**Optimal Defense Theory in an ant-plant mutualism: extrafloral nectar as an
induced defense is maximized in the most valuable plant structures**

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Abstract

1. Plants allocate defenses in order to decrease costs and maximize benefits against herbivores. The Optimal Defense Theory (ODT) predicts that continuously expressed (i.e., constitutive) defenses are expected in structures of high value, whereas defenses that are expressed or that increase their expression only after damage or upon risk of damage (i.e., induced defenses) are expected in structures of low value. Although there are several studies evaluating ODT predictions, few studies have successfully tested them as a way of measuring ecological investment in extrafloral nectary (EFN)-mediated ant-plant interactions.
2. Here, we compared extrafloral nectar production and ant attractiveness to EFNs located on reproductive versus vegetative plant structures on *Qualea multiflora* plants subjected to different levels of simulated herbivory. We then addressed the following predictions emerging from the ODT: (i) extrafloral nectar produced in inflorescence EFNs will have higher volumes and calories and will attract more ants than extrafloral nectar produced in leaf EFNs; (ii) extrafloral nectar production (volume and calories) and ant attendance will increase after simulated herbivory in leaf EFNs but not in inflorescence EFNs; (iii) higher simulated leaf herbivory will induce higher extrafloral nectar production in EFNs on leaves; and (iv) more attractive extrafloral nectar (higher volume and calories) will attract more ants.
3. Extrafloral nectar volume and calorie content, as well as ant abundance, were higher in EFNs of inflorescences compared to EFNs of leaves both before and after simulated herbivory, consistent with one of our predictions. However, EFNs on both leaves and inflorescences, not on leaves only, were induced by simulated herbivory, a pattern opposite to our prediction. Plants subjected to higher levels of leaf damage (i.e., more damage to lower-value tissues) produced more and higher-calorie extrafloral nectar, but showed similar ant abundance, partially consistent with our prediction. Finally, more attractive extrafloral nectar

45 attracted more ants.

46 4. *Synthesis*. Our results show that extrafloral nectar production before and after
47 simulated herbivory, as well as ant recruitment, varies according to the plant structure on
48 which EFNs are located. Our study is the first to show that ant recruitment via extrafloral
49 nectar follows predictions from Optimal Defense Theory, and that the ant foraging patterns
50 may be shaped by the plant part attacked and the level of damage it receives.

51 **Keywords:** optimal defense theory, ant-plant mutualism, extrafloral nectar, induced defense,
52 indirect defense, cerrado, herbivory, mutualism, plant defense, *Qualea multiflora*

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Introduction

Plants and herbivores have interacted for millions of years (Pemberton, 1992; Stowe, Marquis, Hochwender, & Simms, 2000; Thompson, 2005, 2013). Herbivores have evolved to feed effectively, while plants have countered with a wide variety of anti-herbivore defenses (Agrawal, Salminen, & Fishbein, 2009; Mortensen, 2013). Some of these defenses are continuously expressed in plants across ontogeny (constitutive defenses such as secondary metabolites and spines; Boege & Marquis, 2005), whereas other defenses are expressed or increase in expression only after damage or upon risk of damage (inducible defenses such as volatile organic compounds; Chen, 2008; Frost, Mescher, Carlson, & De Moraes, 2008; Zangerl & Rutledge, 1996). Note that this distinction is approximate, and defenses that are mostly constitutive can be partially inducible, while inducible defenses may also be expressed at low constitutive levels. Plant defenses can be also classified as either direct or indirect (Pearse et al., 2020), with indirect defense represented by associations between plants and their herbivores' own predators and parasitoids (Calixto, Lange, & Del-Claro, 2018; Pearse et al., 2020). These associations are mediated by a diversity of plant resources, notably nectar produced in organs located outside of flowers (extrafloral nectaries, or EFNs; Koptur, 1992; Marazzi, Bronstein, & Koptur, 2013).

Extrafloral nectar, a carbohydrate-based liquid containing other dissolved compounds (González-Teuber & Heil, 2009; Nicolson, Nepi, & Pacini, 2007), attracts diverse predators and parasitoids (Calixto, Sousa-Lopes, & Del-Claro, 2018; Koptur, 2005; Taylor & Pfannenstiel, 2009), but especially ants (Calixto, Lange, & Del-Claro, 2018). This interaction is a classic example of protection mutualism, in which ants can reduce foliar herbivory (Lange & Del-Claro, 2014; Rosumek et al., 2009; Trager et al., 2010) and/or increase plant fitness (Nahas, Gonzaga, & Del-Claro, 2012; Nascimento & Del-Claro, 2010; Trager et al., 2010). EFNs are generally active on young leaves (Calixto, Lange, & Del-Claro, 2015;

Dáttilo et al., 2015), which are more vulnerable to damage by herbivores compared to mature leaves (Heil, 2015; Heil, Fiala, Baumann, & Linsenmair, 2000). However, some plant species also produce EFNs on other plant parts, including fruits (Del-Claro, Guillermo-Ferreira, Almeida, Zardini, & Torezan-Silingardi, 2013; Sousa-Lopes, Calixto, Torezan-Silingardi, & Del-Claro, 2020), sepals, flower buds, and inflorescences (Elias, 1983). The presence of EFNs on reproductive structures can directly influence plant fitness, since ants foraging on these structures protect them against seed-eating herbivores, resulting in an increase of fruit production (Del-Claro, Berto, & Réu, 1996).

Optimal Defense Theory (ODT; sensu McKey, 1974, 1979; Rhoades, 1979) is one of the leading theories that seeks to explain patterns of anti-herbivore defense. According to this theory, to minimize costs and maximize defenses against herbivores, plants will allocate their defenses to structures according to their value and probability of attack. In this context, it is predicted that constitutive defenses should be used to protect structures of high value (including buds, flowers and fruits) that are subject to a high probability of attack, whereas induced defenses should be used in structures of low value (such as fully expanded leaves; Karban & Myers, 1989; Zangerl & Rutledge, 1996) that are subject to a low probability of attack. However, only recently have studies experimentally examined indirect defenses such as ant-mediated defense in the context of ODT (Holland, Chamberlain, & Horn, 2009; Radhika, Kost, Bartram, Heil, & Boland, 2008; Rostás & Eggert, 2008; Wäckers & Bonifay, 2004). For instance, support has been found for predictions from ODT in several ant-defended plants, showing different extrafloral nectar production between higher-value and lower-value structures (Holland, Chamberlain, & Horn, 2009; Radhika, Kost, Bartram, Heil, & Boland, 2008; Rostás & Eggert, 2008; Wäckers & Bonifay, 2004).

We can also predict that the extrafloral nectar produced on reproductive structures will be more attractive than that produced on vegetative structures, resulting in greater attraction

of ants. Studies have shown that variation in extrafloral nectar production directly influences the ant community and ant foraging patterns (Bixenmann, Coley, & Kursar, 2011; Lange, Calixto, & Del-Claro, 2017; Pacelhe, Costa, Bronstein, Mello, & Neves, 2019). An increase in extrafloral nectar production attracts more ants and can increase ant aggressiveness, resulting in improved plant defense (Falcão, Dáttilo, & Izzo, 2014; Pacelhe, Costa, Bronstein, Mello, & Neves, 2019). For instance, Pacelhe, Costa, Bronstein, Mello, and Neves (2019) showed that the predatory activity of ants was higher on plants augmented with artificial extrafloral nectar composed of sugar and amino acids than in plants augmented with only sugar or amino acids or water. Thus, these studies show that more concentrated and nutritive extrafloral nectar influences ant foraging patterns.

A few studies have successfully tested ODT predictions as a way of measuring ecological investment in EFN-mediated ant-plant interactions (Holland, Chamberlain, & Horn, 2009; Radhika, Kost, Bartram, Heil, & Boland, 2008; Rostás & Eggert, 2008; Wäckers & Bonifay, 2004). However, while these studies have considered patterns in the production of extrafloral nectar, they have not looked at ant attraction to the EFNs. Extrafloral nectar is the resource provided by plants to mediate the interaction between defensive ants and host plants, but it is essential to assess ant attendance to fully evaluate ODT's predictions concerning indirect defenses (see Pearse et al., 2020). One of the reasons for the rarity of studies empirically evaluating ODT predictions related to indirect defense is the difficulty of finding good experimental plant models, that is, plants bearing EFNs on tissues differing in value to the plant. In this regard, the Brazilian savanna plant *Qualea multiflora* Mart. (Vochysiaceae) offers exceptional advantages. This plant species bears EFNs on both the leaves (Fig. 1a-b) and inflorescences (Fig. 1c); continuous ant availability (Del-Claro, Berto, & Réu, 1996) combined with a phenological separation of leaf and inflorescence rewards (Calixto, Lange, & Del-Claro, 2015) allow us to distinguish ant attraction to the two types of EFNs.

Here, we compared extrafloral nectar production and ant attractiveness to EFNs located on reproductive versus vegetative plant structures on *Q. multiflora* plants subjected to simulated herbivory. We then addressed the following predictions emerging from the ODT: (i) extrafloral nectar produced in inflorescence EFNs will have higher volumes and calories and will attract more ants than extrafloral nectar produced in leaf EFNs, given the relative value of these tissues (Cousens, Dytham, & Law, 2008); (ii) Extrafloral nectar production (volume and calories) and ant attendance will increase after simulated herbivory in leaf EFNs but not in inflorescence EFNs since the latter are expected to produce nectar constitutively (Wäckers & Bonifay, 2004); (iii) higher simulated leaf herbivory will induce higher extrafloral nectar production in EFNs on leaves. Induction of extrafloral nectar depends on the severity of the damage, and the greater the damage, the greater the stimulus (Kwok & Laird, 2012); and (iv) more attractive extrafloral nectar (higher volume and calories) will attract more ants.

Material and Methods

Study area and species evaluated

We carried out this study in the Reserva Ecológica do Clube de Caça e Pesca Itororó de Uberlândia (CCPIU - 48°17'51" W; 18°58'58" S; ~200ha), in Uberlândia, MG, Brazil, from September 2013 to January 2014. The vegetation is characterized by cerrado *sensu stricto*, consisting of trees 2-10 m high and shrubs and grasses in the sub-forest. The region is characterized by rainy summers (from October to March) and dry winters (from April to September). For a fuller characterization of the area, see Alves-Silva, Bächtold, and Del-Claro (2018) and Ferreira and Torezan-Silingardi (2013).

Qualea multiflora (Vochysiaceae) is a deciduous, EFN-bearing species that loses leaves from June to August and produces new leaves at the beginning of the rainy season

(September). Flowers are produced in November and are borne in a terminal inflorescence. EFNs are located on both sides of the young stems, at the base of the leaf petioles (Fig. 1a-b) and in the floral pedicel at the base of each floral bud (Fig. 1c). Leaves are continuously produced from early September until December, and present active EFNs during their early life span. As leaves develop, these EFNs stop producing nectar (Calixto, Lange, & Del-Claro, 2015). Once all leaves are developed and leaf EFNs stop secreting nectar, flowering begins; at that point, EFNs on inflorescences appear and become active (Fig. 1c). Ants patrolling this species do not vary in species identity between the periods of leaf production and flowering (Del-Claro, Berto, & Réu, 1996). In a previous study (Calixto, Lange, & Del-Claro, 2015), we showed that indirect defense is more effective than physical defense (trichomes and foliar toughness) during the intermediate phase of leaf development in *Q. multiflora*. Lange & Del-Claro (2014) have shown that plant individuals without ants experience more herbivory than plants with ants.

Figure 1. Extrafloral nectaries in *Qualea multiflora* (Vochysiaceae). Active extrafloral nectaries a-b) on leaves, and c) on the most basal flower. d) Extrafloral nectaries on the abaxial surface after necrosis (on dead tissues).

Experimental design

We selected 45 plants with similar phenotypic characteristics (1.5-2 m in height, producing leaves with EFNs but not yet producing flowers) and at least 10 m apart. We randomly allocated the plants to one of three treatments (N = 15 plants per treatment; treatments are summarized in Table 1). In the first treatment (Foliar control), no manipulation was conducted. In the other two treatments, herbivory was simulated by cutting the apical part of leaves with scissors. In one treatment (Foliar 10%), 10 % of the leaf area was removed from all leaves (including young and mature leaves) of each plant, In the other treatment (Foliar 40%), we removed 40 % of the leaf area from all leaves in each plant.

Table 1. Experimental treatments on *Qualea multiflora* based on extrafloral nectary location and plant part value according to Optimal Defense Theory (ODT).

Extrafloral nectary location	Plant part value according to ODT	Treatments	Experimental manipulation
	Low value	<i>Foliar control</i>	No manipulation
		<i>Foliar 10%</i>	10% removal of leaf area from all leaves of plant
		<i>Foliar 40%</i>	40% removal of leaf area from all leaves of plant
	High value	<i>Floral control</i>	No manipulation

Floral 10%

10% removal of
flower bud and
flower area from
all flower buds
and flowers of
plant

183
184 In addition, we selected a different set of 30 similar plant individuals (1.5-2 m in
185 height, developed leaves, 10-15 inflorescences), but that were flowering and that had active
186 EFNs on the inflorescences but not on the leaves. We randomly allocated the plants to one of
187 two treatments (N = 15 plants per treatment). In the first treatment (Floral control), no
188 experimental manipulation was conducted; in the second treatment (Floral 10%), we cut 10 %
189 of the apical part of all buds and flowers of each plant with scissors. We did not do a 40 %
190 cutting treatment on buds and flowers as we had on the leaves, due to the small size of floral
191 buds and the associated difficulty in their handling.

192 Leaves, buds and flowers were cut at 2100 h, during the period of highest productivity
193 of extrafloral nectar in *Q. multiflora* (Lange, Calixto, & Del-Claro, 2017). Simulated
194 herbivory has been used in many studies to test induced plant responses, including production
195 of extrafloral nectar (Heil, Fiala, Baumann, & Linsenmair, 2000; Jones & Koptur, 2015;
196 Wäckers & Wunderlin, 1999). In the case of *Q. multiflora*, natural foliar herbivory rates vary
197 from 2.64 ± 1.9 % (mean $\pm$ SD) in ant-attended plants to 8.16 ± 4.08 % in plants without ants
198 (Calixto et al., unpublished). Thus, our treatments mimicked natural herbivory rates.

199 *Data collection*

200 On each individual, we selected one EFN. If studying leaf EFNs, we selected an EFN
201 on the adaxial surface of a young leaf near the apical meristem (Fig. 1b), and if studying
202 inflorescence EFNs, we chose the most basal EFN of an inflorescence (Fig. 1c). The marked
203 EFNs were isolated with a mesh bag and a Tanglefoot resin strip (Tanglefoot®, Grand Rapids,
204 USA), decreasing dilution by rain and dew and reducing access to and removal of nectar by

ants and other arthropods. Both factors (dilution and removal) might influence the amount of nectar present at the time of assessment. Foliar experiments and data collection took place during October, while Floral experiments took place in January.

Nectar produced in all selected EFNs on plants in all five treatments was collected 1, 6, 24, 48, 72, and 96 hours after cutting (method adapted from Heil, Fiala, Baumann, & Linsenmair, 2000). At each census, we measured the volume of nectar produced and the quantity of sugar (Brix % - mg sugar per ml solution) with the aid of 5µL graduated microcapillary tubes and manual refractometer (Eclipse® model, Bellingham & Stanley, Tunbridge Wells, UK). All evaluated EFNs were washed with distilled water and dried with filter paper immediately after simulated herbivory and after each evaluation. During censuses, we recorded ant abundance and richness on plants at the time of nectar collection. An individual of each ant species was collected, fixed in 70% alcohol, and identified, with confirmation by specialists from the Universidade Federal do Paraná, in Curitiba, Brazil. Data on ant identity are presented in Supporting Information (Table S1).

In both experiments (with leaves and with flowers), all data were collected on clear days on which the average daily temperature, humidity, and precipitation for the two periods of collection (October and January) was not significantly different ($P > 0.05$). By collecting data over five consecutive days and due to this similarity between the values of the main environmental variables during the two months of data collection, we reduced the likelihood that any differences in attractiveness to ants between treatments could be attributed primarily to differences in environmental conditions rather than to experimentally manipulated differences in nectar volumes.

Data analysis

Analyses were conducted using RStudio 4.0.0 (R Core Team, 2020). When extrafloral nectar volume was very low ($< 0.1\mu\text{L}$), it was not possible to measure the sugar concentration of the solution. In these cases, only the volume was recorded.

Before conducting analyses, sugar concentration was converted to calories, using the manual refractometer value in % Brix and the volume of nectar (μL). We then calculated the sugar concentration in milligrams (mg) per microliter (μL) using the equation $y = 0.00226 + (0.00937x) + (0.0000585x^2)$, where x is the sugar concentration shown in the refractometer and y is total sugars in $1\mu\text{L}$. Next, as each mg of sugar is equivalent to four calories, the sugar amount found was multiplied by four (Dafni, Kevan, & Husband, 2005; Lange, Calixto, & Del-Claro, 2017).

To test our predictions, we fit different models to different treatments (Table 2). Before testing for significance in each model, we checked the residuals to verify the suitability of the model and overdispersion when applicable. To do this, we first analyzed the fitted *versus* residual values plot, the distribution of residuals in a QQ plot, and the histogram of residuals. Second, we used the “DHARMA” package (Hartig, 2020) to create scaled residuals by simulation from the fit model. We used a parametric bootstrap (250 randomizations) to compare observed residuals against refit residuals, which is indicated for testing overdispersion. Finally, we checked heteroscedasticity with a Breusch-Pagan test using the package “lmtest” (Zeileis & Hothorn, 2002).

Prediction i – To evaluate whether extrafloral nectar produced in inflorescence EFNs has higher volumes and calories and attracts more ants than extrafloral nectar produced in leaf EFNs, we used Linear Mixed Model (LMM) and Generalized Linear Mixed Model (GLMM) followed by Wald chi-square test using the packages “glmmTMB” (Brooks et al., 2017) and

252 “car” (Fox & Weisberg, 2011). For volume and calories as response variables, we used a
253 LMM with Gaussian error distribution, while for ant abundance as the response variable, we
254 used a Zero-Inflated GLMM (ZIGLMM) with Poisson error distribution controlling for zero
255 inflation. Volume was square root transformed to account for heteroscedasticity. For these
256 three models, we used only treatments under control conditions (Foliar control and Floral
257 control) as predictor variable (fixed effect). We added plant individual as a random effect to
258 control for temporal repeated measures (Table 2).

259

260 **Table 2** - Model statistics for extrafloral nectar volume and calories and ant abundance of *Qualea multiflora* individuals in plants with
 261 extrafloral nectaries active on leaves and inflorescences and before and after simulated herbivory.

Predictions	Response	Fixed effects	N	Wald test	P-value	Random effects	Variance	SD
<i>Prediction i</i>	Volume (µL)	Plant part	180	10.771	0.0010	Plant ID	0.005	0.073
	Calories	Plant part	61	7.431	0.0064	Plant ID	4.278 ⁻¹¹	6.541 ⁻⁰⁶
	Ant abundance (number)	Plant part	180	4.996	0.0254	Plant ID	4.735 ⁻¹⁰	2.176 ⁻⁰⁵
<i>Prediction ii and iii</i>	Volume (µL)	Damage level	450	30.127	0.0001	Plant ID	0.001	0.018
	Calories	Damage level	31	52.626	0.0001	Plant ID	3.698 ⁻¹¹	6.081 ⁻⁰⁶
	Ant abundance (number)	Damage level	450	33.536	0.0001	Plant ID	3.399 ⁻⁰⁹	5.83 ⁻⁰⁵
	Volume (µL)	Damage level	180	11.886	0.0001	Plant ID	1.373 ⁻¹¹	3.705 ⁻⁰⁶
	Calories	Damage level	41	4.066	0.0437	Plant ID	3.511 ⁻¹²	1.874 ⁻⁰⁶
	Ant abundance (number)	Damage level	30	11.317	0.0007	Plant ID	1.157 ⁻⁰⁹	3.401 ⁻⁰⁵
	Ant abundance (number)	Volume	72	0.417	0.5181	Plant ID	1.852 ⁻⁰⁹	4.303 ⁻⁰⁵
<i>Prediction iv</i>		Calories	72	9.975	0.0015			
		Volume:Calories	72	5.275	0.0216			

Prediction ii and iii – to test whether extrafloral nectar production and ant attendance will increase after simulated herbivory in leaf EFNs but not in inflorescence EFNs, we conducted two sets of analyses. In the first set we compared only the foliar treatments (Foliar control, Foliar 10%, Foliar 40%), while in the second set we compared only the floral treatments (Floral control and Floral 10%). To compare volume and calories between treatments within each set of analyses, we used a LMM with Gaussian error distribution followed by Wald chi-square test. Volume and calories were square root transformed to account for heteroscedasticity in both models. In the case of ant abundance, we used a GLMM with negative binomial error distribution when comparing foliar treatments, and a ZIGLMM with Poisson error distribution controlling the zero inflation when comparing floral treatments. The selected treatments of each set of analyses were fit as predictor variables (fixed effect). We added plant individual as a random effect to control temporal repeated measures (Table 2).

To test prediction ii regarding foliar treatments, we looked for a significant increase of the response variables (extrafloral nectar volume and calories, and ant abundance) in the simulated treatments compared to the control treatment. We performed pairwise comparisons using Estimated Marginal Means (EMMs) through the package “emmeans” (Lenth, 2018). To test prediction iii, we looked for a significant increase of the response variables among the three treatments, that is, Foliar 40% should significantly increase its values compared to Foliar 10%, which in turn should significantly increase its values compared to Foliar control. To test this, we also used EMMs.

Prediction iv – To assess if more attractive extrafloral nectar (higher volumes and amount of calories) attract more ants, we conducted a GLMM with negative binomial error distribution to control overdispersion followed by a Wald chi-square test. Ant abundance was used as the response variable and the interaction between volume and calories was the explanatory variable (fixed effects) (Table 2).

Results

We observed 13 ant species belonging to five subfamilies, of which Formicinae was the subfamily with the largest number of species (Supporting Information Table S1). *Camponotus renggeri* made up 43-68 % of all ants observed across the five treatments. This ant was commonly seen foraging near the reproductive parts and attacking putative herbivores, as is common for *Camponotus* spp. Below, we consider these results in the context of each of the four predictions we posed.

Prediction i – extrafloral nectar production and ant attendance will be higher in inflorescence EFNs than in leaf EFNs

We observed a significant difference in volume ($\chi^2 = 10.771$, $df = 1$, $P < 0.01$), calories ($\chi^2 = 7.431$, $df = 1$, $P < 0.01$) of extrafloral nectar as well as in ant attendance ($\chi^2 = 4.996$, $df = 1$, $P < 0.05$) between Floral control and Foliar control treatments (Fig. 2). Floral EFNs produced on average 1.73 and 2.93 times more extrafloral nectar (volume and calories respectively) than foliar EFNs. In addition, Floral EFNs attracted on average 2.63 more ants than Foliar control.

Figure 2. Variation in extrafloral nectar volume (a) and calories (b) and in ant abundance (c) of *Qualea multiflora* individuals with extrafloral nectaries active on leaves (Foliar) and inflorescences (Floral) under control conditions. Graphs are represented by bars with mean, hinges and whiskers, raw data (points), and violin plot based on Kernel density function.

Prediction ii – extrafloral nectar production and ant attendance will increase after simulated herbivory in leaf EFNs but not in inflorescence EFNs

Simulated herbivory increased the volume and calories of extrafloral nectar produced in both types of EFNs (Fig. 3a-d); in the leaf treatments, however, this only occurred for volume and calories when simulated herbivory was heavy. Foliar 10% did not significantly

increase production of extrafloral nectar (EMMs – volume: estimate = -0.11, $P = 0.076$;
 calories: estimate = -0.13, $P = 0.061$; Fig. 3a,c), whereas Foliar 40% did (EMMs – volume:
 estimate = -0.28, $P < 0.001$; calories: estimate = -0.33, $P < 0.001$; Fig. 3a,c). Foliar 40%
 EFNs produced on average 2.86 and 6.68 times more extrafloral nectar, by volume and
 calories respectively, than Foliar control EFNs. Furthermore, after simulated herbivory, Floral
 10% also produced significantly higher volumes ($1.88\times$ higher; $\chi^2 = 11.886$, $df = 1$, $P < 0.001$)
 and calories ($1.46\times$ higher; $\chi^2 = 4.066$, $df = 1$, $P < 0.05$) of extrafloral nectar than Floral
 control (Fig. 3b,d).

Simulated herbivory resulted in significantly higher ant numbers than on control
 plants. Foliar 10% attracted on average 3.5 times more ants (EMMs: estimate = -1.24, $P <$
 0.01) and Foliar 40% attracted 3.98 times more ants (EMMs: estimate = -1.38, $P < 0.001$)
 compared to Foliar control (Fig. 3e). Similarly, Floral 10% attracted significantly more ants
 than Floral control ($1.7\times$ more; $\chi^2 = 11.317$, $df = 1$, $P < 0.001$; Fig. 3f).

Figure 3. Variation in extrafloral nectar volume (a-b) and calories (c-d), and in ant abundance (e-f) of *Qualea multiflora* individuals with extrafloral nectaries active on leaves (a, c, e) and inflorescences (b, d, f), and before (Control) and after (10 % or 40 % damage) simulated herbivory. Graphs are represented by bars with mean, hinges and whiskers, raw data (points), and violin plot based on Kernel density function. Different letters differ from each other by estimated marginal means.

Prediction *iii* – the higher the foliar damage, the higher the foliar extrafloral nectar production and ant attraction

Higher levels of leaf damage (Foliar 40%) resulted in significantly higher extrafloral nectar volumes (1.6x higher; EMMs: estimate = -0.17, $P < 0.01$) and calories (2.1x higher; EMMs: estimate = -0.20, $P < 0.001$) compared to the lower damage level (Foliar 10%) (Fig. 3a,c). On the other hand, ant abundance was similar between Foliar 10% and Foliar 40% (EMMs: estimate = -0.13, $P = 0.805$; Fig. 3e).

Prediction *iv* – more attractive extrafloral nectar will attract more ants

We found that higher extrafloral nectar volumes and calories attracted significantly more ants (volume*calories: $\chi^2 = 5.275$, $df = 1$, $P < 0.05$; Fig. 4). Furthermore, when analyzed separately, calories showed a significant and positive influence on ant abundance ($\chi^2 = 9.975$, $df = 1$, $P < 0.01$, but volume did not ($\chi^2 = 0.417$, $df = 1$, $P = 0.518$).

Figure 4 – Positive relationship between ant abundance and volume (a) and calories (b) of extrafloral nectar in *Qualea multiflora*. GLMM (volume*calories: $\chi^2 = 5.275$, $df = 1$, $P < 0.05$; volume: $\chi^2 = 0.417$, $df = 1$, $P = 0.518$; calories: $\chi^2 = 9.975$, $df = 1$, $P < 0.01$).

Discussion

Overview

Optimal Defense Theory seeks to explain patterns of defense against herbivores (McKey, 1974, 1979; Rhoades, 1979). According to this theory, plants minimize costs and maximize defenses against herbivores by allocating those defenses to structures according to their value and probability of attack. In this context, constitutive defenses should be used in structures of high value that are subject to a high probability of attack, whereas induced defenses should be used in structures of low value and probability of attack.

We tested predictions from ODT for one of the most widely distributed indirect defenses of plants, biotic defense mediated by EFNs. EFNs are widespread, diverse, and frequently studied, but little is known about the patterns of constitutive and induced extrafloral nectar production among plant species or among parts of a single plant. A handful of studies have tested the ODT's predictions to evaluate extrafloral nectar investment as an indirect defense (Holland, Chamberlain, & Horn, 2009; Radhika, Kost, Bartram, Heil, & Boland, 2008; Rostás & Eggert, 2008; Wäckers & Bonifay, 2004). All of them have provided support for two of its predictions: (1) more extrafloral nectar is produced on structures of high value and probability of attack, and (2) constitutive secretion of EFN is found in structures of high value and probability of attack, whereas inducible secretion is found in structures of low value and probability of attack. However, these studies are limited in two important respects that should be considered in applying ODT to interpret indirect defenses. First, they have not considered the extent to which ants are attracted to the EFNs. Second, these studies have not used different levels of damage to assess the induced defenses. The type and level of damage can be crucial factors in the induction of EFNs (Kwok & Laird, 2012; Pulice & Packer, 2008), and therefore they must also be evaluated.

We made the following predictions based on expectations from ODT: (i) under control

conditions, EFNs on inflorescences will produce higher extrafloral nectar volumes and calories than EFNs on leaves, attracting more ants; (ii) EFNs on leaves but not inflorescences will be inducible, i.e., will increase extrafloral nectar production after simulated herbivory, leading to an increase in ant attraction, in contrast to EFNs on damaged vs. undamaged inflorescences; (iii) higher simulated leaf herbivory will induce higher extrafloral nectar production in EFNs on leaves; and (iv) more attractive extrafloral nectar (higher volume and calories) will attract more ants. Our results, summarized in Fig. 5, were consistent with predictions *i* and *iv*, but only partially consistent with prediction *ii* and *iii*: nectar production in both foliar and floral EFNs was found to be inducible, and only volume and calories were related to the extent of damage.

Figure 5. Summary of nectar production (volume and calories) by extrafloral nectaries (EFNs) on leaves and inflorescences of *Qualea multiflora*, and of ant abundance at nectaries, under control conditions and after simulated herbivory.

Extrafloral nectar production and ant attractiveness

Under control conditions, EFNs on inflorescences produced more volume and calories of extrafloral nectar and attracted more ants than EFNs on leaves, as we predicted (Prediction *i*). According to the ODT, reproductive parts of plants should be better protected than vegetative ones. Vegetative tissues are generally less valuable and relatively easier to replace compared to flowers and fruits (McKey, 1979; Zangerl & Bazzaz, 1992), which are more valuable due to their direct link with plant reproductive success (Cipollini & Levey, 1997; Holland, Chamberlain, & Horn, 2009). In addition, Floral control EFNs produced higher quality and quantity of extrafloral nectar than Foliar control EFNs under control conditions, attracting more ants. These results are consistent with studies showing that quantity and quality of nectar can influence ant patrolling behavior (Blüthgen, Gottsberger, & Fiedler, 2004; Lange, Calixto, & Del-Claro, 2017; Pacelhe, Costa, Bronstein, Mello, & Neves, 2019), and that higher production of extrafloral nectar leads to higher ant attendance (Bixenmann, Coley, & Kursar, 2011; Falcão, Dáttilo, & Izzo, 2014; Pacelhe, Costa, Bronstein, Mello, & Neves, 2019). Although our results suggest a higher attractiveness of ants to Floral control EFNs than to Foliar control EFNs, other intrinsic (e.g., ant behavior) and extrinsic (e.g., environmental variables) factors to ants might also influence ant abundance. However, at least for temperature, precipitation and humidity, there was no significant difference between the two periods of collection (Foliar experiments in October, and Floral experiments in January), and therefore we suggest that ant foraging in our study is more influenced by extrafloral nectar attractiveness than by environmental variables.

EFNs on both leaves and inflorescences increased the production of extrafloral nectar (volume and calories) after simulated herbivory, showing that both are inducible defenses. This result is contrary to the expectation from ODT. We had expected that EFNs on leaves but not those on inflorescences would be inducible (Prediction *ii*). Several studies have shown that

foliar EFNs can be induced after simulated herbivory or natural damage (e.g., Agrawal & Rutter, 1998; Heil, Fiala, Baumann, & Linsenmair, 2000; Mondor, Tremblay, & Messing, 2006). For instance, Wäckers and Bonifay (2004) showed in *Gossypium hirsutum* that nectar production by EFNs on leaves was induced, whereas that by EFNs on bracts was not (Zangerl & Rutledge, 1996). These studies fit the predictions of ODT: more valuable tissues express constitutive defenses, whereas less valuable ones express induced defense. However, little is known about the rate of secretion of EFNs on reproductive regions as induced defense. It is possible that (1) extrafloral nectar might be expensive to produce, so it should not be wasted by being produced constitutively (O'Dowd, 1979); (2) ants are very abundant on *Q. multiflora* (Lange, Calixto, & Del-Claro, 2017) and so responsive to increases in EFN that they could respond very quickly to a threat and fully protect the inflorescences under attack, allowing EFN to be inducible; and (3) there may be evolutionary and developmental constraints that prevent leaf and floral EFNs on the same plant from being regulated differently. These hypotheses remain to be tested.

After simulated herbivory, extrafloral nectar production in plants with active EFNs on leaves increased as leaf damage increased, as we predicted (Prediction *iii*). Kwok and Laird (2012) showed that *Vicia faba* L. have the ability to recognize the severity of herbivore damage, inducing the production of extrafloral nectar, and consequently recruiting more ants. Several studies on damage recognition by plants have shown how complex and refined these systems can be. Many plants have developed damage recognition systems, in which they are able to identify certain substances or molecules present in insect saliva or eggs (Arimura, Kost, & Boland, 2005; Arimura, Ozawa, & Maffei, 2011; Carrillo, Wang, Ding, & Siemann, 2012). These studies show that recognition systems can be very accurate, and therefore that the intensity of the damage can also be an important factor for the induced response in plants. Although there was an increase in the production of extrafloral nectar as leaf damage

increased, the ant abundance was similar between Foliar 10% and Foliar 40%, which was not consistent with our prediction (Prediction *iii*). It is probable that the difference in nectar production between 10% and 40% foliar damage is not sufficient to trigger a differential response by ants.

Finally, higher volumes and calories of extrafloral nectar attracted more ants (Prediction *iv*) in all of our experiments. Jones & Koptur (2015) showed that an increase in extrafloral nectar production increased the number of ants that attend *Senna chapmanii*. As previously seen, different volumes and concentrations of extrafloral nectar influence ant foraging (Lange, Calixto, & Del-Claro, 2017), where higher volumes and concentrations of nectar attract a greater numbers of ants (also see Bixenmann, Coley, & Kursar, 2011; Falcão, Dáttilo, & Izzo, 2014; Pacelhe, Costa, Bronstein, Mello, & Neves, 2019). Extrafloral nectar is mainly composed of monosaccharides and disaccharides, especially sucrose, fructose and glucose (Koptur, 1994). Since carbohydrates are essential for the metabolism and physiology of ant workers (Davidson, 1998; Davidson, Cook, Snelling, & Chua, 2003), it is expected that more caloric resources (higher concentrations of sugar) will attract more ants. Further, we note that this increase in extrafloral nectar production does not only attract more ants, but specifically the mutualistic ants that will protect the plant. We found that *Camponotus renggeri* was the most common species in all treatments, making up 43-68% of all ant visitors. *Camponotus* are frequent and abundant on plants and are considered the main defense agents of extrafloral nectary-bearing plants in the Cerrado habitat (Anjos et al., 2017; Lange, Calixto, & Del-Claro, 2017; Lange, Calixto, Rosa, Sales, & Del-Claro, 2019; Pires, Calixto, Oliveira, & Del-Claro, 2017).

Final remarks

Several studies have tested the predictions of the ODT in indirect plant defense systems. However, they have not considered attraction to ants of EFNs or the different levels

of damage to assess the induced defenses. In this study, we tested ODT predictions in an EFN-mediated ant-plant mutualism, in which ants attending host plants are also influenced by the plant structure providing the food resource, as well as by the presence and level of damage to these structures. EFNs on vegetative and reproductive plant structures present distinct patterns of secretion: the former exhibit low levels of extrafloral nectar production, whereas the latter present high levels of production. However, extrafloral nectar in both cases can be induced. They respond in different ways, consistent with the value of the plant structure as well as the damage level. Thus, our study directly contributes to the understanding of how indirect plant defenses are allocated.

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Authors' contributions

ESC, DL and KDC conceived the ideas and designed methodology; ESC collected the data; ESC analyzed the data; ESC, DL, HMTS, JB, and KDC interpreted the results; ESC led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Data availability

Data available from the Dryad Digital Repository:

<https://doi.org/10.5061/dryad.stqjq2c0x> (E. S. Calixto, Lange, Bronstein, Torezan-Silingardi, & Del-Claro, 2020)

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