in the 30<sup>th</sup> and 60<sup>th</sup> day.

	Low sugar	Low sugar + aa	High sugar + aa	High sugar + insect
<b>30<sup>th</sup> day</b>				
Workers	4.37 $\pm$ 0.51	4.75 $\pm$ 0.46	5.12 $\pm$ 0.83	5.37 $\pm$ 0.51
Pupae	0.87 $\pm$ 0.83	1.00 $\pm$ 0.75	1.12 $\pm$ 0.83	1.25 $\pm$ 0.70
Larvae	1.87 $\pm$ 2.10	3.75 $\pm$ 1.03	4.62 $\pm$ 1.30	6.37 $\pm$ 1.76
Eggs	2.50 $\pm$ 2.20	4.25 $\pm$ 1.28	4.75 $\pm$ 1.28	5.75 $\pm$ 1.66
<b>60<sup>th</sup> day</b>				
Workers	2.75 $\pm$ 1.75	4.75 $\pm$ 1.38	4.87 $\pm$ 1.64	5.75 $\pm$ 1.03
Pupae	0.50 $\pm$ 0.75	1.12 $\pm$ 1.12	1.62 $\pm$ 1.06	3.12 $\pm$ 1.24
Larvae	0.87 $\pm$ 2.47	5.12 $\pm$ 2.58	8.50 $\pm$ 3.46	10.12 $\pm$ 4.38
Eggs	2.12 $\pm$ 2.10	3.50 $\pm$ 2.67	6.50 $\pm$ 3.33	7.12 $\pm$ 2.90

We found that there is a significant difference of ant colony survival according to the food resource ( $\chi^2 = 25.6$ , d.f. = 3,  $p < 0.001$ , Fig. 5). From a total of eight colonies per treatment used in the experiment, we observed that 50% of Low sugar treatment colonies died in 120 days, while no other colony died in the same period of time. At the end of the analyses (210 days), we observed that one (12%) colony of Low sugar group survived, four (50%) of Low sugar + aa, six (75%) of High sugar + aa, and seven (88%) of High sugar + insect (Fig. 5).

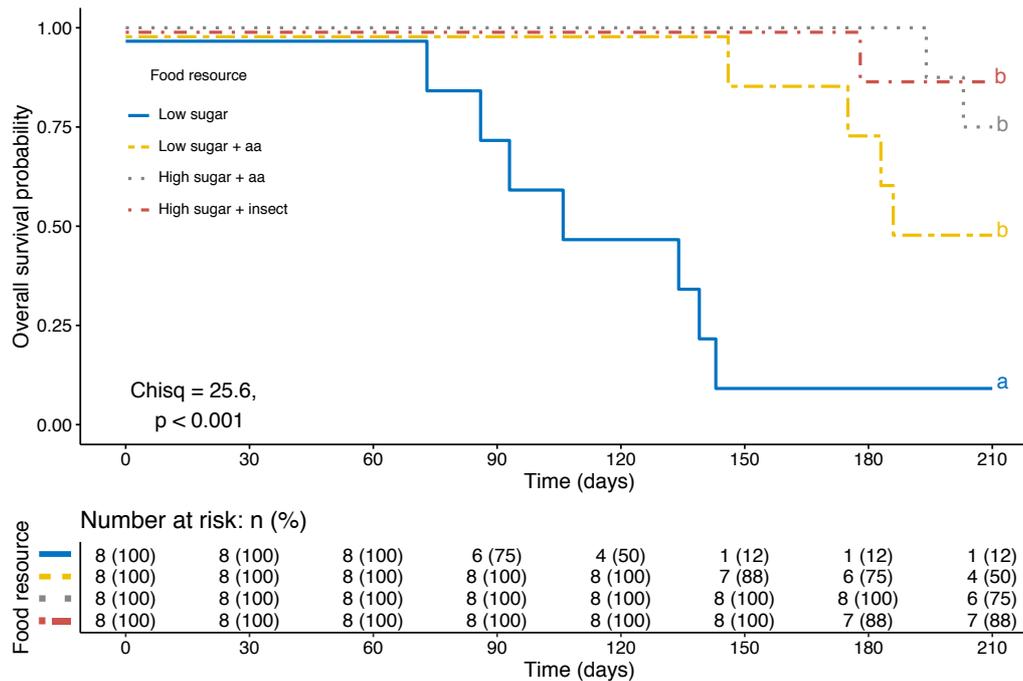


Figure 5 – *Camponotus crassus* ant colony survival among the different treatments of food resource. Difference between survival curves was made with G-rho family followed by Log-Rank test (lowercase letter at the right of each survival curve). Survival curves were fitted with the model survfit (Surv(Colony survival + Time)~Group, where Colony survival is a dummy variable (1 – survived, 0 – not survived).

## Discussion

Our study showed that there are short- and long-term benefits on ant colony fitness (number and weight of individuals) and survival according to specific liquid food resource, corroborating our main hypotheses. We found that the presence and short-term supply of extrafloral nectar influenced positively ant colony fitness, and that the short- and long-term supply of more concentrated carbohydrate and nitrogen food resources enhanced the colony fitness and survival compared to nutrient-poor resources.

Extrafloral nectar is a key resource for the interactions between plants and ants (Del-Claro et al. 2016, Calixto et al. 2018a), and due to its high concentration of sugar, it has been pointed out as main fuel for workers (Koptur 2005), influencing their foraging and defensive behavior, consequently affecting the development of the colony (Davidson 1998). Many studies have evaluated the impact that different food resources [e.g. elaiosomes (Morales and

Heithaus 1998, Gammans et al. 2005, Fokuhl et al. 2007), and secretions of lycaenid larva (Cushman et al. 1994)] have on the colony fitness, or even on specific castes or stages of development. However, few studies have evaluated the “true” benefits that ants have within the ant-EFN-bearing plants relationship, even though this interaction is considered a mutualism. Our study, along with that of Lach et al. (2009) and Byk and Del-Claro (2011), is the first to clearly show short- and long-term net effects of extrafloral nectar and different dietary resources on colony fitness and survival (Cushman et al. 1994).

Ant colonies that fed on nectar presented a greater number and weight of individuals in short time. According to Davidson (1998), carbohydrates, which are the main compound of extrafloral nectar, fuel the foraging activities of adult workers and may indirectly benefit the other castes. Furthermore, in addition to foraging for food for their metabolic balance, adult workers forage also for food items to feed larvae. Therefore, an adequate feeding and development of the larvae will result in a significant growth of pupae and adults, and consequently of the colony (Blüthgen and Feldhaar 2010). According to Wheeler (1994) and Nation (2015), lipids and proteins directly benefit queens and brood, since they are required for larval growth, ovary development, and egg production. In this way, colonies that fed with extrafloral nectar presented greater availability of amino acids reflecting on the weight of the queens and on the growth and weight of the colony as a whole.

Colony fitness and survival were influenced by different treatments with food resources, where higher positive values were found for High sugar + insect treatment. Each food resource presented a certain degree of composition, where we observed extremely nutritious (High sugar + insect) to non-nutritive resources (Low sugar). According to Kay *et al.* (2006), the carbohydrate-protein ratio of the larval ant diet may strongly affect the body composition of carbon, nitrogen, and phosphorous in larvae, which reflects on pupae and adults. Also, an omnivorous ants can present a dynamic response to this carbohydrate-protein

balance (see Flores-Flores et al. 2018, Ribeiro et al. 2019), which can affect from worker size and mortality, colony growth, and the body mass composition, to foraging behaviors, or even aggressiveness (Blüthgen and Feldhaar 2010). In this context, the presence of balanced resources and/or the physiological needs of each ant species are fundamental for the colony fitness and survival.

The need and assimilation of nitrogen is closely linked to the trophic position of each ant species, where larvae of species that are considered primary consumers, present a higher amount of nitrogen from plants than larvae of species that are considered primary and secondary predators (Blüthgen et al. 2003, Davidson et al. 2003). For instance, *Camponotus* (Formicinae) showed the highest dietary contribution of plant-based nitrogen, while species of Dolichoderinae and Ponerinae subfamilies showed moderate and low values, respectively (Davidson et al. 2003). This may be one of the reasons why most colonies of the Low sugar treatment of our second experiment died in just over two months, and why colonies of the High sugar + insect survived at least six months. As the feed resource offered for Low sugar treatment had no amino acid sources and low concentration of sugar, there was a negative impact on queen survival probability and consequently of the colonies (Fig. 5), unlike what occurred for the High sugar + insect treatment. Since the latter showed a high sugar concentration and a high presence of amino acids, as well as other nutrients through the supply of Tenebrionidae larvae, these colonies were better nourished and had better growth and survival rates.

Finally, we expected the Low sugar + aa treatment to present a survival rate similar to the High sugar + aa and High sugar + insect treatments, since up to 120 days of experiment, all three treatments had all colonies alive (Fig. 5). However, we observed between 90 and 120 days that the number of eggs and larvae began to decrease in some colonies, even though those eggs and larvae were already present (ESC pers. obs.). Despite of not directly evaluating that,

this decrease of brood can indicate cannibalism by the queen due to nutrient imbalance stress. The same behavior was pointed out by Buschinger & Pfeifer (1988), which showed that colonies of *Leptothorax acervorum* (Myrmicinae) fed with protein deficient diet cannibalized their pupae and larvae (also see Way 1963, Bourke 1991, Del-Claro and Tizo-Pedroso 2009). These results show us that even with the presence of carbohydrates and amino acids, a specific balance between these compounds is needed to adequately supply the needs of each ant species, as tested by Blüthgen & Fiedler (2004) with carbohydrates and amino acids preference experiment. Furthermore, when the availability of the food resource is changed, some ants can change the foraging behavior and the food resource sought. Anjos et al. (2019) highlighted the role of *Pogonomyrmex naegelii* ant as seasonal invertebrate carrion removers, which showed preference for specific food types in different moment of the year (also see Belchior et al. 2012). In another study, Whitford & Jackson (2007) showed that seed harvester ants increase the foraging rate in the moments of low amount of available seeds and high concentrations of prey, being these alive or dead.

In conclusion, our results have showed one side of the ant-EFN-bearing plant mutualism that has not been highly evaluated. A large number of studies, including reviews and meta-analyses, have been published on the benefits that ants provide for EFN-bearing plants, but very few have assessed the other side of the coin. Thus, in addition to showing that there are benefits in short-and long-term on ant colony fitness and survival according to specific types of food resources, our study provides new evidences that indeed the ant-EFN-bearing plant mutualism can be considered a true mutualism, both partners are indeed benefited. Therefore, we suggest that studies be done evaluating how EFN phenology, and other food resources, influence the growth and survival of ant colonies over the year; since the period of greatest activity of the EFNs of the majority of plants of the Cerrado is concentrated in the

rainy season (Lange et al. 2013), the growth of the colonies of ants that use this resource should be maximized at this time.

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# **Chapter 4**

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**Ants decrease fitness of plants bearing extrafloral nectaries by  
deterring pollinators**

**Ants decrease fitness of plants bearing extrafloral nectaries by deterring pollinators**

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## Abstract

Ants are attracted by different plant structures (e.g., extrafloral nectaries; EFNs) and in return they can act as “bodyguards” against the plant’s natural enemies, decreasing herbivory and increasing seed production. However, due to aggressive patrolling behavior, ants are not always beneficial to the plants. They can repel or even eat beneficial insects, such as pollinators, showing context-dependent outcomes in the ant-plant-pollinator interaction. In this study we tested whether the protective mutualism between ants and EFN-bearing plants negatively influences plant fitness via deterring visitation by pollinators. Our main hypothesis was that the presence of ants on flowers of an EFN-bearing plant decreases plant fitness by dissuading floral visitors. To achieve this aim we conducted experimental manipulations and field observations in an EFN-bearing plant from Brazilian “cerrado” savanna, from November 2017 to February 2018. We evaluated the floral visitor behavior, the amount of floral resources collected by pollinators, and the plant fitness influenced by the ant identity. We observed that the identity of ants, as well as floral visitors, showed different impacts on the plant fitness. We found that ant identity interferes with the behavior of invertebrate and vertebrate pollinators, fruit set, seed weight and seed size, and can indirectly affect the amount of flower resources collected. In addition, we observed that different floral visitors differed in their behavior according to ant identity. In conclusion, we showed experimentally that the presence of ants on flowers decreases plant fitness components by deterring visitation of potential pollinators to the plants. This result indicates a tradeoff between the beneficial effects of ant protection against herbivorous insects and the negative consequences for plant fitness.

**Keywords:** *Qualea multiflora*; extrafloral-nectar; biotic defense; Cerrado; hummingbirds; Bumblebee; Carpenter bee

## 1           **Introduction**

2           A number of plant species have evolved domatia, food bodies, and/or extrafloral  
3 nectaries (EFNs) to attract ants (Koptur 2005). These ants in return serve as “bodyguards”  
4 against the plant’s natural enemies (Del-Claro et al. 2016, Calixto et al. 2018a). As bodyguards,  
5 ants may bother arthropod visitors to the plant, sometimes eating these arthropods (see reviews  
6 in Rosumek et al. 2009, Trager et al. 2010, Heil 2015, Del-Claro et al. 2016, Calixto et al.  
7 2018a). As a result of these aggressive behaviors, ants can reduce herbivory (e.g., Lange and  
8 Del-Claro 2014), often resulting in increased fruit and seed production (e.g., Nascimento and  
9 Del-Claro 2010, Del-Claro and Marquis 2015). However, ant-plant interactions are not always  
10 beneficial to plants (e.g., Baker-Méio and Marquis 2012, Pires and Del-Claro 2014b, Ibarra-  
11 Isassi and Oliveira 2017, Melati and Leal 2018). Part of this conditionality might be due to ants  
12 repelling or otherwise eating beneficial insects, namely pollinators (e.g., Ness 2006, Assunção  
13 et al. 2014, Villamil et al. 2018). Thus, if negative interactions between ants and pollinators  
14 occur, the overall impact of ant defense on plant fitness would be a balance between the positive  
15 effects of ant protection versus the negative effects of pollinator distraction.

16           The presence of arthropod predators on or near flowers can influence the behavior of  
17 potential pollinators about to visit those flowers. Pollinator response to the presence of crab  
18 spiders (Thomisidae) varies greatly depending on pollinator identity, from avoiding flowers in  
19 which the spiders are waiting to completely ignoring them, sometimes to their own peril (Dukas  
20 and Morse 2003, Gonçalves-Souza et al. 2008, Brechbühl et al. 2010). In instances in which  
21 spiders repel or consume pollinators, reduced seed production can result (Louda 1982). The  
22 role of ants in deterring pollinators is much less studied than that of spiders. Some aggressive  
23 ants, for instance, are similar to crab spiders in their behavior towards pollinators: they are  
24 known to repel, deter, or even prey on pollinators, sometimes reducing seed set (e.g., Ness  
25 2006, Assunção et al. 2014, Ibarra-Isassi and Oliveira 2017, Villamil et al. 2018).

26           The variable responses of pollinators to the presence of crab spiders suggest that a  
27 similar range in reactions to ant presence may occur in pollinators. Some studies have shown  
28 that pollinators can potentially detect specific predator traits, such as shape and odor, and as  
29 result, the pollinator will avoid visiting flowers inhabited by ants (Ballantyne and Willmer  
30 2012, Assunção et al. 2014). In other cases, pollinators will still visit the flower but spend less  
31 time in it, or even change their behavior, so that they perform pollination, but without being  
32 attacked by ants (see Ness 2006, Assunção et al. 2014, Barônio and Del-Claro 2017, Aguirre-  
33 Jaimes et al. 2018). The potential range of interactions is further complicated by the fact that  
34 foliage-dwelling ants show a range of aggression towards other insects. Some species are non-  
35 aggressive and docile, for example, *Cephalotes pusillus* and *Brachymyrmex* sp. (Davidson et  
36 al. 2003, Byk and Del-Claro 2010); others are aggressive predators, as is often reported for  
37 *Camponotus crassus* and *Ectatomma tuberculatum* (Wheeler 1986, Anjos et al. 2017, Fagundes  
38 et al. 2017). If pollinators can recognize species differences in ant aggressiveness, then their  
39 response to the presence of ants near or on flowers may vary depending on the ant species.

40           Here we tested whether the protective mutualism between ants and EFN-bearing plants  
41 negatively influences plant fitness via deterring visitation by pollinators. We chose as our focal  
42 plant species, *Qualea multiflora* (Vochysiaceae), whose flowers are visited by invertebrates and  
43 vertebrates, and whose EFNs are visited by a number of ant species that range in level of  
44 aggression towards other arthropods. Most importantly the phenology and architecture of the  
45 plant are such that when the plant is flowering, only the EFNs in inflorescences are actively  
46 producing nectar, not those on leaves. This allowed us to isolate the negative effects of ant  
47 foraging at EFNs on plant fitness via deterrence of pollinators from the potentially confounding  
48 positive effects of ants on plant fitness via deterrence of herbivores. The latter have been  
49 previously quantified by Del-Claro et al. (1996). Specifically, we addressed the following  
50 questions: (i) Does the timing of ant activity overlap with the timing of visitation of pollinators

51 to flowers? (ii) Do flower visitors vary in their response to the presence of ants in flowers? (iii)  
52 Do ant species vary in their deterrence of floral visitors? (iv) Is the amount of pollen and nectar  
53 removal by floral visitors affected by the presence of ants in flowers? (v) Does the presence of  
54 ants reduce fruit set and seed quality via changes in pollinator behavior?

55 Our main hypothesis was that the presence of ants on flowers of *Q. multiflora* decreases  
56 fruit set and therefore plant fitness by dissuading floral visitors. Based on our understanding of  
57 the natural history of the system, we predicted that: (i) ant activity and flower visitation would  
58 overlap greatly in time; (ii) that flower visitors would vary in their response to the presence of  
59 ants; (iii) that different ant species would have different impacts on floral visitors depending on  
60 known differences in their aggressiveness; (iv) that pollinator deterrence would be manifested  
61 as differences in remaining pollen and nectar levels in visited flowers, depending on which ant  
62 species was present; (v) that ultimately plant fitness components would be reduced but the level  
63 of impact would depend upon ant species. In answering these questions, our goal was to  
64 quantify the relatively unexplored negative effects of ant visitation to extrafloral nectaries.

65

## 66 **Materials and Methods**

### 67 *Study area, study plant and ant species*

68 Fieldwork was conducted in the Reserva Ecológica do Clube de Caça e Pesca Itororó  
69 de Uberlândia (CCPIU - 18°58'59"S; 48°17'53"W), in Uberlândia, Brazil, from November  
70 2017 to February 2018. The ~200 ha reserve is located in the Cerrado biome. The cerrado  
71 vegetation at CCPIU ranges from open areas, with sparse coverage of shrubs and small trees,  
72 to more closed-canopy areas, with trees reaching 12-15 m in height. The climate of the region  
73 is subtropical, characterized by a rainy summer from October to March and a dry winter from  
74 April to September. The mean annual temperature varies from 18°C to 28°C and the mean  
75 annual rainfall from 800 to 2,000 mm (Ferreira and Torezan-Silingardi 2013).

76 *Qualea multiflora* Mart. (Vochysiaceae), a common EFN-bearing plant in the study  
77 area, was the focus of our study. Reproductive individuals of this species are 1-4 m tall. New  
78 leaves are produced in a single flush at the beginning of the rainy season, September-October.  
79 This species is in flower and fruit from November to February at the study area. EFNs are  
80 present at the bases of leaf petioles and along the inflorescences at the base of each flower  
81 pedicel. Leaves subtend inflorescences but are not found within inflorescences. EFNs on leaves  
82 are active when leaves are one-half to fully grown, but cease activity when the leaves become  
83 full size (Calixto et al. 2015). When all leaves have completed development and their EFNs  
84 have ceased secreting nectar, reproduction begins. EFNs located at the base of flowers can  
85 secrete nectar from the floral bud stage until after flower has fallen (ESC, pers. obs.). Previous  
86 studies have shown that the EFNs on leaves of this species attract ants that protect the plant  
87 against insect herbivores, and as a result, increase fruit set (Del-Claro et al. 1996). As such, the  
88 foliar EFNs of *Q. multiflora* represent an important defense against insect herbivores during  
89 leaf development (Calixto et al. 2015).

90 We used three ant species in the experimental manipulation, all of which are frequent  
91 visitors to the EFNs of *Q. multiflora* (Lange and Del-Claro 2014, Lange et al. 2017, also see  
92 results): *Cephalotes pusillus* (Klug, 1824), *Camponotus crassus* Mayr, 1862 and *Ectatomma*  
93 *tuberculatum* (Olivier, 1792). Of the three ant species, *C. pusillus* is the smallest (~ 0.6 cm) and  
94 least aggressive (Byk and Del-Claro 2010). *Camponotus crassus* is slightly larger (~ 0.7 cm)  
95 than *C. pusillus*, is very agile and aggressive, and is a demonstrated biotic defender of EFN-  
96 bearing plant species in cerrado (Anjos et al. 2017, Lange et al. 2017). *Ectatomma tuberculatum*  
97 is the largest of the three (~ 1.0 cm), is very aggressive, sometimes attacks and eats pollinators  
98 (Assunção et al. 2014; ESC, pers. obs.), and also is common on EFN-bearing plants in Brazilian  
99 savannah (see Assunção et al. 2014). Based on their characteristics and behavior, we predicted  
100 that *E. tuberculatum* would have the greatest negative impact on floral visitors (see

101 experimental design below), followed by *C. crassus* and *C. pusillus*, respectively. We predicted  
102 this ranking because *E. tuberculatum* and *C. crassus* are more aggressive, and as a result are  
103 likely to dissuade more floral visitors than *C. pusillus*. However, *E. tuberculatum* is larger than  
104 *C. crassus*, so we expected that the former would be more easily seen and therefore avoided by  
105 floral visitors.

#### 106 *Experimental design*

107 *Floral phenology* – We marked 10 individuals of *Q. multiflora* (at least 10 m apart), all  
108 similar in size (1.5-2.5 m height and 10-15 inflorescences per plant). On each plant we selected  
109 24 flower buds (n = 240 flowers). From previous observations, we observed that flowers last 2-  
110 3 days. However, at the end of the first day, the flowers begin to lose characteristics related to  
111 color (yellow/brown spots appear on the corollas), odor (which weakens), and shape (the flower  
112 begins to wither). Because of these changes, we observed flower anthesis, stigma receptivity,  
113 and pollen availability only on the first day on which a flower was open. All flowers were  
114 bagged in the floral bud stage with a mesh until the moment of stigma and pollen analysis.

115 During each day of the experimental period, we observed flower anthesis, stigma  
116 receptivity, and pollen availability on a single flower at 30 min intervals from 6:30-18:00 h. To  
117 test for stigma receptivity (on one flower per plant each 30 min), the stigma surface was dipped  
118 in a 3% hydrogen peroxide solution. The stigma was considered to be receptive if bubbles  
119 appeared (Dafni and Maués 1998). Pollen availability was measured by observing whether the  
120 single anther in a flower had dehisced (Fig. 1a–b). If so, we then gently pressed that anther  
121 against black paper (one flower per plant each 30 min; Fig. 1c).



122

123 Figure 1 – Anther with available (a) and non-available pollen (b), and scale to evaluate  
 124 the availability and amount of pollen present in the anther (c). “0” represents a low amount of  
 125 pollen, and “1” represents a high amount of pollen.

126

127 *Treatments* – To verify if plant fitness is influenced by the identity and the presence of  
 128 ants to flowers, and their impact on pollinator behavior, we tagged 20 individuals of *Q.*  
 129 *multiflora* with similar characteristics (1.5–2.5 m height and 10–15 inflorescences per plant).  
 130 On each plant, we selected five flowers (one per inflorescence), one each assigned to one of  
 131 five treatments (Table 1). There were two control treatments. The first (Control) was a  
 132 procedural control. It tested for the effect on plant reproduction of using Tanglefoot to exclude  
 133 ants in the other four treatments, and for the effect of the presence of the insect pin in treatments  
 134 3-5. In this treatment, Tanglefoot was placed around the half of the full diameter of the  
 135 inflorescence rachis, and a pin was stuck in a flower. In the second control (No-ants), all ants  
 136 were excluded using Tanglefoot to test for the effect of presence of any ants on plant  
 137 reproduction, and a pin was stuck in a flower, again to control for the effect of the presence of  
 138 the pin only. The three remaining treatments tested for the effect of the presence of the three  
 139 ant species, when near or on flowers, on the behavior of visitors to flowers, and resulting plant  
 140 reproduction (Table 1). A single, freshly killed ant, pierced with an insect pin, was affixed to  
 141 the sepal of each experimental flower, and subsequent floral visitor behavioral near or on that  
 142 flower was monitored.

143 Table 1. Treatment division in *Qualea multiflora* (Vochysiaceae) based on ant access to  
 144 the inflorescence, application of Tanglefoot, and flower manipulation.  
 145

Treatments	Ant access	Tanglefoot	Flower manipulation
<i>Control</i>	Yes	Half of the inflorescence stem base	Insect pin only
<i>No-ants</i>	No	Around the inflorescence stem base	Insect pin only
<i>Cephalotes pusillus</i>	No	Around the inflorescence stem base	Freshly killed and pinned <i>C. pusillus</i>
<i>Camponotus crassus</i>	No	Around the inflorescence stem base	Freshly killed and pinned <i>C. crassus</i>
<i>Ectatomma tuberculatum</i>	No	Around the inflorescence stem base	Freshly killed and pinned <i>E. tuberculatum</i>

146

147 All flowers were tagged at the bud stage and enclosed with a voile bag until the actual  
 148 experimental setup, still pre-anthesis. We removed the bags from flowers between 06:30–07:00  
 149 h, and then immediately conducted the experimental manipulations. The bag covered the entire  
 150 inflorescence so that no bud or flower was exposed to herbivore attack.

151 For the three treatments with pinned ants, in the morning and before observations, we  
 152 collected three individuals of each ant species, always from the same nest. We then immediately  
 153 killed the ant by pinching its thorax, and then inserted an insect pin (size “0”) into its thorax  
 154 (see Sendoya et al. 2009). For these treatments, we inserted these pins with their ants (or an  
 155 insect pin only in the case of the Control and the No-ants treatment) through a sepal of an  
 156 experimental flower, always standardizing the placement (near the central region of the flower  
 157 sepal), the height of the ant on the pin (in the middle of the pin), the height of the ant near the  
 158 flower (in the middle of stamen height), and the side of the flower (always opposite to the floral  
 159 nectary). We frequently checked the position of the pins on the flower to ensure that the pin  
 160 and the ants were in the correct position, since floral visitors, while visiting a flower, could  
 161 touch the pin, changing its position, or they could break the pinned ants (in this last case, we  
 162 exchanged the broken ant with a new one).

163 *Floral visitor behavior* – To verify the floral visitor and their respective behaviors, we  
164 observed the plants from 07:00–18:00 h. We started observations at 07:00, when flowers first  
165 began to open. When possible, we recorded the time spent by floral visitors in the flowers using  
166 a stopwatch or we recorded videos for *a posteriori* time analysis. We always observed all five  
167 experimental flowers on a plant on the same day, that is, we used plants with at least five freshly  
168 opened flowers, totaling 20 days of experimentation, one day per plant.

169 We classified each floral visitor approach as either: 1) a ‘visit’, or 2) ‘avoidance’. To be  
170 considered a visit, the floral visitors needed to approach the flower and touch the reproductive  
171 parts and/or probe the floral tube; to be considered an avoidance, the floral visitor needed to  
172 perform any of the following avoidance behaviors: remain hovering in the air analyzing the  
173 flower, ultimately without landing on the flower (Video 1; <https://youtu.be/T0ZxGNeEGLs>),  
174 or land on the flower but quickly leave without touching reproductive parts or probing the floral  
175 tube.

176 As a result of their usual behavior, we classified each species of floral visitor as a  
177 potential pollinator (effective or occasional pollinators), or non-pollinator, following Alves-  
178 dos-Santos et al. (2016). We classified a visitor species as an effective pollinator if they  
179 contacted the anther and stigma on most visits, and frequently visited experimental plants  
180 compared to all the other visitors; as occasional pollinators, if they contacted the anther and  
181 stigma few times, and were similar in frequency of visits to that of effective pollinators; and as  
182 non-pollinators, if they did not touch the reproductive parts, but collected either floral nectar  
183 and/or pollen. We collected individuals of floral visitors, except hummingbirds, for further  
184 identification at the Universidade Federal de Uberlândia, where voucher specimens were  
185 deposited. When it was not possible to collect specimens (e.g., hummingbirds), we filmed  
186 and/or took photos of some species for later identification by specialists.

187        *Ant composition* – To quantify the abundance and composition of ant species visiting  
188 plants at the time of the experiment, we identified to species and counted the number of ants on  
189 one to three branches near to the inflorescences containing Control treatment flowers at hourly  
190 intervals. We collected individuals of each ant species for further identification at the  
191 Universidade Federal de Uberlândia, where voucher specimens were deposited.

192        *Floral resources and plant reproduction* – To determine the indirect impact of ants on  
193 the pollination process via disturbance of the floral visitors, we measured the volume and  
194 concentration of floral nectar, as well as the amount of pollen, remaining in each experimental  
195 flower after pollinator observations had been completed (18:00 h). To quantify the volume and  
196 concentration of nectar (Brix°, sugar contents) at the end of each experimental day, we used a  
197 5µL-graduated capillary tube and a handheld refractometer (Eclipse®), respectively. To  
198 evaluate the amount of pollen present at the end of each experimental day (or the inverse, the  
199 amount of pollen removed during the day), we pressed the anther (this species has only one  
200 anther) against a piece of black paper containing a previously prepared scale (Fig. 1c). This  
201 scale contained two categories: 0 - represents a low amount of pollen, and 1 - represents a high  
202 amount of pollen.

203        After assessing pollen and nectar availability on each of the experimental flowers, we  
204 re-bagged the flowers and then followed each flower until fruit production. After fruit  
205 maturation, seeds were collected, counted, air-dried, and weighed with a digital precision  
206 analytical balance to the nearest 1.000 g. Lastly, we measured seed surface area with the  
207 program ImageJ 1.52a (Calixto et al. 2015).

208        *Data analysis*

209        All statistical analyses were performed using RStudio 3.5.1 at 5% of probability. Graphs  
210 were produced using Graphpad Prism 7.0. For analyses, we used Linear Mixed-Effect Models  
211 (LMM) or Generalized Linear Mixed Models (GLMM), using the error distribution according

212 to each response variable. We then checked the residuals to verify the suitability of the model  
213 and overdispersion when applicable.

214 We used Pearson correlation to evaluate if the average number of floral visitor  
215 approaches (number of visits and avoidances) matches with the average number of ants per  
216 plant per hour. To compare the number of total flowers visited and avoided per day among  
217 treatments, we used a GLMM with Poisson error distribution followed by Wald chi-square test  
218 using the “lme4” package (Bates et al. 2015), and “car” package (Fox and Weisberg 2011),  
219 respectively. For this, treatments were considered as fixed factor, plants as random block factor,  
220 and number of visits or avoidances as count response variable. Pairwise comparison was  
221 conducted using Tukey’s HSD test with “multcomp” package (Hothorn et al. 2008).

222 To evaluate if there was variation in behavior among species visiting flowers, checking  
223 for visitors more and less likely to visit flowers with ants, we used visit proportion data  
224 (hereafter “relative visit”), which is:  $\text{number of visits}/(\text{number of visits} + \text{number of}$   
225  $\text{avoidances})$ . This analysis allowed us to determine if the identity of the visitor is important to  
226 the plant. If we find a higher value of relative visits by pollinators in relation to non-pollinators,  
227 then we can suggest that the former are more likely to visit the flower with ants than the latter,  
228 and that the presence and identity of ants can act as a filter, “allowing” the use of floral resources  
229 only by potential pollinators. Also, regarding only pollinators, this analysis can show which  
230 pollinators are more likely to visit flowers with ants. To compare the relative visits between  
231 pollinators and non-pollinators, and only among pollinators, we fit a GLMM with Beta  
232 distribution and “logit” link function, followed by Wald chi-square test, using the package  
233 “glmmTMB” (Brooks et al. 2017). Pairwise comparisons was performed using Estimated  
234 Marginal Means (EMMs) with “emmeans” package (Lenth 2018). For that, we used only data  
235 from treatments with pinned ants, since they were the treatments that most affected the behavior  
236 of visitors (see results). Then, treatments were considered as fixed factor, plants as random

237 block factor, and relative visits per plant as response variable. We used 0.1 as the relative visit  
238 value when the number of visits was equal to zero ( $n = 4$ ), since the package “glmmTMB” does  
239 not accept zeros.

240 To compare the time spent on flowers by floral visitors among treatments, we fit a  
241 GLMM with Poisson distribution and a Wald chi-square test, followed by Tukey’s HSD test.  
242 Treatment was considered as a fixed factor, plant as a random block factor, and time spent  
243 (seconds) by visitors per flower as count response variable.

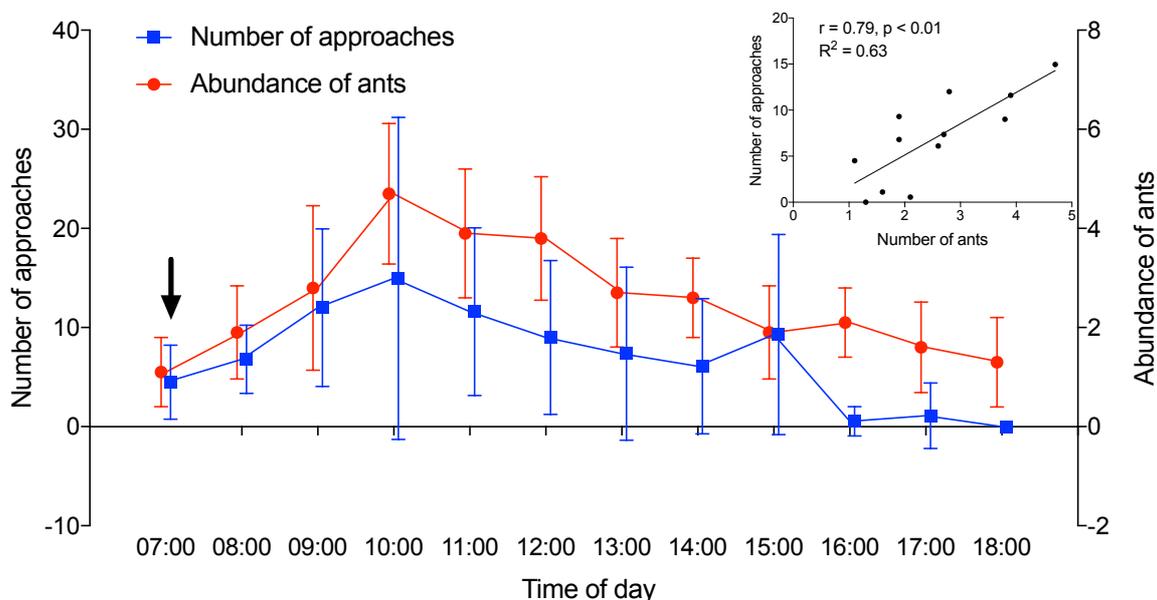
244 We ran a LMM and Wald chi-square test, followed by Tukey’s HSD test using nectar  
245 volume and concentration per flower as the continuous response variable, treatments as fixed  
246 factor and plants as random block factor, to compare the volume and sugar concentration of  
247 nectar among treatments. The amount of pollen, as well as the fruit production, among  
248 treatments was compared using a GLMM with binomial distribution followed by Wald chi-  
249 square test. Treatments were considered as fixed factor, plants as random block factor, and  
250 pollen amount and fruit production as response variable. Pairwise comparison was conducted  
251 using Tukey’s HSD test. We used a GLMM with Poisson distribution followed by Wald chi-  
252 square test to compare the number of seeds produced among treatments. In this case, we  
253 considered treatment as fixed factor, fruit as random factor, and the number of seeds produced  
254 per fruit as the response variable. To analyze differences in the seed weight and seed size among  
255 treatments, we ran a LMM and Wald chi-square test, followed by Tukey’s HSD test. Treatment  
256 was considered as a fixed factor, fruit as a random block factor, and seed weight and seed size  
257 as response variables.

258

## 259 **Results**

260 *Floral phenology* – Flowers of our experimental plants began to open around 06:30,  
261 with 100% of flowers opened by 07:30 am (Fig. 2). Before 06:30, no flowers had receptive

262 stigmas and no anthers had dehisced. By 07:30, pollen of all stamens was available to  
 263 pollinators (all anthers had dehisced); by 08:00, 100% of the stigmas tested were receptive (Fig.  
 264 2). Floral visitors began to arrive at flower anthesis (prior to when pollen was available and  
 265 stigmas were receptive), increasing in number throughout the morning until about 10:00, and  
 266 then gradually tapering off through the afternoon until 17:00, after which there were no more  
 267 visits (Fig. 2). Most importantly, the temporal pattern of ant abundance on the plants coincided  
 268 with the peak of floral visitor visitation (Pearson correlation:  $r = 0.79$ ,  $p < 0.01$ ), beginning,  
 269 peaking, and declining at a similar time (Fig. 2). The three ant species used in the experiment  
 270 were equally active throughout the day (Table 2).



271

272 Figure 2 – Average ( $\pm$  SD) number of approaches (visits and avoidances) by floral  
 273 visitors and average ( $\pm$  SD) number of ants per plant per hour in *Qualea multiflora*. Black arrow  
 274 represents the moment at which we found 100% of stigmas to be receptive ( $n = 10$  sampled  
 275 every 30 minutes), 100% of stamens with available pollen ( $n = 10$  sampled every 30 minutes),  
 276 and 100% of flowers opened ( $n = 10$  sampled every 30 minutes). The upper right graph shows  
 277 a positive correlation between the mean abundance of ants and mean number of approaches per  
 278 plant per hour ( $F_{[1,10]}=17.0$ ,  $p < 0.01$ ,  $R^2 = 0.63$ ;  $Y = 3.397 * X - 1.669$ ).  
 279

280

Table 2. Diversity, absolute abundance, absolute frequency, and relative abundance of ants in *Qualea multiflora* at the study site.

Ant species (subfamily/species)	Total abundance of ants												Number of plants presenting the specific ant	Relative abundance (%)
	Time of day													
	07	08	09	10	11	12	13	14	15	16	17	18		
<b>Ectatomminae</b>														
<i>Ectatomma tuberculatum</i> (Olivier, 1792)	7	7	8	12	13	14	12	10	10	10	8	5	5	19.1
<b>Formicinae</b>														
<i>Brachymyrmex</i> sp.	2	4	7	12	11	7	5	5	0	2	0	3	3	9.6
<i>Camponotus blandus</i> (Smith, F., 1858)	1	2	8	10	8	6	6	4	5	2	2	1	3	9.1
<i>Camponotus crassus</i> Mayr, 1862	7	19	25	43	36	36	24	27	20	22	14	15	11	47.4
<i>Camponotus</i> sp.	0	0	0	1	1	1	0	1	0	0	0	0	1	0.7
<b>Myrmicinae</b>														
<i>Cephalotes pusillus</i> (Klug, 1824)	3	3	6	8	3	5	4	3	3	3	4	1	5	7.6
<i>Crematogaster</i> sp.	2	3	2	6	6	6	4	2	3	3	2	1	1	6.6
<b>Total</b>													<b>20*</b>	<b>100.0</b>

281

\*Some ants were found in the same individual and therefore, the total absolute frequency is the total of plants analyzed (20 plants).

282

283 *Floral visitors and ant composition* – We recorded 17 species of floral visitors (seven  
284 bee species, six Lepidoptera species, and two hummingbird species) (Table 3). Based on their  
285 behavior and frequency at treatments flowers, two of the bee species were considered to be  
286 effective pollinators, three we classified as occasional pollinators, and the remaining species  
287 were considered non-pollinators (Table 3). The two species considered to be effective  
288 pollinators were the bees *Bombus morio* (Fig. 3a) and *Xylocopa* sp. (Fig. 3b). Three occasional  
289 pollinators were: a wasp of the genus *Campsomeris* (Scoliidae; Fig. 3c), and two hummingbird  
290 species, *Amazilia fimbriata* (Fig. 3d) and *Eupetomena macroura*. These five potential  
291 pollinators represented approximately 82% of the total visits (Table 3) and 67% of the cases of  
292 avoidance, with emphasis on *B. morio*, which represented 27% of the recorded avoidances (see  
293 Video 1). Some species visited infrequently or had no contact with reproductive parts, as was  
294 the case of the bee *Exomalopsis* sp. (Fig. 3e) and the Lepidoptera *Aellopos* sp., respectively;  
295 others visited a few times and had no contact with reproductive parts but did take nectar (*e.g.*,  
296 *Anteos* sp., Fig. 3f).

297 During the course of the experiment, we encountered seven ant species belonging to  
298 three subfamilies. *Cephalotes pusillus* and *E. tuberculatum* were found on five plants,  
299 comprising 25% of the total plants, and *C. crassus* was found on 11 plants, comprising 55% of  
300 the total plants (Table 2). Also, *C. crassus*, *C. pusillus*, and *E. tuberculatum* made up 47.4, 7.6  
301 and 19.1% of all ants encountered, respectively (Table 2), totaling almost 75% of the total  
302 abundance.

303 Table 3 – Number of visits and avoidances of floral visitors in each treatment of *Qualea multiflora* flowers at the study site.

Species	Control		No-ants		<i>Cephalotes pusillus</i>		<i>Camponotus crassus</i>		<i>Ectatomma tuberculatum</i>	
	V	A	V	A	V	A	V	A	V	A
<b>Hymenoptera</b>										
Apidae										
<i>Apis mellifera</i> Linnaeus, 1758	0	0	0	0	0	0	3	0	0	0
<i>Bombus morio</i> <sup>1</sup> (Swederus, 1787)	41	0	51	0	57	0	43	13	18	56
<i>Centris</i> sp.	11	0	18	0	6	0	2	7	4	21
<i>Euglossa</i> sp.	3	0	0	0	0	0	0	0	0	3
<i>Exomalopsis</i> sp.	3	0	8	0	8	3	2	6	3	20
<i>Trigona spinipes</i> (Fabr., 1793)	6	0	3	0	0	0	0	0	6	0
<i>Xylocopa</i> sp. <sup>1</sup>	56	0	84	0	33	0	32	8	16	28
Halictidae										
<i>Augochloropsis</i> sp.	0	0	0	0	0	0	0	0	0	3
Scoliidae										
<i>Campsomeris</i> sp. <sup>2</sup>	47	0	71	0	50	0	41	0	13	31
<b>Lepidoptera</b>										
Sphingidae										
<i>Aellopos</i> sp.	32	0	23	0	25	0	32	3	8	34
Lycaenidae										
<i>Calycopis</i> sp.	1	0	0	0	0	0	6	0	0	0
Hesperiidae										
Hesperiidae sp.1	5	1	1	0	0	0	4	0	0	2
Hesperiidae sp.2	3	0	0	0	0	0	0	0	3	0
Pieridae										
<i>Anteos</i> sp.	4	0	2	0	0	0	1	0	3	0
Pieridae sp.	2	0	0	0	2	0	4	0	2	0
<b>Hummingbirds</b>										
Trochilidae										
<i>Amazilia fimbriata</i> <sup>2</sup> (Gmelin, 1788)	55	0	67	0	34	0	36	0	12	36
<i>Eupetomena macroura</i> <sup>2</sup> (Gmelin, 1788)	30	0	36	0	29	0	26	0	14	35
<b>Total</b>	<b>299</b>	<b>1</b>	<b>364</b>	<b>0</b>	<b>244</b>	<b>3</b>	<b>232</b>	<b>37</b>	<b>102</b>	<b>269</b>

304 V – total number of visits. A – total number of avoidances. <sup>1</sup>Effective pollinators. <sup>2</sup>Occasional pollinators. Species without superscript numbers represent non-pollinators.

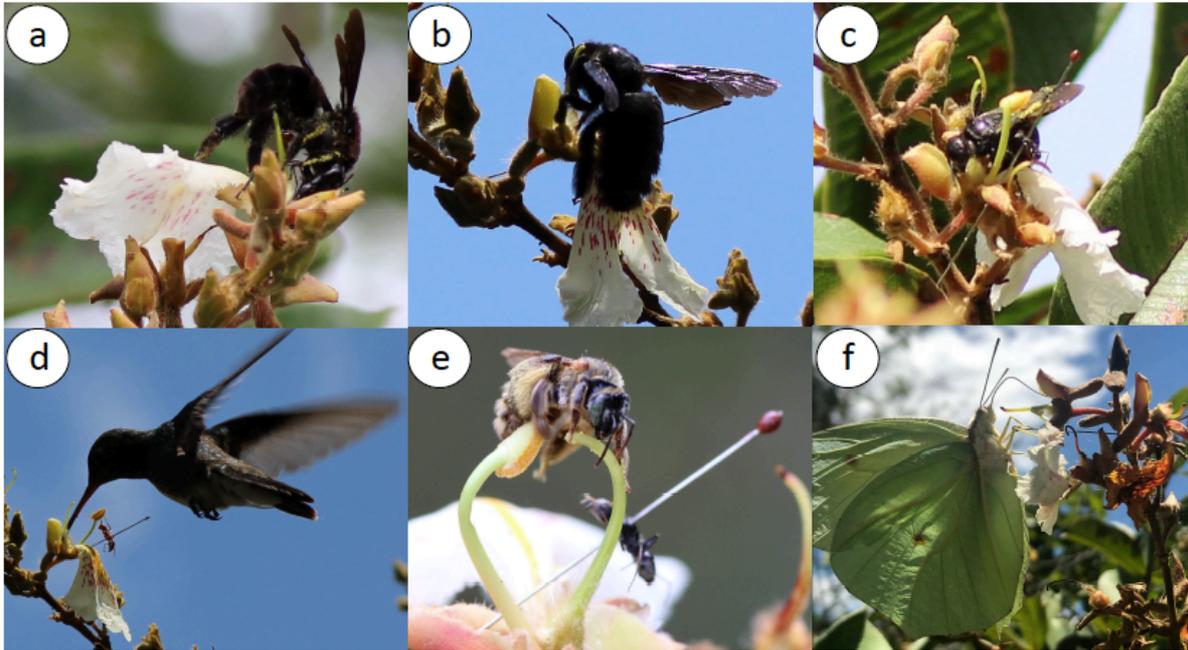


Figure 3 – Some examples of visitors to experimental flowers of *Qualea multiflora* at the study site. (a) *Bombus morio* visiting a flower of the No-ants treatment; (b) *Xylocopa* sp. visiting a flower of the *Ectatomma tuberculatum* treatment; (c) *Campsomeris* sp. visiting flower of the No-ants treatment; (d) *Amazilia fimbriata* visiting a flower of the *E. tuberculatum* treatment and avoiding contact with the ant and consequently with the reproductive parts; (e) *Exomalopsis* sp. visiting a flower of the *Camponotus crassus* treatment; and (f) *Anteos* sp. visiting the No-ants treatment. Pollen grains adhering to the body of floral visitors can be seen in (a) and (c).

*Floral visitor behavior* – We found a significant treatment effect on the average number of visits per flower per plant (GLMM:  $\chi^2 = 39.352$ ,  $p < 0.001$ ; Fig. 4a). Flowers of the No-ants treatment were the most visited, followed by the Control, *C. pusillus*, *C. crassus*, and *E. tuberculatum* treatments, respectively (Fig. 4a). Similarly, there was a statistical difference in the average number of avoidances per flower per plant by treatment (GLMM:  $\chi^2 = 58.404$ ,  $p < 0.001$ ; Fig. 4b). The *E. tuberculatum* treatment was the most avoided treatment, followed by *C. crassus*, *C. pusillus*, Control, and No-ants treatments (Fig. 4b).

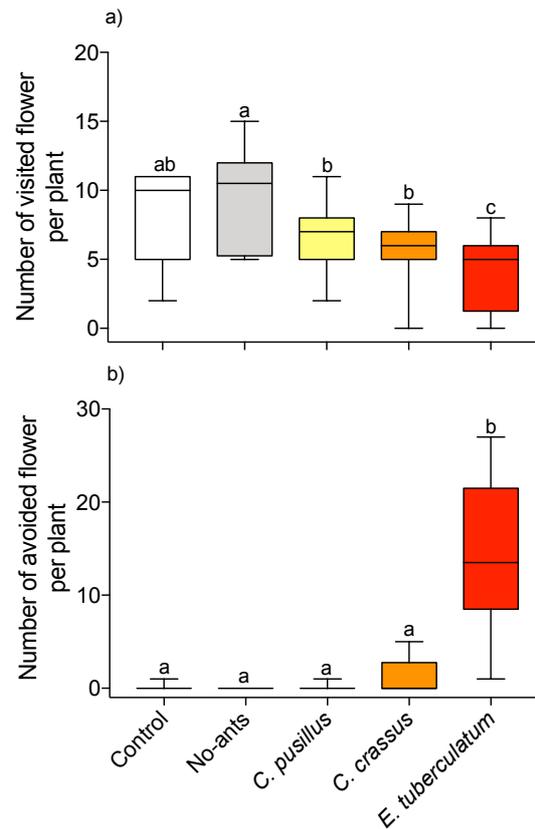


Figure 4 – Number of visited (a) and avoided (b) flower per plant in *Qualea multiflora* at the study site according to each treatment. GLMM:  $\chi^2 = 39.352$ ,  $p < 0.001$  (a); and GLMM:  $\chi^2 = 58.404$ ,  $p < 0.001$  (b). Letters represent statistic difference among treatments by the Tukey's HSD test. The boxes represent median values and minimums and maximums.

We observed a significant difference in the relative visits per plant of pollinators versus non-pollinators (GLMM:  $\chi^2 = 4.596$ ,  $p < 0.05$ ), in which pollinators were more likely to land on flowers with ants than non-pollinators (Fig. 5a). We also observed a significant difference in the likelihood of visiting a flower with ants among the five potential pollinator species (GLMM:  $\chi^2 = 14.019$ ,  $p < 0.01$ ; Fig. 5b): *Campsomeris* sp. was the most likely to visit flowers with ants.

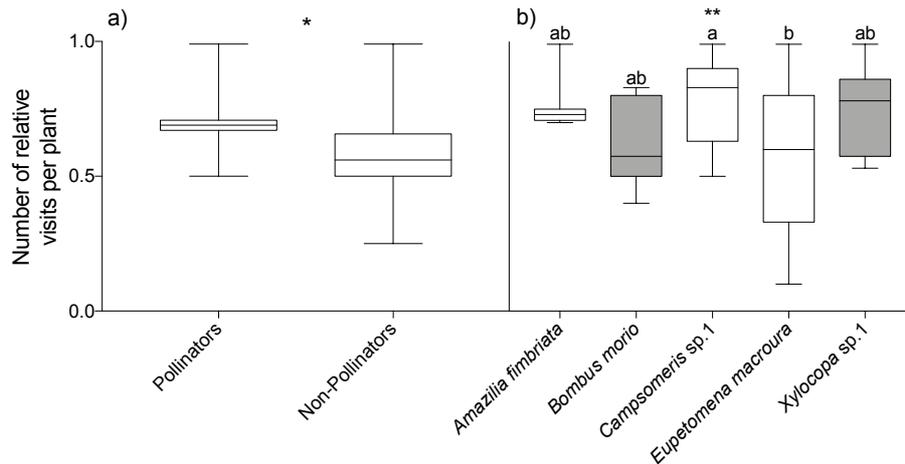


Figure 5 – Number of relative visits per plant between pollinators (effective and occasional pollinators) and non-pollinators (a), and only among pollinators (b) in *Qualea multiflora* at the study site. GLMM:  $\chi^2 = 4.596$ ,  $p < 0.05$  (a); and GLMM:  $\chi^2 = 14.019$ ,  $p < 0.01$  (b). The boxes represent median values and minimums and maximums. \* represents  $p < 0.05$ , and \*\* represent  $p < 0.01$ . Letters represent statistic difference among treatments by Estimated Marginal Means (EMMs). Gray bars (b) show the effective pollinators. The number of relative visits was from treatments with pinned ants, since they were the treatments that most affected visitor behavior.

*Time spent on flower* – We observed statistical differences in the time spent by all floral visitors per flower among treatments (GLMM:  $\chi^2 = 10.89$ ,  $p < 0.05$ ; Fig. 6). Floral visitors stayed longest in the No-ants treatment, followed by the Control, *C. pusillus*, *C. crassus*, and *E. tuberculatum* treatments, respectively (Fig. 6).

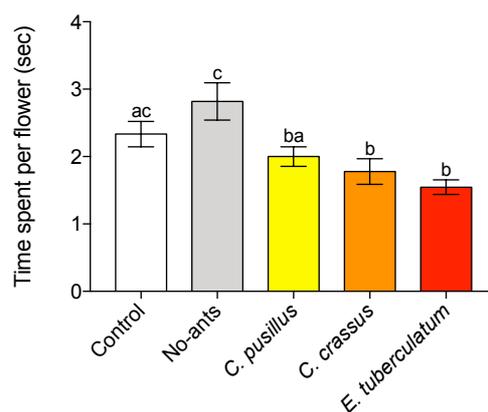


Figure 6 – Time spent by floral visitors per flower among treatments (means  $\pm$  SE). GLMM:  $\chi^2 = 10.89$ ,  $p < 0.05$ . Letters represent statistic differences between the treatments by the Tukey's HSD test.

*Floral resources* – Differences in the number and length of pollinator visitations translated into differences in the amount of remaining nectar and pollen. We observed a statistical difference in the volume of floral nectar still available at the end of the day among treatments (LMM:  $\chi^2 = 41.658$ ,  $p < 0.001$ ; Fig. 7a), where the *E. tuberculatum* and *C. crassus* treatments had the largest volume of nectar. In addition, we found a statistical difference in the sugar concentration of the nectar (LMM:  $\chi^2 = 10.502$ ,  $p < 0.05$ ; Fig. 7b). The Control treatment had the highest nectar concentration, followed by *E. tuberculatum*, *C. crassus*, *C. pusillus*, and No-ants, respectively. Finally, we observed the highest amount of pollen in the *E. tuberculatum* treatment and a low amount in the other treatments (GLMM:  $\chi^2 = 17.779$ ,  $p < 0.01$ ; Fig. 7c).

*Plant fitness components* – The treatments ultimately affected a measure of plant fitness, i.e., the number of fruits per treatment and the quality of seeds. We observed a statistical difference in the fruit set among treatments (GLMM:  $\chi^2 = 10.306$ ,  $p < 0.05$ ; Fig. 8a). The No-ants treatment produced the most fruits (13 fruits), followed by *C. pusillus* (9 fruits), Control (8 fruits), *C. crassus* (7 fruits) and *E. tuberculatum* (3 fruits), which represents for each treatment a probability of a flower producing a fruit of 65%, 45%, 40%, 35% and 15%, respectively. The mean number of seeds per fruit did not vary among treatments (GLMM:  $\chi^2 = 1.658$ ,  $p < 0.798$ ). However, there was a significant difference in seed mass (LMM:  $\chi^2 = 49.645$ ,  $p < 0.001$ ; Fig. 8b) and seed size (LMM:  $\chi^2 = 45.146$ ,  $p < 0.001$ ; Fig. 8c) among treatments. On average, flowers of the No-ants treatment produced the heaviest seeds, followed by *C. pusillus*, Control, *C. crassus*, and *E. tuberculatum* (Fig. 8b). In the case of seed size, again flowers of the No-ants treatment had the largest seeds, followed by Control, *C. pusillus*, *C. crassus*, and *E. tuberculatum* (Fig. 8c).

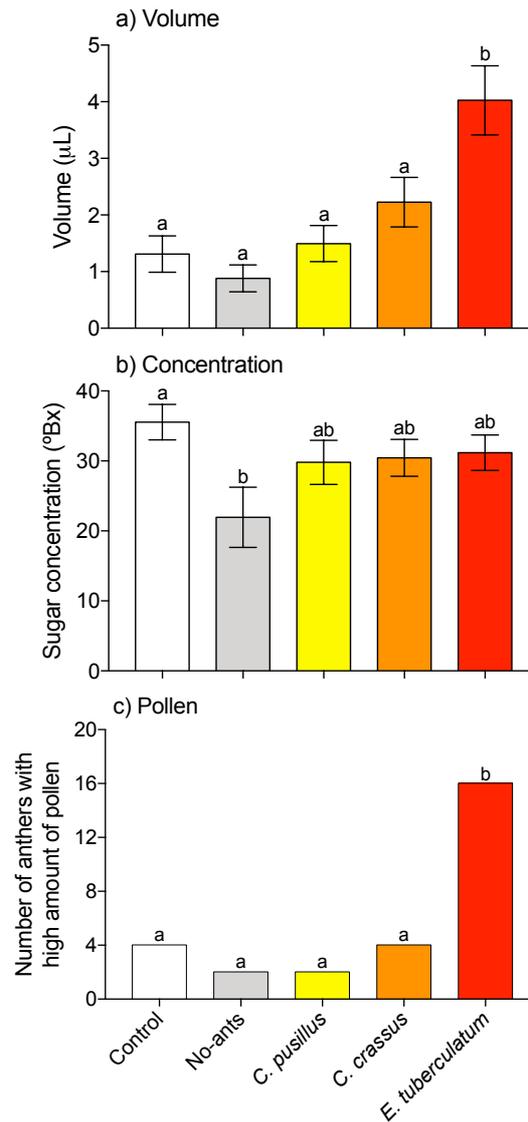


Figure 7 – Volume (a) and sugar concentration of floral nectar (b), and number of anthers with high amount of pollen per treatment (c). LMM:  $\chi^2 = 41.658$ ,  $p < 0.001$  (a); LMM:  $\chi^2 = 10.502$ ,  $p < 0.05$  (b); GLMM:  $\chi^2 = 17.779$ ,  $p < 0.01$  (c); Letters represent statistic difference between the treatments by the post-hoc of Tukey's HSD. Bars in (a) and (b) represent mean and SE.

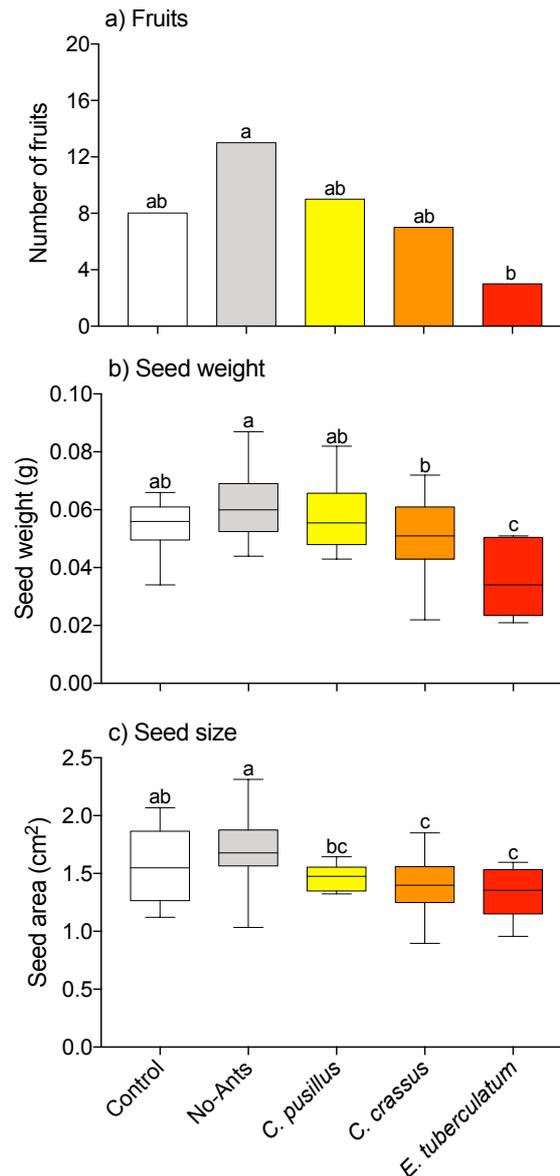


Figure 8 – Number of fruit set (a), seed weight (b) and seed size (c) per treatment. GLMM:  $\chi^2 = 10.063$ ,  $p < 0.05$  (a); LMM:  $\chi^2 = 49.645$ ,  $p < 0.001$  (b); LMM:  $\chi^2 = 45.146$ ,  $p < 0.001$  (c). Letters represent statistic difference between the treatments by the post-hoc of Tukey's HSD. Bars in (b) and (c) represent median and minimums and maximums.

## Discussion

Our experiments demonstrate that the presence of ants on flowers can decrease plant fitness components by deterring visitation of potential pollinators to the plants, including vertebrates, as we have hypothesized. The strength of this negative impact depended on both the identity of ants and the identity of the floral visitors to *Qualea multiflora*. Importantly, we

found that the hourly activity of ants and pollinators overlapped. As a result, depending on the identity of the ant species and that of the floral visitor, ants (i) interfered with the behavior of invertebrate (especially bees) and vertebrate (hummingbirds) pollinators, (ii) affected the amount of floral resources collected (nectar volume and pollen), and (iii) reduced fruit set, seed weight, and seed size. Our study is the first experimental study to show a tradeoff between the beneficial effects of ant protection against herbivorous insects on the one hand (supported by Del-Claro et al. 1996), and the negative consequences for pollination on the other in the same plant species. Previous studies that tested for ant effects on pollinator behavior either did not test for an effect on plant fitness (Villamil et al. 2018), showed an effect on plant fitness but were not experimental (Ness 2006), or showed an effect on pollinator behavior and plant fitness but involved Membracidae instead of extrafloral nectaries (Ibarra-Isassi and Oliveira 2017).

The presence and identity of ants were limiting factors for plant fitness, floral resources, and pollinator behavior. Other studies have shown similar results, in which pollinators were able to recognize ant identity leading to a change in their behavior (see Ness 2006, Ibarra-Isassi and Oliveira 2017). For instance, Villamil et al. (2018) showed that pollinators of *Turnera velutina* (Passifloraceae) behaved differently, as quantified by duration of the visit, the time inspecting the flower, and the number of visits, depending on the ant species present. The most aggressive ant, *Dorymyrmex bicolor*, had the strongest effect on pollinator behavior, especially on honeybees (Villamil et al. 2018). Similarly, Assunção et al. (2014) showed flowers with an ant-shaped plastic figure in them were less likely to be visited by pollinators compared to control flowers containing a circular plastic figure (also see Barônio and Del-Claro 2017).

We observed a decrease in the impacts of ants on plant fitness, floral resources and pollinator behavior, as ant size and ant aggressiveness decreased, as we hypothesized. *Ectatomma tuberculatum*, the largest and most aggressive species had the greatest negative effect, while *C. pusillus*, the smallest and the least aggressive species had the smallest negative

effect. Ibarra-Isassi and Oliveira (2017) showed that *Ectatomma brunneum* (tending Membracidae in *Byrsonima intermedia*) had a greater negative impact than *Camponotus rufipes* on floral visitor behavior. This result is similar to ours, in that we saw a greater negative effect on plant fitness and pollinator behavior by *Ectatomma* species compared to the other two ant species. In fact, *E. tuberculatum* is a larger and very aggressive ant (Reu and Del-Claro 2005, Sanz-Veiga et al. 2017), disrupting pollinators or even eating them (Assunção et al. 2014). Despite being similar in size, we predicted that *C. crassus* would have a greater impact than *C. pusillus*, since the former is more aggressive. *Camponotus crassus* displays aggressive behaviors towards herbivores (Anjos et al. 2017, Fagundes et al. 2017), while *C. pusillus* is docile and is not aggressive (Quinet et al. 2005, Byk and Del-Claro 2010). It is important to mention that, beyond ant shape and size, ant scent markings could also have influenced the pollinator behavior. For instance, some floral visitors may learn to use ant scent markings as informative signals (Cembrowski et al. 2014), as shown in laboratory trials with naïve bumblebees by Ballantyne and Willmer (2012). Despite not directly testing the influence of scent mark on pollinator repellence in the present study, we suggest that both ant shape and scent markings are important to the ant-plant-pollinator interaction.

Our results suggest that ants determine the subset of the visitors to flowers that are actual pollinators, and that this ant effect is related to the body size of the visitors. Pollinators were larger and more robust ( $> 2\text{cm}$ ) than the non-pollinators ( $< 2\text{cm}$ ), except for some lepidopterans (e.g. *Aellopos* sp.). Previous studies suggest that smaller bees tend to approach more carefully flowers containing ants, while larger bees appear to be unaffected by the presence of ants (González et al. 2013, Assunção et al. 2014, Barônio and Del-Claro 2017). In regard to pollinators alone, *Campsomeris* sp. and *Xylocopa* sp. were more likely to visit an ant-tended flower than the other pollinators. Specifically, *Xylocopa* bees were not as affected by ant presence as were *Exomalopsis* bees (Chi-squared = 4.04,  $p = 0.044$ ) (Table 3). In the

case of *Campsomeris* sp., we observed that for the majority of visits, the wasp landed on an inflorescence and passed from one flower to another by walking, which seems to influence the visibility of the experimental ant, since it was pinned at a higher level than that of the wasp. Although *Campsomeris* sp. was more likely than the other species to visit a flower with ants, this wasp did not always touch the reproductive parts, thereby qualified as an occasional pollinator. In this way, there seems to be a filter or selection of floral visitors influenced by ant identity, which can impact plant fitness.

Plants with the most aggressive ant bodyguards produced fruits with lighter and smaller seeds. In a similar study, Ness (2006) showed that seed mass per fruit, seed size, and seed number were negatively correlated with the aggressiveness of the ant. Ness (2006) suggested this result is a consequence of the decrease in pollinator visitation rate and in flower foraging time where the most aggressive ant was present; and also the author proposed that this result could be also due to changes in the quantity or quality of pollen deposited to the plant (although this is unknown). We agree with Ness' (2006) proposals since some studies have shown that the amount of pollen deposited may influence the number and quality of fruits, seeds and consequently the vigor of the seedlings (see "pollen competition hypothesis" and "gametophyte competition hypothesis") (Mulcahy 1979, Lee 1984, Stephenson et al. 1986, Visser et al. 1988), even though we did not evaluate this factor. Although we did not measure the amount of pollen deposited on stigmas, we believe that the visitation rate and pollinator behavior can influence the pollen grain quantity and quality on stigmas, translating in a lower number and quality of fruits and seeds.

Our results, together with those of previous studies, suggest that there is a tradeoff in *Q. multiflora* between the beneficial effects of ant protection against herbivorous insects on the one hand, and the negative consequences for pollination on the other. Del-Claro et al. (1996) showed that *Q. multiflora* individuals with ants suffered less damage to leaves, buds, and

flowers, and produced more fruits than plants from which ants were experimentally excluded. Thus ants provide protection against herbivores in this system. Our results, however, suggest that this protective effect comes at the cost of decreased pollination, and as a result, reduced seed quality. The identity of the ant species is also likely to be important. Xu and Chen (2010) showed that more aggressive ant species visiting extrafloral nectaries of two species of *Passiflora* reduced herbivory to a greater extent. In our system, however, the more aggressive the ant species was, the greater the negative effect on pollinator visitation (Fig. 4) and seed production (Fig. 8). The relative strengths of these beneficial versus detrimental effects are apparently unstudied for any one system. Thus the balance of this tradeoff is not known. Our results do suggest that studies of the impacts of ants on plant fitness components via protection against herbivores may underestimate the positive effects of those ants if plants have active extrafloral nectaries when the plant is reproductive. In our study, we were able to isolate the negative effects of ants on plant fitness via pollinator distraction because EFNs on leaves of *Q. multiflora* are not active while the plant is in flower. We also suggest that we underestimated the impact of ant presence on pollinator behavior because the ants we used were dead, and therefore not able to respond aggressively to approaching floral visitors.

Some plant traits may mitigate this suggested tradeoff: (i) EFN placement – *Q. multiflora* produces EFNs at the base of leaf petioles, resulting in ant protection of leaves from herbivores (Calixto et al. 2015); production of EFNs at the base of each flower pedicel may be related to reproductive part protection or to the distraction of ants from flowers (flower-distraction hypothesis), consequently decreasing the direct conflict between ants and pollinators (see Calixto et al. 2018a, Villamil et al. 2019). However, as far as we know, no study has tested these latter two hypotheses in plants bearing EFNs in vegetative and reproductive parts. Since we observed ants foraging on flowers in the Control treatment, and consuming floral nectar, we believe that these structures are more related to the distraction of

the ants than to the defense; (ii) Flower phenology – we believe that *Q. multiflora* produces many flowers (~30 per inflorescence) and inflorescences (~15 per plant), and a daily sequential flower opening (1-5 per inflorescence per day) as a strategy to increase the likelihood of producing a fruit in response to the negative impact of pollinator deterrence. Effective pollinators are bumblebees and carpenter bees. These solitary bees present a specific body size, which fits the length of the stamen and carpel of *Q. multiflora*. In addition, they have a visitor rate lower than that of social bees, such as honeybees (see Courcelles et al. 2013). In this sense, the floral visitor size, the frequency of visits, the time of visits, among other traits, appear to be prime factors in the pollinating process of *Q. multiflora*. Therefore, there seems to be a complex pollination system, in which small changes can result in a non-production of fruits.

In conclusion, we showed experimentally that the presence of ants on flowers decreases plant fitness components by deterring visitation of potential pollinators to the plants. This result indicates a tradeoff between the beneficial effects of ant protection against herbivorous insects and the negative consequences for plant fitness. Then, in order to further our understanding of multitrophic interactions, we suggest that further studies should be undertaken addressing the following: what is the evolutionary role of EFNs located in reproductive parts of the EFN-bearing plants? What are the means found by plants to reduce the costs of dissuasion of pollinators by protective ants? What are the net effects of the tradeoff between protection and pollinator repellence by ants?

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# Conclusion

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Our study pointed out that (i) in recent years the scientific and academic areas have been working to provide and improve the knowledge about insect-plant relationships, particularly the mutualism between ants and plants mediated by EFNs, where most studies have shown the protective effect of ants, and others have shown small peculiarities and conditionalities of the system; (ii) the activity of EFNs influences the ant-herbivore relationship leading to patterns of seasonality and synchronism of herbivore and ant populations, with different herbivore predation rates, and impacts on plant fitness; (iii) short-and long-term provision of extrafloral nectar and artificial food source, especially those rich in carbohydrates and proteins, benefits the ant colony fitness (number and weight of individuals) and survival; (iv) the protective mutualism between ants and EFN-bearing plants may negatively influence plant fitness via deterring visitation by pollinators.

We showed that extrafloral nectar is a key factor in the regulation and structuring of ecological interactions, directly influencing the growth and survival of ant colonies, as well as in the interaction of ants with herbivores and pollinators in plants bearing extrafloral nectars. In this perspective, we should study each ecological system in detail, knowing all interacting beings, as well as their respective natural histories, to understand the patterns of interaction, the structuring of ecological networks, and the net effect of presenting aggressive ants as “bodyguards”, which can directly influence the natural ecosystems dynamic.