

# Nectar quality affects ant aggressiveness and biotic defense provided to plants

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## Abstract

An excellent model to investigate how biological attributes of species affect interaction outcomes are plants bearing extrafloral nectaries and the ants that feed at them. As plant-provided resources are essential components of ant diets, plants that offer more nutritious food to ants should be better defended in return, as a result of more aggressive behavior towards natural enemies. We tested this hypothesis in a field experiment by adding artificial nectaries to individual plants of the species *Vochysia elliptica* (Vochysiaceae). Ants were offered one of four liquid foods of different nutritional quality: amino acids, sugar, sugar + amino acids, and water (control). We used live termites (*Nasutitermes coxipoensis*) as model herbivores and observed ant behavior towards them. In 88 h of observations, we recorded 1,009 interactions with artificial nectaries involving 1,923 individual ants of 26

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species. We recorded 381 encounters between ants and termites, of which 38 percent led to attack. In addition, 61 percent of these attacks led to termite exclusion from the plants. Recruitment and patrolling were highest when ants fed upon nectaries providing sugar + amino acids, the most balanced and nutritious food. This increase in recruitment and patrolling led to higher encounter rates between ants and termites, more frequent attacks, and faster and larger termite removal. Our results are consistent with the hypothesis that plant biotic defense is mediated by resource quality. We highlight the importance of qualitative differences in nectar composition for the outcome of ant-plant interactions.

**Keywords:** ant-plant interactions; behavioral ecology; Brazil; chemical ecology; extrafloral nectar; mutualism; Serra do Cipó.

## Resumo

Um excelente modelo para investigar como os atributos biológicos das espécies afetam o resultado das interações são plantas com nectários extraflorais e as formigas que se alimentam delas. Como os recursos fornecidos pelas plantas são componentes essenciais na dieta de formigas, as plantas que oferecem alimento mais nutritivo às formigas devem ser melhor defendidas em retorno, como resultado de um comportamento mais agressivo em relação aos inimigos naturais. Nós testamos esta hipótese em um experimento de campo adicionando nectários artificiais em plantas individuais da espécie *Vochysia elliptica* (Vochysiaceae). As formigas receberam um dos quatro alimentos líquidos de diferentes qualidades nutricionais: aminoácidos, açúcar, açúcar + aminoácidos e água (controle). Utilizamos cupins vivos (*Nasutitermes coxipoensis*) como modelo de herbívoro e observamos os comportamentos das formigas em relação a eles. Em 88 h de observações, registramos 1.009 interações com nectários artificiais envolvendo 1.923 indivíduos de 26 espécies de formigas. Registramos 381 encontros entre formigas e cupins, dos quais 38% levaram ao ataque. Além disso, 61% desses ataques levaram à exclusão dos cupins das plantas. Recrutamento e patrulhamento foram maiores quando as formigas se alimentaram de nectários com açúcar + aminoácidos, o alimento mais equilibrado e nutritivo. Esse aumento no recrutamento e

patrolhamento leva a uma maior taxa de encontro entre formigas e cupins, ataques mais frequentes e uma maior e mais rápida remoção de cupins. Nossos resultados são consistentes com a hipótese de que a defesa biótica das plantas é mediada pela qualidade dos recursos. Além disso, destacamos a importância das diferenças qualitativas na composição do néctar para o resultado das interações entre formigas e plantas.

HOW THE BIOLOGICAL ATTRIBUTES OF DIFFERENT SPECIES AFFECT THE OUTCOMES OF THEIR INTERACTIONS IS ONE OF THE MAIN OPEN QUESTIONS IN ECOLOGY (Sutherland *et al.* 2013). This question has no simple answer, as thousands of species with different features interact with one another and establish relationships that vary from positive to neutral to negative (Chamberlain *et al.* 2014). It is necessary to disentangle this complexity to understand the processes that drive interaction outcomes and their consequences for ecological functions and evolutionary dynamics.

Interactions between ants and plants with extrafloral nectaries (secretory glands not related to pollination; see Marazzi *et al.* 2013) are a good model in which to study context-dependence of interaction outcomes (Bronstein 1998, Del-Claro *et al.* 2016). These interactions are generally assumed to be mutually beneficial, since plants provide food for ants, which in turn deter herbivores and other natural enemies (Rosumek *et al.* 2009). This defensive biotic response can be explained by increased ant visitation and “ownership” behavior on plants that provide a predictable and valuable food source (Ness *et al.* 2009, Fagundes *et al.* 2017). The defense provided by ants can have strong effects on plant fitness, as suppression of herbivory conserves resources that might then be allocated to vegetative growth and reproduction (Nascimento & Del-Claro 2010, Rosumek *et al.* 2009). Although the chemical composition of extrafloral nectar has been studied in detail (Heil 2015), little is known about how nectar components might drive the aggressive behavior of ants that visit plants (González-Teuber & Heil 2009a).

The effects of ants on plants are commonly context-dependent, ranging from positive (Sendoya *et al.* 2016) to negative (Chamberlain & Holland 2009), as a function of biotic (Koch *et al.* 2016) and abiotic (Jones *et al.* 2017) conditions. For the outcome to be positive for the plants, the cost of producing nutritious extrafloral nectar

0 must not exceed the benefits of the biotic defense provided in return (Bronstein 2001). Production of extrafloral  
1 nectar has been presumed to be inexpensive (O'Dowd 1979), which makes it likely that even occasional ant  
2 defense might be worth the investment. However, the assumption that a high-quality reward may drive ant  
3 behavior, improving plant defense, has rarely been tested (but see Flores-Flores *et al.* 2018). The physiological  
4 costs of nectar production may vary not only genetically (Rutter & Rausher 2004), but also plastically, in  
5 response to local availability of nutrients and water (Reich *et al.* 2003). Furthermore, from the ants' perspective,  
6 low-quality nectar might not be worth finding, collecting, and defending, if alternative resources are available  
7 (Stadler & Dixon 2008).

8 In nature, the chemistry of nectar is extremely complex, with a wide variety of sugars and amino acids  
9 differing in concentration across species (Blüthgen *et al.* 2004, Heil 2011). To understand the potential influence  
10 of the nutritional quality of extrafloral nectar on the outcome of ant-plant interactions, we must first consider the  
11 importance of different nutrients. Ants require a balanced diet of sugars and amino acids, and ant species that  
12 normally collect carbohydrate-rich nectar may exhibit a strong preference for protein- or amino acid-rich  
13 resources (Kay 2002). Evidence suggests that consumption of extrafloral nectar alters the optimal balance of  
14 carbohydrates and proteins in ant diets, leading ants to require additional protein intake (Ness *et al.* 2009). This  
15 protein deficit is usually resolved by consuming other plant visitors (including herbivores), which in turn boosts  
16 biotic defense (Ness *et al.* 2009). Consistent with these observations, cafeteria trials indicate that ants generally  
17 prefer artificial mixtures containing both sugar and amino acids over those with sugar only (Blüthgen & Fiedler  
18 2004b). In addition, evidence suggests that nectar composition plays a central role in determining ant competitive  
19 hierarchies, with the most dominant species monopolizing better plant rewards (Blüthgen & Fiedler 2004a). Thus,  
20 offering high quality nectar, which we assume here is a more nutritionally balanced reward, can confer benefits to  
21 the host plant. In addition, different lines of evidence have led to the hypothesis that plants that offer higher-  
22 volume and more sugar-rich extrafloral nectars receive better biotic defense in return, mostly due to the  
23 dominance of competitively superior ants (Fagundes et al. 2017, Flores-Flores *et al.* 2018).

14 A common feature in the study of mutualism is a consideration of the ‘effectiveness’ of mutualistic partners  
15 in conferring benefits, measured by both quantitative and qualitative parameters (González-Teuber *et al.* 2012,  
16 Dáttilo *et al.* 2015, Lange *et al.* 2017, Fagundes *et al.* 2017, Flores-Flores *et al.* 2018). However, the meaning of  
17 effectiveness, quantity, and quality vary greatly across studies. The conceptual framework proposed by Schupp *et al.*  
18 *al.* (2017) contributes to clarifying these concepts. They offer a general effectiveness framework for viewing the  
19 processes and outcomes of mutualistic interactions, based on a consistent terminology and a restricted range of  
20 metrics, which apply to both sides in any type of mutualism. This framework encompasses two central  
21 components that might predict mutualist efficiency: (1) the quantity component of effectiveness, which measures  
22 the number of immediate outcomes of the interaction; and (2) the quality component of effectiveness, which  
23 incorporates post-interaction delayed outcomes. A way to apply this concept to ant-plant protection mutualisms  
24 would be measure the number of enemies removed, whether by killing or repelling, as a function of the number of  
25 visitors attending the plant and the probability that an attending visitor removes an enemy, interpreted as the  
26 quantity component, and evaluate the proportional increase in fruit production per enemy removed, being a  
27 function of the amount of resources saved per enemy removed and the proportional increase in fruit production  
28 per unit resource, representing the quality component (for more details, see figure 2c in Schupp *et al.* 2017).

29 Here, we report on an experiment in which we manipulated the two most important chemical components  
30 of extrafloral nectars: sugar and amino acids. Specifically, we tested whether the nutritional value of nectar,  
31 defined based on the levels of those two main nutrients, influences the quantity component of the plant protection  
32 mutualism (Schupp *et al.* 2017). We carried out a field experiment using treatments with artificial extrafloral  
33 nectar of different qualities and termites as model herbivores. We considered a nectar rich in sugar and amino  
34 acids to be more nutritious and balanced from the ants’ perspective than a nectar with only sugar or amino acids.  
35 We expected that, on plants offering artificial extrafloral nectar enriched with sugar plus amino acids, ants would:  
36 (1) exhibit higher recruitment and patrolling; (2) attack termites more frequently and remove them in larger  
37 numbers; and (3) find and remove termites from the plants more quickly. We demonstrate experimentally that

resource quality influences the effectiveness of a plant protection mutualism, offering insights into how nectar composition affects consumer behavior and biotic defense.

## METHODS

**STUDY AREA.** —The study was carried out at Serra do Cipó National Park (hereafter Serra do Cipó), located in the southern region of Espinhaço Range, state of Minas Gerais, Brazil (19°20'55.93" S, 43°37'10.23" W). The region is markedly seasonal, with rainy summers and dry winters, and an average annual rainfall of 1450 mm (Alvares *et al.* 2013). Our experiment was carried out along a 3-km-long trail, locally known as Capão dos Palmitos (19°20'57.78" S, 43°37'00.46" W and 19°21'59.08" S, 43°37'12.78" W, 832-937 m a.s.l.). Capão dos Palmitos represents an ecotone between Cerrado (savannas) and Campos Rupestres (rupestrian grasslands) occupied by species of both environments, such as plants of the families Vochysiaceae, Asteraceae, and Velloziaceae (Mota *et al.* 2017).

**EXPERIMENTAL DESIGN.** —We used as a model the plant *Vochysia elliptica* Mart. (Vochysiaceae), one of the most abundant small trees in Serra do Cipó (Shimizu & Yamamoto 2012). This species lacks extrafloral nectaries and other secretory structures unrelated to pollination (Stafleu 1948). We specifically chose a plant lacking extrafloral nectaries so as to isolate the effects of the variable we were attempting to test (the chemical content of extrafloral nectar). The use of artificial nectaries allowed us to control temporal (Baker-Méio & Marquis 2012, Falcão *et al.* 2014, Dáttilo *et al.* 2015) and ontogenetic (Heil *et al.* 2000) variations in qualitative and quantitative nectar properties. Furthermore, our method excluded natural variation in production and quality of natural nectar influenced by ant consumption (Heil *et al.* 2000) and by the production of volatile organic compounds (VOCs) produced by plants that are being consumed (Heil & Bueno 2007). Further, we used only plants at non-reproductive phases, since inflorescences might attract ants (Romero 2002) and create a confounding factor.

We selected 40 individual plants up to 2 m in height spaced at least 5 m apart. As the absence of EFN is not a constraint to ant presence on plants since different ant species commonly forage on plant surface looking for different resource types (Costa *et al.* 2016), before installing artificial nectaries, we did 30 observations of 3-min (a diurnal and other nocturnal) on 15 individual plants for three d (13-15 January 2015) to sample the set of ant species that naturally forage in surface of *V. elliptica*. As we expected, very few ants were observed to be foraging on plant. We found only six ant occurrences represented by a single individual each: *Brachymyrmex* sp1 and *Crematogaster prox. erecta* once, and *Camponotus crassus* and *Ectatomma tuberculatum* twice.

After these initial observations, we arbitrarily placed five artificial nectaries on each plant, one on the main branch (trunk) and the other four on the medial part of different secondary branches (between the trunk and the apex). The artificial nectaries were 2 ml Eppendorf vials with a cotton thread extending from each. The vials were filled with the solution defined for each treatment. Artificial EFNs were used as suggested by Blüthgen & Fiedler (2004b), but we modified their distribution. Instead of placing pairs of Eppendorf vials at a single location or distributing ten pairs along the tree trunk with different concentrations (which was not possible due to the small size of *V. elliptica*), we chose to distribute the Eppendorf vials throughout the plant and to offer different concentrations on different individuals. This way, we could distribute the resources evenly on the plants, to attract ants homogeneously, allowing us to observe their behavior while controlling for resource quality. We established four treatments with ten plants assigned to each: amino acids (hereafter A) – 3 percent of amino acid solution (1% glutamine, 1% proline, and 1% threonine); sugar (hereafter S) - 30 percent of sucrose solution; sugar + amino acids (hereafter S+A) – 20 percent sucrose + 3 percent of amino acid solution (using the same amino acids as in the A treatment); and filtered water (hereafter W) as a control. Treatments were arbitrarily assigned to plants sequentially from north to south of the trail.

Few studies have assessed the chemical profile of natural EFN. Among those, there is information on the chemistry of nectar at the species (Shenoy *et al.* 2012), clade (González-Teuber & Heil 2009b), and community levels (Blüthgen *et al.* 2004). The composition and concentration of sugars and amino acids vary extensively

across species. As we found no studies at the community level that traced the chemical profile of EFN in the Cerrado, we had to consider the scant information available to define the experimental concentrations. By analyzing the data presented by Blüthgen et al. (2004), we found that, among 16 plants sampled in the rainforest at North Queensland, Australia, the concentration of total sugars ranged from 7 percent (*Macaranga tanarius*; Euphorbiaceae) to 76 percent (*Entada phaseoloides*; Fabaceae), with an average value of  $21 \pm 8\%$ . In addition, the total concentration of amino acids ranged from 0.02 percent (*Homalanthus novoguinnensis*; Euphorbiaceae) to 2.67 percent (*Smilax australis*; Smilacaceae) with an average value of  $0.3 \pm 0.07\%$ . Our nectar concentrations were defined taking into account the mean concentration of nectars produced by all plants sampled by Blüthgen et al. (2004), excluding those belonging to families that do not occur in the Brazilian Cerrado. Our objective here was not to mimic the complex composition of natural nectars, but to see how ants responded to qualitative variation in nectar composition, considering the two most prominent components of nectar in nature: sugar and amino acids.

We used sucrose because most ant species prefer it to glucose or fructose (Blüthgen & Fiedler 2004b), and glutamine, proline and threonine because they are the amino acids found most frequently and in the highest concentration in extrafloral nectar of several plant species (Blüthgen et al. 2004). In nature, when nectar composition is more complex (i.e., when there is a wider variety in chemical components), there is a balance in chemical concentration: high-sugar nectars have a lower variety of amino acids, and nectars with a higher variety of amino acids are lower in sugar (Blüthgen et al. 2004). Therefore, we used a lower sugar concentration in S+A treatment compared to the S treatment. In addition, a pilot experiment carried out in the study site revealed that 20 percent and 30 percent sucrose solutions did not differ in attractiveness to ants (richness: GLMM - deviance (1,19) = 27.7,  $R^2 = 0.3$ ,  $\chi^2 = 0.55$ ,  $P = 0.45$ ; abundance: GLMM - deviance (1,19) = 67.5,  $R^2 = 0.03$ ,  $\chi^2 = 0.55$ ,  $P = 0.45$ ).

ANT BEHAVIOR TOWARDS MODEL HERBIVORES. — After installing the artificial nectaries, we filled and monitored them daily for 12 d (20 - 31 January 2015), until their contents were consumed or evaporated. During



0 this period, we recorded ant richness and abundance, as well as occurrence and frequency of interactions. After  
1 this period, we noticed a stabilization in the frequencies of ant visitation and their abundances and “ownership”  
2 behavior near the food source. We only began the behavioral experiment with termites after this preliminary test.  
3 As stated above, the artificial nectar offered in our experiment was a simplification of natural EFN, and was  
4 composed of the main nutrients observed in natural EFN. Nevertheless, there is evidence that our experimental  
5 conditions induced natural behaviors. Even using a relatively simple food, we obtained similar results as other  
6 studies that measured the behavior of ants while interacting with natural EFNs or hemipteran honeydew  
7 (Katayama & Suzuki 2005, Campos & Camacho 2014).

8 To evaluate the biotic defense provided by ants, on each *V. elliptica* plant we placed one live *Nasutitermes*  
9 *coxipoensis*, the most common termite species in the study area (Nunes *et al.* 2017). Termites are commonly used  
10 to simulate herbivores in studies of ant-plant interactions (e.g., Oliveira et al. 1987; Campos and Camacho 2014),  
11 as they can be added in controlled numbers and ants readily attack them; here, the goal was simply to evaluate the  
12 behavior of different ant species towards a standardized threat. The termite was released on the apex of the  
13 secondary branch most distant from the trunk, so it could move freely around the plant. We observed the host  
14 plant it for 30 min, recording every 5 min the number of ants (defined as recruitment), the number of ants that  
15 interacted with the termite, and the ants’ behavior towards the termite.

16 We considered an ant-termite interaction to be an *attack* when the ant injured or consumed the termite, and  
17 a *touch* when the ant touched the termite with its antennae without injuring it. The outcome was considered  
18 *neutral* when the ant did not interact with the termite (following Katayama and Suzuki 2005). We also recorded  
19 the number of attacks after first contact and whether these attacks led to termite exclusion from the plant.

20 We stopped observing the ants after 30 min or when the termite was consumed, left the plant, or fell from  
21 the plant due to vibrations caused by wind or ant attacks. The observations were made for 26 d (2-27 February  
22 2015). We carried out a total of eight observations on each plant, four diurnal (from 0600 to 1200 h) and four  
23 nocturnal (from 1800 to 2400 h), totaling 320 observation events and 88 h of observations in field. For nocturnal

4 observations, we used a headlamp with a red plastic filter to avoid disturbing the ants. We sampled the spectrum  
5 of ants over 24 h, because the composition of ant species interacting with extrafloral nectary-bearing plants is  
6 known to differ between day and night (Dáttilo *et al.* 2014, Anjos *et al.* 2016).

7 To measure biotic defense, we calculated the following metrics during each observation event: encounter  
8 rate, minimum time to encounter, attack rate, exclusion success, and efficiency of exclusion (see Table 1 and  
9 Katayama and Suzuki 2005 for detailed information about defense metrics). We then compared these metrics  
0 across the four nectar-quality treatments.

1  
2 STATISTICAL ANALYSIS. — We used generalized linear mixed models (GLMM, Crawley 2013) to test the  
3 first two predictions, that ants should (1) exhibit higher recruitment and patrolling and (2) attack termites more  
4 frequently and remove them in larger number on plants whose extrafloral nectar has been enriched with sugar  
5 plus amino acids,. We calculated those models using the *lmer* function of the package *lme4* (Bates *et al.* 2014) for  
6 R (R Development Team 2015). In these models, we assumed temporal pseudoreplication, and considered metrics  
7 related to biotic defense as dependent variables and the treatment as the fixed factor. Plant identity was used as  
8 random factor in our models since each individual plant was observed eight times.

9 To test prediction 3, that ants should remove termites faster on plants whose extrafloral nectar was  
0 enriched with sugar and amino acids, we used a survival analysis with Weibull distribution (Weibull 1951), inside  
1 package *survival* (Therneau 2015) for R. For this analysis, we used termite exclusion from host plant (binary  
2 variable: 1 = exclusion and 0 = no exclusion) and the time spent for exclusion as response variables, and  
3 treatment as explanatory factor.

4 For all GLMM models, when significant differences were observed among treatments, the models were  
5 submitted to contrast analysis by aggregating different levels of treatment variable and testing whether the model  
6 with the new arrangement is different from the previous one (Crawley 2013). Thus, if the level of aggregation did

not alter the deviance explained by the previous model ( $p > 0.05$ ), the levels were pooled and the model was adjusted.

## RESULTS

We recorded 1,009 interactions with artificial nectaries, involving 1,923 individual ants from 26 species and six subfamilies, on the experimental plants. The richest ant subfamilies, Myrmicinae and Formicinae, were represented by nine species each. *Camponotus* was the most frequent genus, with five species (Table S1).

The treatment with sugar (S) attracted the largest number of ant species (19, 73% of all ant species observed). Most of species were observed foraging on the experimental plants in fewer than 10 of the 320 observations, representing only 23 percent of the interactions observed with the artificial nectars (Table S1), without displaying defensive behavior. Eight ant species (31%) attacked and either excluded or consumed the termites: *Brachymyrmex* sp1, *Camponotus blandus*, *C. crassus*, *C. rufipes*, *C. renggeri*, *C. vittatus*, *Ectatomma tuberculatum*, and *Pseudomyrmex gracillis*. The species *Brachymyrmex* sp1, *C. vittatus* and *P. gracillis* displayed attack and exclusion behaviors only two times each in nine, ten and five observations, respectively. The other five ant species frequently displayed attack and exclusion behaviors. They were responsible for 96 percent of attack observations and 93 percent of exclusion occurrences, being numerically and behaviorally dominant (as defined by Cerdá *et al.* 2013) in all treatments, only varying among treatments in their recruitment rates (Table S2). We recorded 381 encounter events between ants and termites. Out of those encounters, 38 percent led to attack behavior and 61 percent of these attacks led to termite exclusion from plants.

Ants recruited to the sugar (S) and sugar + amino acids (S+A) treatments twice as often as to the amino acid (A) treatment, and ten times more than to the water (W) treatment (Deviance<sub>(5,315)</sub> = 1967.3,  $R^2 = 0.22$ ,  $\chi^2 = 31.32$ ,  $P < 0.001$ ; Fig. 1). Supporting prediction 1, the S+A treatment showed the highest encounter rate: the encounter rate was 1.4 times higher in S+A than in the S treatment, and 3.5 times higher than in the A or W

1 treatments (Deviance<sub>(4,316)</sub> = 163.3,  $R^2 = 0.05$ ,  $\chi^2 = 9.55$ ,  $P = 0.001$ ; Fig. 1). Supporting prediction 2, ants attacked  
2 termites more frequently on plants assigned the S+A treatment. Attack rate was twice as high in the S+A  
3 treatment than in either the S or A treatment and 7.6 times higher than in the W treatment (Deviance<sub>(5,315)</sub> = 165,  
4  $R^2 = 0.19$ ,  $\chi^2 = 11.25$ ,  $P = 0.003$ ; Fig. 1). Termite removal from the plant was also higher in the S+A treatment,  
5 with an exclusion success rate 2.5 times higher than in the S or A treatment and 16 times higher than in the W  
6 treatment (Deviance<sub>(5,315)</sub> = 254.9,  $R^2 = 0.17$ ,  $\chi^2 = 30.93$ ,  $P < 0.001$ ; Fig. 1).

7 Supporting prediction 3, ants found the termites twice as fast in the S+A treatment than in either the S or A  
8 treatment and 2.6 times faster than in the W treatment (Deviance<sub>(5,315)</sub> = 2462.7,  $R^2 = 0.22$ ,  $\chi^2 = 26.3$ ,  $P < 0.001$ ).  
9 In addition, ants removed termites from the plants twice as fast in S+A than in either the S or the A treatments.  
0 We found no difference in the time taken by ants to exclude live termites in the S and A treatments, but both  
1 differed from the control (W treatment; Deviance<sub>(2,316)</sub> = 54.85;  $P < 0,001$ ; Fig. 2). Thus, in the S+A treatment,  
2 ants removed 50 percent of the termites in half the time (14 min) it took in the other treatments (S = 27 min; A =  
3 31 min and W = 67 min).

## 5 DISCUSSION

6  
7 Our results are consistent with the hypothesis that biotic defense depends on the quality of the reward offered by  
8 plants. Specifically, our results suggest that plants that offer more nutritious extrafloral nectar receive better biotic  
9 defense in return, due to increased aggressiveness. Consumption of high-quality nectar composed of sugar +  
0 amino acids was associated with higher ant recruitment, higher ant aggressiveness, and higher exclusion  
1 efficiency of a surrogate herbivore compared to nectar containing only sugar or amino acids.

2 Five ant species were dominant in all treatments and are considered as "core" in the studied environment  
3 (see Ribeiro *et al.* 2018). These ants have traits that define dominance, such as massive recruitment and high  
4 aggressiveness, suggesting that they can be considered "mutualists of high quantitative value", one of the

5 components of the plant protection effectiveness framework (Schupp *et al.* 2017). We observed a marked  
6 dominance hierarchy in the S and S+A treatments, with the five numerically and behaviorally dominant species  
7 using the resources, foraging, attacking termites and defending the plants. The other ant species arrived in smaller  
8 numbers and fed from nectaries occupied by dominant species, but never showed defensive behaviors. In sum,  
9 our observations show that in this system, attracting more high-quality mutualists is more important than  
0 attracting more species. These results are in agreement with Miller (2007), who showed that EFN-bearing plants  
1 visited by multiple ant species received reduced benefits relative to those associated with a single, high quality  
2 mutualist species (see also Mody & Linsenmair 2004, Del-Claro & Marquis 2015).

3 In nature, the chemistry of nectar is highly complex, with a wide variety of sugars and amino acids  
4 occurring in different concentrations in different plant species (Heil 2011). Our study shows that even a quite  
5 simple food, containing either only sugar or only amino acids, can attract ants and promote biotic defense.  
6 Although the nectar composition used in our experiment was a simplified version of the composition observed in  
7 nature, we obtained similar results as other studies that have studied ants interacting with EFNs and hemipteran  
8 honeydew (Katayama & Suzuki 2005, Campos & Camacho 2014), suggesting that we were observing natural  
9 behaviors.

0 Although in nature, extrafloral nectar is never composed of amino acids alone, our results call attention to  
1 the importance of this nutrient in eliciting mutualistic ant behaviors. Nectars composed by a single nutrient (S or  
2 A) produced similar levels of defense (same capacity to encounter, attack, and exclude termites), while the  
3 treatment with both nutrients led to improved biotic defense. This suggests that the effects of those two nutrients  
4 might be synergistic (as proposed by Raubenheimer & Simpson 1997). In fact, S+A effects are higher than the  
5 effects of S and A summed (see Fig. S1). This synergistic response may be related to the nutritional requirements  
6 to sustain brood growth, growth rate, body mass, survival, and indicators of colony fitness (Byk & Del-Claro  
7 2011). Moreover, at the colony level, energy requirements differ among individuals. While worker ants require  
8 mainly carbohydrates for their activities, larval development requires consumption of proteins and amino acids

(Sorensen *et al.* 1981). Consequently, nectars that contain a broader spectrum of nutrients are thought to be more balanced and valuable for the ant colony (Heil 2011).

Even though carbohydrates are an important driver of ant foraging (Rudolph & Palmer 2013) and aggressiveness (Grover *et al.* 2007), evidence from our study and from the literature (see Blüthgen & Fiedler 2004a, Heil 2011) shows that amino acids are critical. The mechanisms responsible for triggering different ant behaviors remain unclear, as information on the role of amino acids in ant metabolism is scarce. We know that sugars and amino acids contribute independently to nectar attractiveness (González-Teuber & Heil 2009a), but amino acids can act as a flavor enhancer, stimulating different chemoreceptors in insects and influencing their perception of nectar taste (Gardener & Gillman 2002). Considering that ants need to maximize their intake of carbon and nitrogen (Ness *et al.* 2009), a more aggressive behavior upon consumption of amino-acid-enriched nectar may be a result of interspecific competition (Davidson 1998) driven by the low availability of these resources in nature. Alternatively, an increase in energy availability in nectar may lead directly to higher ant aggressiveness (Kay *et al.* 2010).

In conclusion, we have presented new evidence that a nectar that combines carbohydrates and amino acids leads to an increase in ant aggressiveness compared to nectar with only one of those nutrients. Consequently, amino acid-enriched nectar boosts ant efficiency in removing herbivores, which potentially improves the quality of the biotic defense received by the plants. A successful mutualism should maximize the cost-benefit relationship for both ants and plants. Our study suggests that, from the plant's perspective, including amino acids in extrafloral nectaries might increase the chance of a positive outcome in their interactions with ants. This relationship should be especially strong in nitrogen-limited environments, such as Cerrado and Campo Rupestre, where carbon is abundant but nitrogen is scarce (Oliveira *et al.* 2016). As our results suggest that a more balanced and nutritious nectar with amino acids, although being more expensive to the plants, improves ant defense behavior, plants should secrete a valuable nectar in situations in which they might benefit from better protection (Smith *et al.* 1990). As extrafloral nectar secretion follows optimal defense theory (Holland *et al.* 2009) and

highly aggressive ants may tilt the balance to a negative outcome for plants (Melati & Leal 2018), natural selection should favor secretion of enriched nectar in situations in which the benefits provided by ants surpass the costs, as periods when herbivore pressure is higher (Millán-Cañongo *et al.* 2014).

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## DATA AVAILABILITY

The data used in this study are archived at the Dryad Digital Repository:

## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

6 TABLE S1. *List of ant species found visiting the experimental plants (Vochysia elliptica) subjected to different*  
7 *nectar quality treatments.*

8 TABLE S2. *Parameters used to calculate the protection effectiveness of eight ant species that attacked and either*  
9 *excluded or consumed the termites.*

10 FIGURE S1. *Synergistic effects of S + A treatment compared to treatments S and A.*

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**TABLES**

TABLE 1. Biotic defense metrics and their interpretation.

Defense metric	Calculation	Interpretation
Encounter rate	Total number of encounters between ants and termites divided by observation time.	A proxy for ant recruitment and patrolling on plants, which represents the probability of encounter between ants and termites.
Minimum time for encounter	Time spent for the first encounter between one ant and the termite.	A proxy for patrolling efficiency of ants on plants.
Attack rate	Total number of ant attacks to termites divided by the total number of encounter during observation.	A proxy for ant aggressiveness, and an estimate of the probability of an attack event when the ant encounters the termite.
Exclusion success rate	Events of termite exclusion (binary variable, being 1 when there is exclusion and 0 when there is no exclusion) divided by the total number of ant attacks to termites.	A proxy for ant aggressiveness, which shows how many ant attacks are needed to exclude the termite from the plant.
Exclusion efficiency	Events of termite exclusion (binary variable, being 1 when there is exclusion and 0 when there is no	A proxy for the time spent by the ant to exclude the termite from the plant.

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exclusion) divided by observation

time.

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## FIGURE LEGENDS

FIGURE 1. Relationship between nectar quality (treatment) and ant defense behavior, estimated in a field experiment with artificial extrafloral nectaries, using live termites as model herbivores. Treatments: A = amino acids, S = sugar, S+A = sugar + amino acids, W = water (control). (A) The S and S+A treatments showed higher ant recruitment than the other treatments. (B) Ants showed higher probability of finding termites in the S+A treatment. (C) Ants showed higher probability of attacking termites in the S+A treatment and (D) a smaller number of attacks were needed to exclude termites in the S+A treatment. Bars represent median values with quartiles. Letters indicate statistical differences between treatments.

FIGURE 2. Time spent by ants to exclude live termites from plants subjected to different treatments of nectar quality. Treatments: A = amino acids, S = sugar, S+A = sugar + amino acids, W = water (control). The proportion of excluded termites in each treatment was measured for 30 min. Termite exclusion was fastest in the S+A treatment, in which 14 min were needed to exclude 50% of the termites from the plants (dashed horizontal line). In the A and S treatments, which lead to similar results, it took the ants 27 and 31 min, respectively, to exclude 50% of the termites from the plants.

**FIGURES**

FIGURE 1.

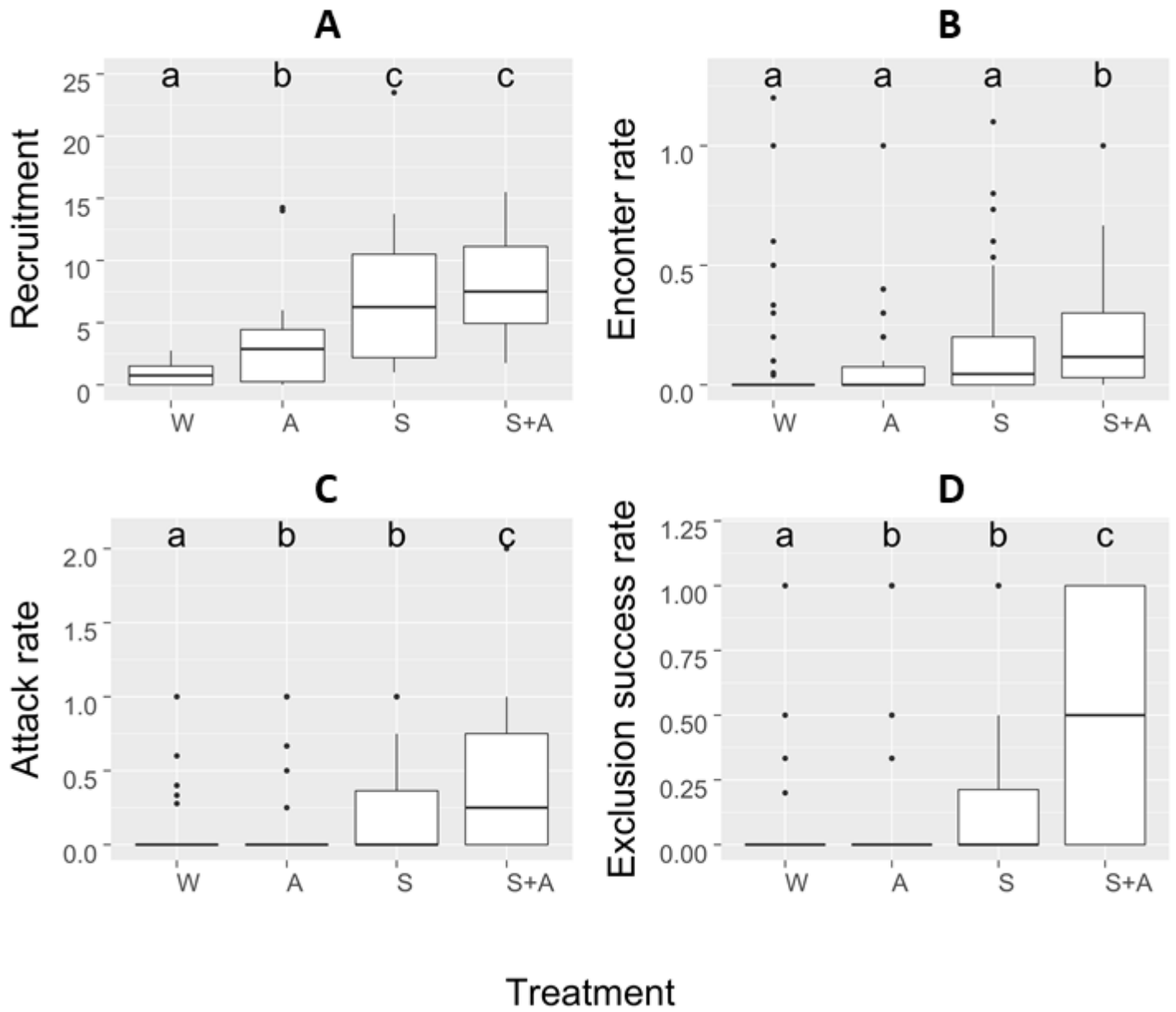
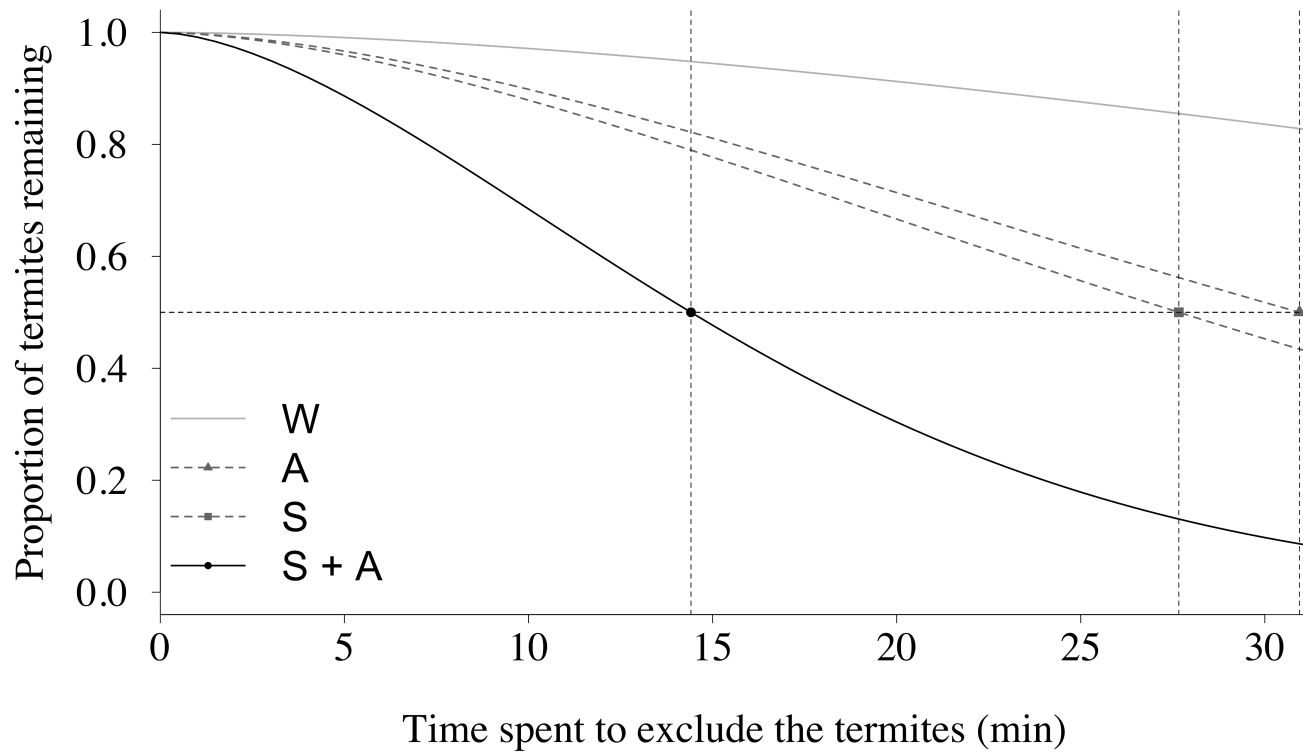


FIGURE 2.



## SUPPORTING INFORMATION

Table S1. List of ant species found visiting the experimental plants (*Vochysia elliptica*) subjected to different nectar quality treatments. The first four columns represent the number of plants visited by ants species according to the nectar quality. **T-Occur.** means the total number of observations by ant species in relation to the total observations; **T-Abun.** means total recruitment of workers ants in all observations; **Abun-Int.** means the total number of interactions between workers ants and artificial nectaries.

Ants species	Treatments				T-Occur.	T-Abun.	Abun-Int.
	W	A	S	S + A			
	<b>Occurrence of ants</b>						
<b>Subfamily Dolichoderinae</b>							
<i>Dorymirmex</i> sp1	-	-	1/10	-	2/320	5	1
<i>Forelius maranhaoensis</i>	-	-	2/10	1/10	5/320	71	59
<i>Tapinoma</i> sp1	-	-	1/10	-	1/320	12	12
<i>Tapinoma</i> sp2	-	-	1/10	-	1/320	3	3
<b>Subfamily Ecitoninae</b>							
<i>Neivamyrmex</i> sp1	-	-	1/10	-	1/320	3	3

### Occurrence of ants

Ants species	Treatments				T-Occur.	T-Abun.	Abun-Int.
	W	A	S	S + A			
<b>Subfamily Ectatomminae</b>							
<i>Ectatomma tuberculatum</i>	5/10	2/10	4/10	4/10	51/320	288	143
<b>Subfamily Formicinae</b>							
<i>Brachymyrmex</i> sp1	1/10	2/10	3/10	-	9/320	77	56
<i>Brachymyrmex</i> sp2	1/10	2/10	6/10	3/10	13/320	53	52
<i>Camponotus blandus</i>	3/10	2/10	4/10	2/10	29/320	243	115
<i>Camponotus crassus</i>	5/10	7/10	7/10	7/10	93/320	712	365
<i>Camponotus renggeri</i>	1/10	2/10	2/10	2/10	12/320	134	40
<i>Camponotus rufipes</i>	2/10	2/10	2/10	2/10	29/320	112	48
<i>Camponotus vittatus</i>	2/10	2/10	2/10	3/10	10/320	14	3
<i>Myrmelachista</i> sp1	1/10	-	-	-	2/320	65	4

### Occurrence of ants

Ants species	Treatments				T-Occur.	T-Abun.	Abun-Int.
	W	A	S	S + A			
<b>Subfamily Formicinae</b>							
<i>Myrmelachista sp2</i>	-	-	1/10	-	2/320	4	4
<b>Subfamily Myrmicinae</b>							
<i>Cephalotes eduarduli</i>	1/10	1/10	-	-	3/320	4	2
<i>Cephalotes pusillus</i>	1/10	-	-	-	1/320	1	1
<i>Crematogaster prox. erecta</i>	-	-	1/10	-	2/320	4	4
<i>Nesomyrmex sp1</i>	-	-	1//10	-	2/320	3	1
<i>Pheidole sp5</i>	-	-	1/10	-	1/320	12	7
<i>Pheidole sp14</i>	1/10	-	-	1/10	2/320	2	2
<i>Solenopsis sp3</i>	-	-	-	1/10	1/320	50	50
<i>Solenopsis substitute</i>	-	2/10	1/10	1/10	5/320	38	23

## Occurrence of ants

Ants species	Treatments				T-Obs	T-Abun	Abun-Int
	W	A	S	S + A			
<b>Subfamily Pseudomyrmecinae</b>							
<i>Pseudomyrmex gracillis</i>	1/10	1/3	1/10	-	5/320	5	4
<i>Pseudomyrmex pallidus</i>	1/10	1/10	-	-	3/320	3	3
<i>Pseudomyrmex termitarius</i>	2/10	-	-	1/10	4/320	5	4
<b>Total</b>	10/10	10/10	10/10	10/10	320/320	1923	1009

Table S2. Parameters used to calculate the protection effectiveness of eight ant species that attacked and either excluded or consumed the termites.

<b>Species</b>	<b>Recruitment of workers</b>	<b>Observation time (min)</b>	<b>Encounter with Termites</b>	<b>Termites attacked</b>	<b>Termites removed</b>	<b>Exclusion efficiency</b>
<i>Brachymyrmex sp1</i>	77	12 ± 2.5	2	2	2	0.08 ± 0.02
<i>Camponotus blandus</i>	243	8.9 ± 3.1	34	18	9	0.13 ± 0.05
<i>Camponotus crassus</i>	712	10.5 ± 4.7	189	69	32	0.12 ± 0.06
<i>Camponotus renggeri</i>	134	8.3 ± 2.3	24	10	6	0.13 ± 0.05
<i>Camponotus rufipes</i>	112	8.42 ± 4	49	24	19	0.14 ± 0.06
<i>Camponotus vittatus</i>	14	12.5 ± 7.5	3	2	2	0.12 ± 0.05
<i>Ectatomma tuberculatum</i>	288	5.1 ± 4.9	78	19	17	0.12 ± 0.06
<i>Pseudomyrmex gracillis</i>	5	7.5 ± 2.5	2	2	2	0.15 ± 0.05
<b>Total</b>	1585	-	381	146	89	-



Figure S3. Synergistic effects of the S + A treatment compared to the treatments S and A summed (i.e., S + A). Statistical parameters from final models indicate that the effect size of the S+A treatment is higher than the effect sizes of the S and A treatments summed (S + A). For Encounter Rate (A), the final GLMM model shows that there is no difference in effect size between the S, A and W treatments and that altogether they are even lower than the S + A treatment. In other words, the effect of S+A is higher than the sum of isolated effect of S, A and W. For Attack Rate (B), Exclusion Success Rate (C) and Exclusion Efficiency (D), the GLMM final model shows that the effect size of the W treatment is lower than those of the S and A treatments, that are also lower than the effect of the S + A treatment. Thus, the effect of S+A is higher than the sum of S and A effects in all biotic defense metrics. The results of Exclusion Efficiency (D) survival model are similar, but they should be interpreted in the opposite way, once this metric represent the time spent to exclude termites by ants, which means that less time spent is better (i.e., lower effect size). Dots and bars represent medium values and standard errors, respectively.

