



## Optimal Defense Theory in an ant-plant mutualism: Extrafloral nectar as an induced defence is maximized in the most valuable plant structures

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

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18

19

20           **Abstract**

21    1.       Plants allocate defenses in order to decrease costs and maximize benefits against  
22 herbivores. The Optimal Defense Theory (ODT) predicts that continuously expressed (i.e.,  
23 constitutive) defenses are expected in structures of high value, whereas defenses that are  
24 expressed or that increase their expression only after damage or upon risk of damage (i.e.,  
25 induced defenses) are expected in structures of low value. Although there are several studies  
26 evaluating ODT predictions, few studies have successfully tested them as a way of measuring  
27 ecological investment in extrafloral nectary (EFN)-mediated ant-plant interactions.

28    2.       Here, we compared extrafloral nectar production and ant attractiveness to EFNs  
29 located on reproductive versus vegetative plant structures on *Qualea multiflora* plants  
30 subjected to different levels of simulated herbivory. We then addressed the following  
31 predictions emerging from the ODT: (i) extrafloral nectar produced in inflorescence EFNs  
32 will have higher volumes and calories and will attract more ants than extrafloral nectar  
33 produced in leaf EFNs; (ii) extrafloral nectar production (volume and calories) and ant  
34 attendance will increase after simulated herbivory in leaf EFNs but not in inflorescence EFNs;  
35 (iii) higher simulated leaf herbivory will induce higher extrafloral nectar production in EFNs  
36 on leaves; and (iv) more attractive extrafloral nectar (higher volume and calories) will attract  
37 more ants.

38    3.       Extrafloral nectar volume and calorie content, as well as ant abundance, were higher in  
39 EFNs of inflorescences compared to EFNs of leaves both before and after simulated  
40 herbivory, consistent with one of our predictions. However, EFNs on both leaves and  
41 inflorescences, not on leaves only, were induced by simulated herbivory, a pattern opposite to  
42 our prediction. Plants subjected to higher levels of leaf damage (i.e., more damage to lower-  
43 value tissues) produced more and higher-calorie extrafloral nectar, but showed similar ant  
44 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*

53

## 54           **Introduction**

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

71           Extrafloral nectar, a carbohydrate-based liquid containing other dissolved compounds  
72 (González-Teuber & Heil, 2009; Nicolson, Nepi, & Pacini, 2007), attracts diverse predators  
73 and parasitoids (Calixto, Sousa-Lopes, & Del-Claro, 2018; Koptur, 2005; Taylor &  
74 Pfannenstiel, 2009), but especially ants (Calixto, Lange, & Del-Claro, 2018). This interaction  
75 is a classic example of protection mutualism, in which ants can reduce foliar herbivory  
76 (Lange & Del-Claro, 2014; Rosumek et al., 2009; Trager et al., 2010) and/or increase plant  
77 fitness (Nahas, Gonzaga, & Del-Claro, 2012; Nascimento & Del-Claro, 2010; Trager et al.,  
78 2010). EFNs are generally active on young leaves (Calixto, Lange, & Del-Claro, 2015;

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

87 Optimal Defense Theory (ODT; sensu McKey, 1974, 1979; Rhoades, 1979) is one of  
88 the leading theories that seeks to explain patterns of anti-herbivore defense. According to this  
89 theory, to minimize costs and maximize defenses against herbivores, plants will allocate their  
90 defenses to structures according to their value and probability of attack. In this context, it is  
91 predicted that constitutive defenses should be used to protect structures of high value  
92 (including buds, flowers and fruits) that are subject to a high probability of attack, whereas  
93 induced defenses should be used in structures of low value (such as fully expanded leaves;  
94 Karban & Myers, 1989; Zangerl & Rutledge, 1996) that are subject to a low probability of  
95 attack. However, only recently have studies experimentally examined indirect defenses such  
96 as ant-mediated defense in the context of ODT (Holland, Chamberlain, & Horn, 2009;  
97 Radhika, Kost, Bartram, Heil, & Boland, 2008; Rostás & Eggert, 2008; Wäckers & Bonifay,  
98 2004). For instance, support has been found for predictions from ODT in several ant-defended  
99 plants, showing different extrafloral nectar production between higher-value and lower-value  
100 structures (Holland, Chamberlain, & Horn, 2009; Radhika, Kost, Bartram, Heil, & Boland,  
101 2008; Rostás & Eggert, 2008; Wäckers & Bonifay, 2004).

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

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

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

129 Here, we compared extrafloral nectar production and ant attractiveness to EFNs  
130 located on reproductive versus vegetative plant structures on *Q. multiflora* plants subjected to  
131 simulated herbivory. We then addressed the following predictions emerging from the ODT:  
132 (i) extrafloral nectar produced in inflorescence EFNs will have higher volumes and calories  
133 and will attract more ants than extrafloral nectar produced in leaf EFNs, given the relative  
134 value of these tissues (Cousens, Dytham, & Law, 2008); (ii) Extrafloral nectar production  
135 (volume and calories) and ant attendance will increase after simulated herbivory in leaf EFNs  
136 but not in inflorescence EFNs since the latter are expected to produce nectar constitutively  
137 (Wäckers & Bonifay, 2004); (iii) higher simulated leaf herbivory will induce higher  
138 extrafloral nectar production in EFNs on leaves. Induction of extrafloral nectar depends on the  
139 severity of the damage, and the greater the damage, the greater the stimulus (Kwok & Laird,  
140 2012); and (iv) more attractive extrafloral nectar (higher volume and calories) will attract  
141 more ants.

142

## 143 **Material and Methods**

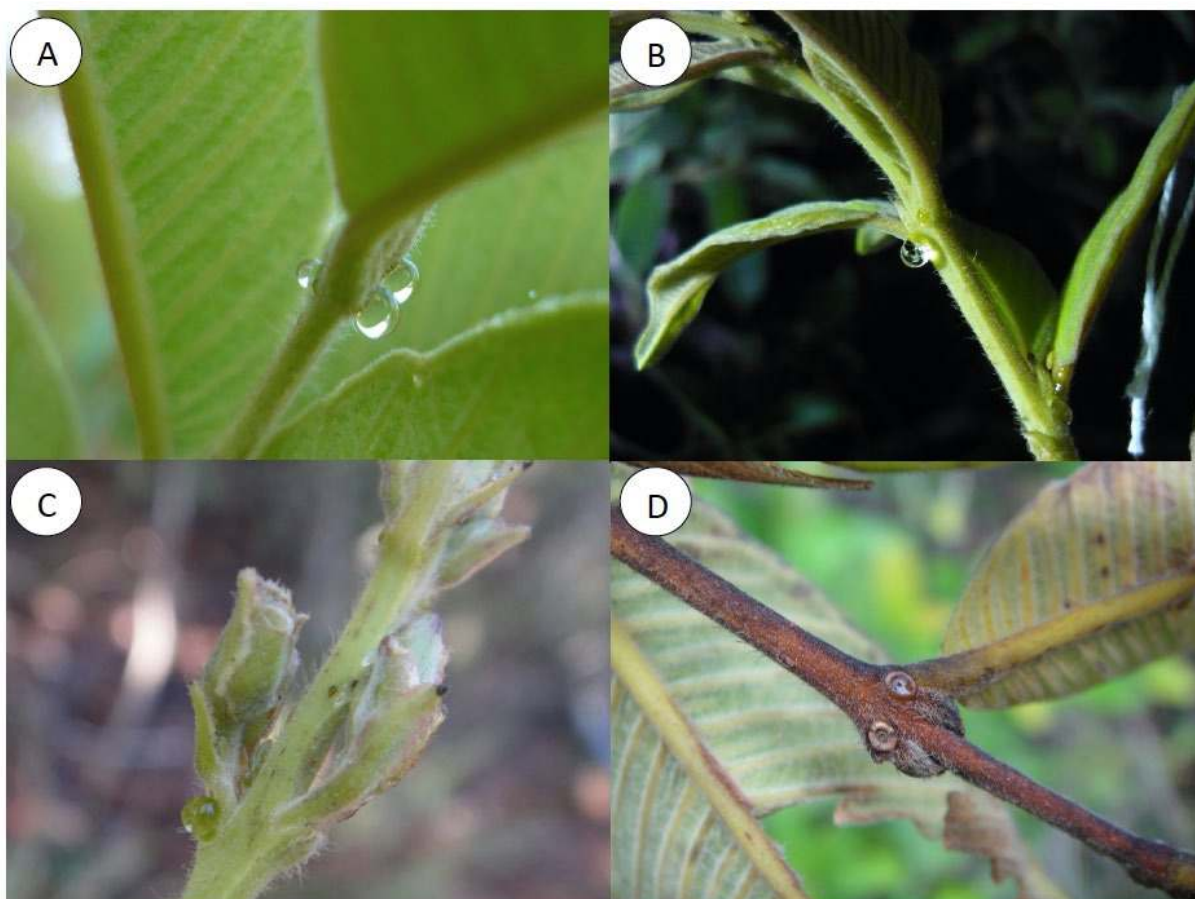
### 144 *Study area and species evaluated*

145 We carried out this study in the Reserva Ecológica do Clube de Caça e Pesca Itororó  
146 de Uberlândia (CCPIU - 48°17'51" W; 18°58'58" S; ~200ha), in Uberlândia, MG, Brazil,  
147 from September 2013 to January 2014. The vegetation is characterized by cerrado *sensu*  
148 *stricto*, consisting of trees 2-10 m high and shrubs and grasses in the sub-forest. The region is  
149 characterized by rainy summers (from October to March) and dry winters (from April to  
150 September). For a fuller characterization of the area, see Alves-Silva, Bächtold, and Del-Claro  
151 (2018) and Ferreira and Torezan-Silingardi (2013).

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



154 (September). Flowers are produced in November and are borne in a terminal inflorescence.  
155 EFNs are located on both sides of the young stems, at the base of the leaf petioles (Fig. 1a-b)  
156 and in the floral pedicel at the base of each floral bud (Fig. 1c). Leaves are continuously  
157 produced from early September until December, and present active EFNs during their early  
158 life span. As leaves develop, these EFNs stop producing nectar (Calixto, Lange, & Del-Claro,  
159 2015). Once all leaves are developed and leaf EFNs stop secreting nectar, flowering begins; at  
160 that point, EFNs on inflorescences appear and become active (Fig. 1c). Ants patrolling this  
161 species do not vary in species identity between the periods of leaf production and flowering  
162 (Del-Claro, Berto, & Réu, 1996). In a previous study (Calixto, Lange, & Del-Claro, 2015), we  
163 showed that indirect defense is more effective than physical defense (trichomes and foliar  
164 toughness) during the intermediate phase of leaf development in *Q. multiflora*. Lange & Del-  
165 Claro (2014) have shown that plant individuals without ants experience more herbivory than  
166 plants with ants.



167



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

171

### 172 *Experimental design*

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

181 **Table 1.** Experimental treatments on *Qualea multiflora* based on extrafloral nectary  
 182 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  $2.64 \pm 1.9$  % (mean  $\pm$  SD) in ant-attended plants to  $8.16 \pm 4.08$  % 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<sup>®</sup>, Grand Rapids,  
204 USA), decreasing dilution by rain and dew and reducing access to and removal of nectar by

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

208 Nectar produced in all selected EFNs on plants in all five treatments was collected 1,  
209 6, 24, 48, 72, and 96 hours after cutting (method adapted from Heil, Fiala, Baumann, &  
210 Linsenmair, 2000). At each census, we measured the volume of nectar produced and the  
211 quantity of sugar (Brix % - mg sugar per ml solution) with the aid of 5 $\mu$ L graduated  
212 microcapillary tubes and manual refractometer (Eclipse<sup>®</sup> model, Bellingham & Stanley,  
213 Tunbridge Wells, UK). All evaluated EFNs were washed with distilled water and dried with  
214 filter paper immediately after simulated herbivory and after each evaluation. During censuses,  
215 we recorded ant abundance and richness on plants at the time of nectar collection. An  
216 individual of each ant species was collected, fixed in 70% alcohol, and identified, with  
217 confirmation by specialists from the Universidade Federal do Paraná, in Curitiba, Brazil. Data  
218 on ant identity are presented in Supporting Information (Table S1).

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

227

228 *Data analysis*

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

232 Before conducting analyses, sugar concentration was converted to calories, using the  
233 manual refractometer value in % Brix and the volume of nectar ( $\mu\text{L}$ ). We then calculated the  
234 sugar concentration in milligrams (mg) per microliter ( $\mu\text{L}$ ) using the equation  $y = 0.00226 +$   
235  $(0.00937x) + (0.0000585x^2)$ , where  $x$  is the sugar concentration shown in the refractometer and  
236  $y$  is total sugars in  $1\mu\text{L}$ . Next, as each mg of sugar is equivalent to four calories, the sugar  
237 amount found was multiplied by four (Dafni, Kevan, & Husband, 2005; Lange, Calixto, &  
238 Del-Claro, 2017).

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

248 *Prediction i* – To evaluate whether extrafloral nectar produced in inflorescence EFNs  
249 has higher volumes and calories and attracts more ants than extrafloral nectar produced in leaf  
250 EFNs, we used Linear Mixed Model (LMM) and Generalized Linear Mixed Model (GLMM)  
251 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 ( $\mu\text{L}$ )	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 <sup>-11</sup>	6.541 <sup>-06</sup>
	Ant abundance (number)	Plant part	180	4.996	0.0254	Plant ID	4.735 <sup>-10</sup>	2.176 <sup>-05</sup>
<i>Prediction ii and iii</i>	Volume ( $\mu\text{L}$ )	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 <sup>-11</sup>	6.081 <sup>-06</sup>
	Ant abundance (number)	Damage level	450	33.536	0.0001	Plant ID	3.399 <sup>-09</sup>	5.83 <sup>-05</sup>
	Volume ( $\mu\text{L}$ )	Damage level	180	11.886	0.0001	Plant ID	1.373 <sup>-11</sup>	3.705 <sup>-06</sup>
	Calories	Damage level	41	4.066	0.0437	Plant ID	3.511 <sup>-12</sup>	1.874 <sup>-06</sup>
	Ant abundance (number)	Damage level	30	11.317	0.0007	Plant ID	1.157 <sup>-09</sup>	3.401 <sup>-05</sup>
<i>Prediction iv</i>	Ant abundance (number)	Volume	72	0.417	0.5181	Plant ID	1.852 <sup>-09</sup>	4.303 <sup>-05</sup>
		Calories	72	9.975	0.0015			
		Volume:Calories	72	5.275	0.0216			

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

274           To test prediction ii regarding foliar treatments, we looked for a significant increase of  
275 the response variables (extrafloral nectar volume and calories, and ant abundance) in the  
276 simulated treatments compared to the control treatment. We performed pairwise comparisons  
277 using Estimated Marginal Means (EMMs) through the package “emmeans” (Lenth, 2018). To  
278 test prediction iii, we looked for a significant increase of the response variables among the  
279 three treatments, that is, Foliar 40% should significantly increase its values compared to Foliar  
280 10%, which in turn should significantly increase its values compared to Foliar control. To test  
281 this, we also used EMMs.



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

287

## 288 **Results**

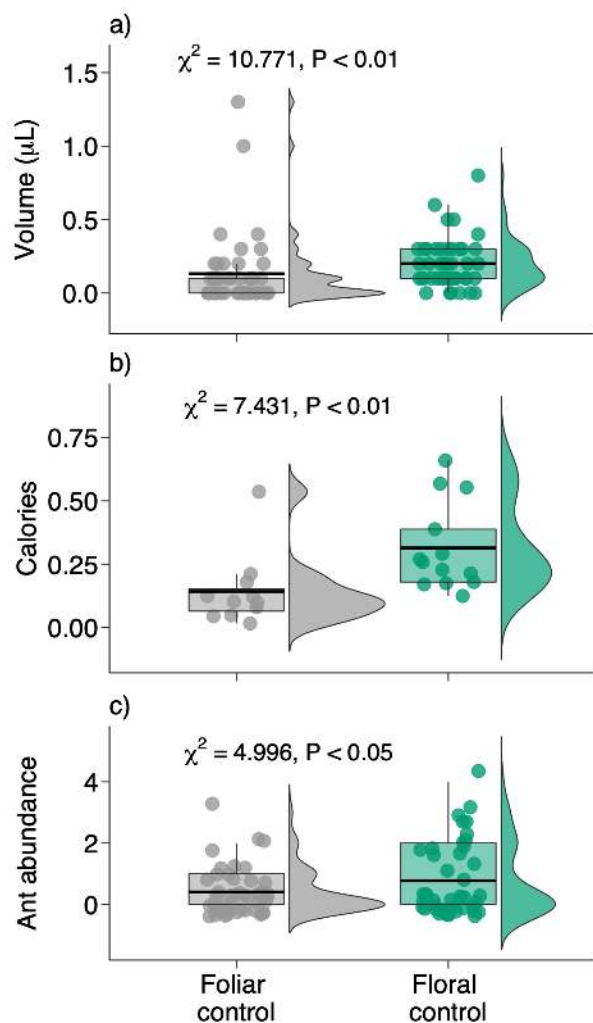
289 We observed 13 ant species belonging to five subfamilies, of which Formicinae was  
290 the subfamily with the largest number of species (Supporting Information Table S1).

291 *Camponotus renggeri* made up 43-68 % of all ants observed across the five treatments. This  
292 ant was commonly seen foraging near the reproductive parts and attacking putative  
293 herbivores, as is common for *Camponotus* spp. Below, we consider these results in the  
294 context of each of the four predictions we posed.

295

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

298 We observed a significant difference in volume ( $\chi^2 = 10.771$ ,  $df = 1$ ,  $P < 0.01$ ),  
299 calories ( $\chi^2 = 7.431$ ,  $df = 1$ ,  $P < 0.01$ ) of extrafloral nectar as well as in ant attendance ( $\chi^2 =$   
300  $4.996$ ,  $df = 1$ ,  $P < 0.05$ ) between Floral control and Foliar control treatments (Fig. 2). Floral  
301 EFNs produced on average 1.73 and 2.93 times more extrafloral nectar (volume and calories  
302 respectively) than foliar EFNs. In addition, Floral EFNs attracted on average 2.63 more ants  
303 than Foliar control.



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**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.

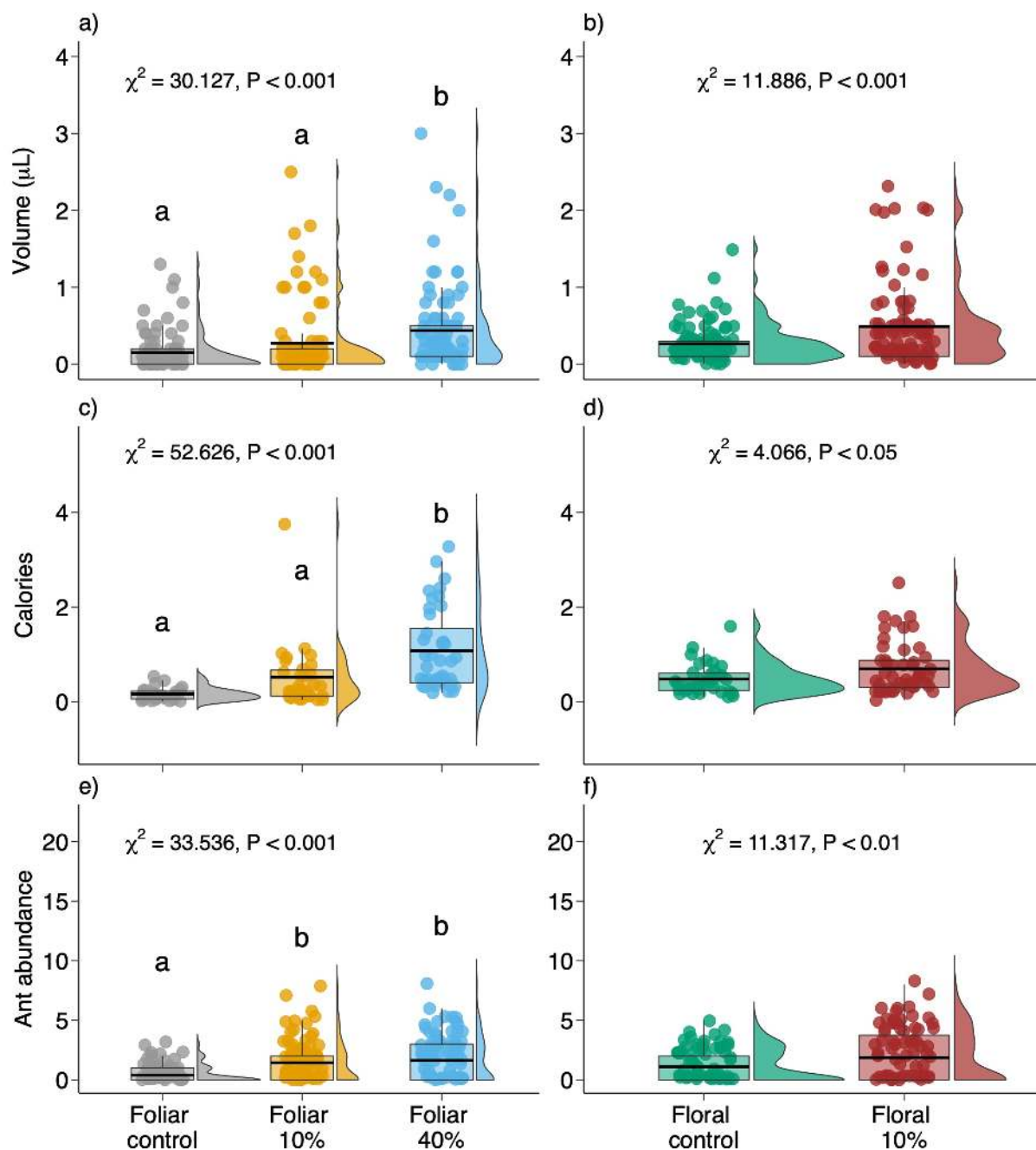
*Prediction ii* – extrafloral nectar production and ant attendance will increase after simulated herbivory in leaf EFNs but not in inflorescence EFNs

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

316 increase production of extrafloral nectar (EMMs – volume: estimate = -0.11,  $P = 0.076$ ;  
317 calories: estimate = -0.13,  $P = 0.061$ ; Fig. 3a,c), whereas Foliar 40% did (EMMs – volume:  
318 estimate = -0.28,  $P < 0.001$ ; calories: estimate = -0.33,  $P < 0.001$ ; Fig. 3a,c). Foliar 40%  
319 EFNs produced on average 2.86 and 6.68 times more extrafloral nectar, by volume and  
320 calories respectively, than Foliar control EFNs. Furthermore, after simulated herbivory, Floral  
321 10% also produced significantly higher volumes (1.88x higher;  $\chi^2 = 11.886$ ,  $df = 1$ ,  $P < 0.001$ )  
322 and calories (1.46x higher;  $\chi^2 = 4.066$ ,  $df = 1$ ,  $P < 0.05$ ) of extrafloral nectar than Floral  
323 control (Fig. 3b,d).

324 Simulated herbivory resulted in significantly higher ant numbers than on control  
325 plants. Foliar 10% attracted on average 3.5 times more ants (EMMs: estimate = -1.24,  $P <$   
326 0.01) and Foliar 40% attracted 3.98 times more ants (EMMs: estimate = -1.38,  $P < 0.001$ )  
327 compared to Foliar control (Fig. 3e). Similarly, Floral 10% attracted significantly more ants  
328 than Floral control (1.7x more;  $\chi^2 = 11.317$ ,  $df = 1$ ,  $P < 0.001$ ; Fig. 3f).

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332 **Figure 3.** Variation in extrafloral nectar volume (a-b) and calories (c-d), and in ant333 abundance (e-f) of *Qualea multiflora* individuals with extrafloral nectaries active on leaves (a,

334 c, e) and inflorescences (b, d, f), and before (Control) and after (10 % or 40 % damage)

335 simulated herbivory. Graphs are represented by bars with mean, hinges and whiskers, raw

336 data (points), and violin plot based on Kernel density function. Different letters differ from

337 each other by estimated marginal means.

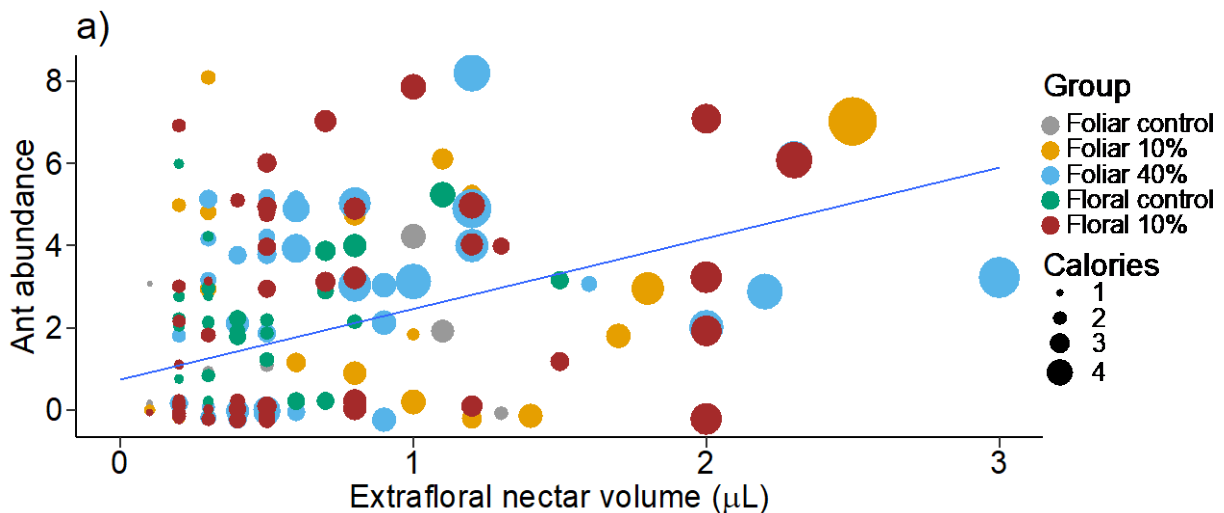
338 Prediction *iii* – the higher the foliar damage, the higher the foliar extrafloral nectar  
 339 production and ant attraction

340 Higher levels of leaf damage (Foliar 40%) resulted in significantly higher extrafloral  
 341 nectar volumes (1.6x higher; EMMs: estimate = -0.17,  $P < 0.01$ ) and calories (2.1x higher;  
 342 EMMs: estimate = -0.20,  $P < 0.001$ ) compared to the lower damage level (Foliar 10%) (Fig.  
 343 3a,c). On the other hand, ant abundance was similar between Foliar 10% and Foliar 40%  
 344 (EMMs: estimate = -0.13,  $P = 0.805$ ; Fig. 3e).

345

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

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



352 **Figure 4** – Positive relationship between ant abundance and volume (a) and calories  
 353 (b) of extrafloral nectar in *Qualea multiflora*. GLMM (volume\*calories:  $\chi^2 = 5.275$ ,  $df = 1$ ,  $P$   
 354  $< 0.05$ ; volume:  $\chi^2 = 0.417$ ,  $df = 1$ ,  $P = 0.518$ ; calories:  $\chi^2 = 9.975$ ,  $df = 1$ ,  $P < 0.01$ ).

355

356           **Discussion**

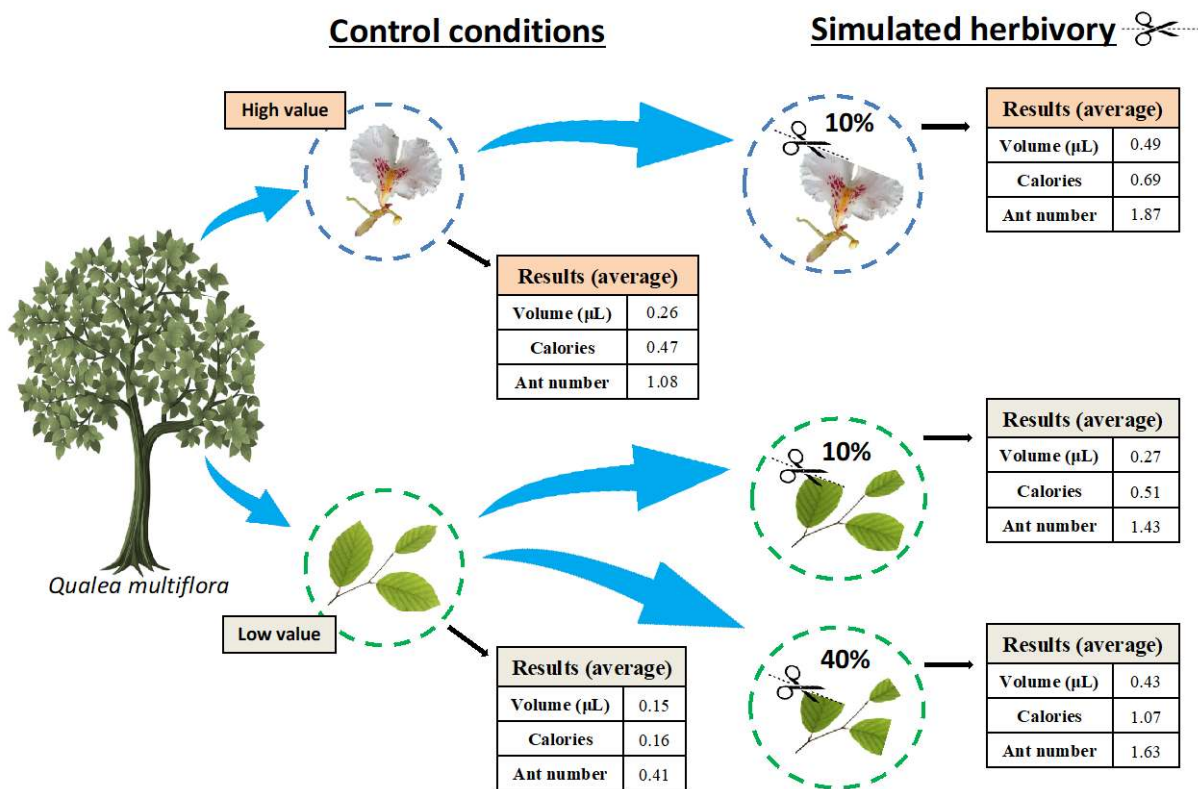
357           *Overview*

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

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

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

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



391  
 392 **Figure 5.** Summary of nectar production (volume and calories) by extrafloral nectaries  
 393 (EFNs) on leaves and inflorescences of *Qualea multiflora*, and of ant abundance at nectaries,  
 394 under control conditions and after simulated herbivory.

395

396 *Extrafloral nectar production and ant attractiveness*

397 Under control conditions, EFNs on inflorescences produced more volume and calories  
398 of extrafloral nectar and attracted more ants than EFNs on leaves, as we predicted (Prediction  
399 *i*). According to the ODT, reproductive parts of plants should be better protected than  
400 vegetative ones. Vegetative tissues are generally less valuable and relatively easier to replace  
401 compared to flowers and fruits (McKey, 1979; Zangerl & Bazzaz, 1992), which are more  
402 valuable due to their direct link with plant reproductive success (Cipollini & Levey, 1997;  
403 Holland, Chamberlain, & Horn, 2009). In addition, Floral control EFNs produced higher  
404 quality and quantity of extrafloral nectar than Foliar control EFNs under control conditions,  
405 attracting more ants. These results are consistent with studies showing that quantity and quality  
406 of nectar can influence ant patrolling behavior (Blüthgen, Gottsberger, & Fiedler, 2004; Lange,  
407 Calixto, & Del-Claro, 2017; Pacelhe, Costa, Bronstein, Mello, & Neves, 2019), and that higher  
408 production of extrafloral nectar leads to higher ant attendance (Bixenmann, Coley, & Kursar,  
409 2011; Falcão, Dáttilo, & Izzo, 2014; Pacelhe, Costa, Bronstein, Mello, & Neves, 2019).  
410 Although our results suggest a higher attractiveness of ants to Floral control EFNs than to  
411 Foliar control EFNs, other intrinsic (e.g., ant behavior) and extrinsic (e.g., environmental  
412 variables) factors to ants might also influence ant abundance. However, at least for  
413 temperature, precipitation and humidity, there was no significant difference between the two  
414 periods of collection (Foliar experiments in October, and Floral experiments in January), and  
415 therefore we suggest that ant foraging in our study is more influenced by extrafloral nectar  
416 attractiveness than by environmental variables.

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



421 foliar EFNs can be induced after simulated herbivory or natural damage (e.g., Agrawal &  
422 Rutter, 1998; Heil, Fiala, Baumann, & Linsenmair, 2000; Mondor, Tremblay, & Messing,  
423 2006). For instance, Wäckers and Bonifay (2004) showed in *Gossypium hirsutum* that nectar  
424 production by EFNs on leaves was induced, whereas that by EFNs on bracts was not (Zangerl  
425 & Rutledge, 1996). These studies fit the predictions of ODT: more valuable tissues express  
426 constitutive defenses, whereas less valuable ones express induced defense. However, little is  
427 known about the rate of secretion of EFNs on reproductive regions as induced defense. It is  
428 possible that (1) extrafloral nectar might be expensive to produce, so it should not be wasted  
429 by being produced constitutively (O'Dowd, 1979); (2) ants are very abundant on *Q. multiflora*  
430 (Lange, Calixto, & Del-Claro, 2017) and so responsive to increases in EFN that they could  
431 respond very quickly to a threat and fully protect the inflorescences under attack, allowing  
432 EFN to be inducible; and (3) there may be evolutionary and developmental constraints that  
433 prevent leaf and floral EFNs on the same plant from being regulated differently. These  
434 hypotheses remain to be tested.

435         After simulated herbivory, extrafloral nectar production in plants with active EFNs on  
436 leaves increased as leaf damage increased, as we predicted (Prediction *iii*). Kwok and Laird  
437 (2012) showed that *Vicia faba* L. have the ability to recognize the severity of herbivore  
438 damage, inducing the production of extrafloral nectar, and consequently recruiting more ants.  
439 Several studies on damage recognition by plants have shown how complex and refined these  
440 systems can be. Many plants have developed damage recognition systems, in which they are  
441 able to identify certain substances or molecules present in insect saliva or eggs (Arimura, Kost,  
442 & Boland, 2005; Arimura, Ozawa, & Maffei, 2011; Carrillo, Wang, Ding, & Siemann, 2012).  
443 These studies show that recognition systems can be very accurate, and therefore that the  
444 intensity of the damage can also be an important factor for the induced response in plants.  
445 Although there was an increase in the production of extrafloral nectar as leaf damage

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

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

#### 468 *Final remarks*

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

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

480

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488

#### 489 **Authors' contributions**

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

494

#### 495 **Data availability**

496 Data available from the Dryad Digital Repository:  
 497 <https://doi.org/10.5061/dryad.stqjq2c0x> (E. S. Calixto, Lange, Bronstein, Torezan-Silingardi,  
 498 & Del-Claro, 2020)

499

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