



Nectar quality affects ant aggressiveness and biotic defense provided to plants

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Complete List of Authors:	Pacelhe, Fábio Túlio; Universidade Federal de Minas Gerais Costa, Fernanda; Universidade Federal de Ouro Preto Instituto de Ciencias Exatas e Biologicas, Department of Biodiversity Evolution and Environment Neves, Frederico; Universidade Federal de Minas Gerais, Department of General Biology Bronstein, Judith; University of Arizona, Ecology and Evol. Biology Mello, Marco; Universidade de Sao Paulo Instituto de Biociencias, Department of Ecology
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To Prof. Emilio Bruna
Editor-in-Chief, Biotropica
Manuscript ID BTR-18-081

Dear Prof. Bruna,

We would like to thank you for the evaluation of our manuscript “Nectar quality affects ant aggressiveness and biotic defense provided to plants”, authored by Fábio Pacelhe, Fernanda Costa, Frederico Neves, Judith Bronstein, and Marco Mello.

Please find attached a revised version of our manuscript. In the following pages you will find point-by-point responses to the issues raised by the reviewers. The review process has improved our manuscript substantially, for which we are deeply grateful. We have addressed all issues and are confident that this new version reads better and presents our arguments in a stronger way.

We look forward to hearing about your decision. Should there be any questions, please do not hesitate to contact us.

We affirm that our manuscript has not been published and is not under review elsewhere.

Yours sincerely,

Fábio Túlio Pacelhe
Ecological Synthesis Lab
University of São Paulo
São Paulo, SP, Brazil
E-mail: fabiotulio.bio@gmail.com
Mobile: +55 31 99227 1730

Response to comments made by the Subject Editor:

“All three reviewers are generally positive about the manuscript. Reviewer 1 does raise some concerns about the validity of putting EFNs on plant that does not already have them. However, I do not think this is too much of a major problem. I can see that running these experiments on a plant species that already has EFNs would be more challenging, as one would have to remove these before replacing them artificially. Plus, my suspicion is that the interaction at EFNs is sufficiently non-specialised that what the authors are reporting in the paper really does reflect what is going on in the wild. However, the rationale for this aspect of the experiment must be strengthened further, and also all of the other points raised by all three reviewers must also be addressed.”

Response: Thank you very much for your assessment. Unfortunately, it would be prohibitively complicated to control the main variables related to our hypothesis using plants that naturally bear extrafloral nectaries (EFNs). In the revised version of our manuscript, as well as in this response letter, we explain in detail why using a plant without EFNs in our experiment made it much simpler to focus on nectar quality, the main factor tested, without altering the studied phenomenon in a way that could hinder drawing solid conclusions about its mechanisms.

Response to the reviewers**Reviewer: 1**

“Although plants bearing extrafloral nectaries are very common in Cerrado, authors used as a model a plant that does not bear such structures and did not give a good reason for that.”

Response: We agree, and explain our reasons for using a species that lacks EFNs in the revised version of our manuscript (L132–L141). Our decision was aimed at allowing us to isolate the effects of the variables we were attempting to test (the presence of sugar and amino acids in EFN). Natural EFN shows daily (Baker-Méio & Marquis 2012, Dáttilo *et al.* 2015, Flores-Flores *et al.* 2018), seasonal (Falcão *et al.* 2014), and ontogenetic variations, as well as variations that occur after the ants consume the nectar (Heil *et al.* 2000). Controlling such qualitative and quantitative variation in nectar properties allowed us to infer more rigorously that the observed behavioral changes were caused exclusively by differences in nectar quality.

“I can see three main problems with the use of such plant species. First, doing so, authors neglect the chemical interface mediating ant interaction with EFN plants. Plants bearing such structures can release volatile compounds that may have some influence on ant attraction and behavior. Although few studies have evaluated the role of such volatiles in EFN plants, several studies have investigated the role of such volatiles in pollination attraction (e.g. Pichersky & Gershenzon 2002 The formation and function of plant volatiles: perfumes for pollination attraction and defense. Current opinion in Plant Biology) and in ant attraction in myrmecophytes (e.g. Agrawal 1998. Leaf damage and associated cues induce aggressive ant recruitment in a Neotropical ant-plant. Ecology). Therefore, it is plausible ants interacting with EFN plants are able to respond to chemical signs other than just extrafloral nectar.”

Response: Thank you for suggesting additional literature. Indeed, the productivity and quality of natural EFN is influenced by the production of volatile organic compounds by the plant after consumption by herbivores (Heil & Bueno 2007), which triggers the biotic defense. This

corroborates our decision to use of artificial EFNs to control qualitative and quantitative variation in nectar properties. We have now included this explanation in the text (L138-L140).

“Second, I am aware that the absence of EFN is not a constraint to ant presence on such plants as different ant species commonly forage on plant surface. However, only a subset of the ant species foraging in plant surface engage in a long-term interaction with EFNs and I am not sure if authors were able to simulate it using the artificial extrafloral nectaries as they are simulating a condition that does not occur naturally in such plants in nature.”

Response: Thank you very much for your urging us to more fully explain our methods. Before installing artificial nectaries, we conducted 30 3-min observations (a diurnal and other nocturnal) on 15 individual *Vochysia elliptica* for three days (13-15 January 2015). We recorded only six ant occurrences: *Brachymyrmex* sp1 and *Crematogaster prox. erecta* once and *Camponotus crassus* and *Ectatomma tuberculatum* twice. As we expected, except during reproductive phases when inflorescences might attract ants (Romero 2002), there were few ants foraging on *V. elliptica*. We have now included this explanation in the text (L142-L149). After the first observations, we installed the artificial EFNs, and refilled and monitored them daily for 12 days (20 - 31 January 2015), when we recorded ant richness and abundance, as well as occurrence and frequency of interactions. After this period, we noticed a stabilization in the frequencies of ant visitation and their abundances and a “ownership” behavior near the food source. We only began the behavioral experiment with termites after this preliminary test. Therefore, we believe our experimental setup did not alter significantly the natural behavior of ants. We have included this explanation in the text (L192-L196).

“Finally, authors did not give any clue about how similar their artificial extrafloral nectar is from extrafloral nectar produced by EFN-bearing plants.”

Response: We agree that the chemical profile of natural EFN is complex and its composition and concentration vary strongly between species (Blüthgen *et al.* 2004). However, only a few studies have assessed the chemical profile of natural EFN. Within this literature, there is information on the chemistry of nectar at the species (Shenoy *et al.* 2012), clade (González-Teuber & Heil 2009), and community levels (Blüthgen *et al.* 2004). The composition and concentration of sugars and amino acids vary largely between species. As we did not find any 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. Additionally, even similar and simpler formulas were already efficiently used to explore the role of extrafloral nectar to structure arboreal ant community in a similar approach in the Cerrado (see Camarota *et al.* 2015). Although it does not emulate the complexity of natural EFN, the components offered in the artificial EFN are among the most common in nature: sucrose, the main sugar used by ants (Blüthgen & Fiedler 2004), and glutamine, proline and threonine, the amino acids found most frequently in the EFN of several plant species (Blüthgen *et al.* 2004). We have now included this explanation in the text (L164-L190).

“I am afraid that, offering a food source that is different from real extrafloral nectar in a plant species that does not bear extrafloral, only allow the authors to evaluate arboreal ant response to variation in food sources. Of course, from ant response to sugar and/or protein food sources it is possible to speculate some mechanisms driving the interaction between arboreal ants and EFN-bearing plants. However, the main objective of the manuscript it is to evaluate the role of extrafloral nectar quality in ant behavior and, consequently, in the quality of plant defense. Then, I am not sure if they are able to directly evaluate it using those methods.”

Response: As already commented, the artificial nectar offered in our experiment was a simplification of the natural EFN, and it was composed of the main nutrients observed in natural EFN. Despite this simplification, we believe that our experimental conditions induced natural behaviors. Even using a relatively simple food, we obtained similar results as other studies that measured the behavior of ants while interacting with natural EFNs or hemipteran honeydew (Katayama & Suzuki 2005, Campos & Camacho 2014). The fact that the artificial EFN was able to attract ants, which defended the experimental plants for 30 days, suggests that our formula is effective and adequate to be used in field experiments. We have now included this justification in the text (L197-L201).

“Finally, I believe that discussion is still superficial. Below, I present some aspects that were neglected by the authors and give some tips to improve the quality of this section.”

Response: We have rewritten the discussion and included considerations on those additional aspects.

Additional comments:

“L55: Understanding processes driving interaction outcomes may indirectly influence ecosystems services only when ecosystems services depend on the outcome of such interactions. Additionally, I did not understand why the authors introduced here disturbed habitats as a particular case. Authors are talking about general theoretical rules regulating the functioning of interactions between species. As the theory is a generalization about specific situations, it is expected that such rules should be similar in both environments. Additionally, authors are not exploring habitat influence on interaction outcome. Therefore, it is just bringing the reader's attention to a subject that does not matter for the manuscript.”

Response: This sentence was indeed confusing, so we have rephrased it (L54-L56).

“L56-57: Some plants have EFNs located on sepals, that are part of the flowers. EFN definition is more related to its function than with its location. Therefore, it is more appropriate to say that EFNs are glands not related to pollination. Additionally, I suggest including Bronstein JL 1998 The contribution of ant-plant protection studies to our understanding of mutualism. Biotropica.”

Response: We agree and rephrased this sentence. Furthermore, we added Bronstein (1998) to the literature cited and Marazzi *et al.* (2013) as supplementary literature (L57-L59).

“L66: Please, include the word “ants” in “...explained by increased ANTS visitation...”

Response: Included (L61).

“L100-106: Authors are presenting here a mix between hypothesis and predictions. It reduces the theoretical value of what has been tested in this study. We can expect that species offering better resources benefit more from its mutualistic partner in any mutualism, not only in ant-plant interactions. Therefore, I would like to see a more theoretical hypothesis followed by its respective predictions, more related to ant-plant interaction parameters used by the authors.”

Response: We have rephrased this paragraph, so that our working hypothesis is now clearly presented and based on theoretical variables (L110-L112), and our predictions are written using precise operational variables (L115-L117).

“L126: Which was the modification done in the method proposed by Blüthgen & Fiedler 2004b?”

Response: Artificial EFNs were used as suggested by Blüthgen & Fiedler (2004), 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 over the entire plant. We also chose to offer different concentrations on different individuals. This way, we could distribute the resources evenly on the plants so to attract ants homogeneously, allowing us to observe their behavior while controlling for resource quality. We have now included this explanation in the text (L153-L158).

“L127-128: Where did those concentrations come from? How are they related to the composition of natural extrafloral nectar?”

Response: As already mentioned, only a few studies have assessed the chemical profile of natural EFN. Among these, there is information on the chemistry of nectar at the species (Shenoy *et al.* 2012), clade (González-Teuber & Heil 2009), and community levels (Blüthgen *et al.* 2004). As we did not find any studies at the community level that traced the chemical profile of EFN in the Cerrado, we had to use the scant information available to define the experimental solutions and their 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 $0.3 \pm 0.07\%$. Additionally, even similar and simpler formulas were already efficiently used to explore the role of extrafloral nectar to structure arboreal ant community in a similar approach in the Cerrado (please see Camarota *et al.* 2015). 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. We have now included this explanation in the text (L164-L180).

“L134-136: But if you are simulating an EFN composition completely different to the ones observed in nature, you are eliciting an ant response that would not be observed on ants interacting with true EFN bearing plants. It would compromise your ability to discuss the relationship between your results and the functioning of EFN-ant interactions.”

Response: As explained above, the nectar composition used in our experiment was a simplified version of the composition observed in nature. Nevertheless, we believe that our experimental conditions induced natural behaviors. After the first 12 days of observations, we noticed a stabilization in the frequencies of ant visitation and their abundances and a “ownership” behavior near the food source. Even using a relatively simple food, we obtained similar results as other studies that have measured the behavior of ants while interacting with natural EFNs or hemipteran honeydew (Katayama & Suzuki 2005, Campos & Camacho 2014). Finally, our experiment

demonstrated how small qualitative differences may change the outcome of ant-plant interactions. We have now included this explanation in the text (L192-L201).

“L141-143: Any statistical test supporting it? What was the sample size used to calculate such percentages?”

Response: Yes. The pilot study was based on a small sample, but its aim was to test the artificial EFNs, especially whether variations in sucrose and amino acid concentrations influenced the richness and abundance of visiting ants. After three days and 20 individual observations, we found no differences in attractiveness between the 20 and 30% sucrose concentrations (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, $R^2 = 0.03$, $\chi^2 = 0.55$, $P = 0.45$). We have added information on sample size and test results in the revised version of our manuscript (L187-L190).

“L151: How ant recruitment was recorded? Number of ants observed at each 5 min?”

Response: We have added further detail on how we recorded ant recruitment in the revised version of our manuscript (L207-209).

“L192-202: The paragraph is full of subjective expressions as “rarely”, “high frequency”. I think it is more informative to present the values observed by authors, supporting such expressions.”

Response: We have now included recorded values and percentages to support our statements (L249-L260).

“L198: Two times from how many appearances of each species?”

Response: We have added further details about those observations (L254-L255).

“L199: How did you classify ant dominance? It did not appear in the methods.”

Response: We have added the original citation (Cerdá *et al.* 2013) in L258.

“L203-212: Where are the results from water treatments? Why is it not presented here?”

Response: We now describe the water treatment results (L261-L277).

“L214-215: Please, remove the sentence “In addition, ants removed termites from the plants twice as fast in S+A than in either the S or the A treatments.” It is redundant with the previous one.”

Response: We would prefer to leave the text as it currently reads, as we do not consider those sentences redundant. In the first sentence we are referring to “minimum time for encounter” (Table 1) or to “time spent for the first encounter”. In the second sentence we are referring to “exclusion efficiency” or to “time spent by the ant to exclude the termite from the plant”. However, we will make this change if the Editor requests it (L271-L273).

“Discussion: An interesting point neglected by authors in the discussion is that despite ants are more aggressive in S + A treatments, sugar treatments were more attractive to ants as more ant species were attracted to it. I believe that it is an important aspect to understand the dynamic of plant protection by ants. Investing in sugar-rich nectar, plants can count with a more diverse set of

ant species foraging on its surface while investing in a more complex nectar, plants attract fewer ant species that guarantee a more efficient defense. Is it possible to think in contexts in which each of such strategies could be favored? The large majority of extrafloral nectar is a protein-poor resource. In which situation secreting protein-rich extrafloral nectar could be advantageous?"

Response: We agree that there might be situations when secreting protein-rich EFN could be advantageous. 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 could benefit from better protection (Smith *et al.* 1990). As EFN 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 would favor secretion of enriched nectar in situations in which the benefits provided by ants surpass the costs, for instance, when herbivore pressure is higher (Millán-Cañongo *et al.* 2014). We have now included this explanation in the text (L333-L339). However, regardless of nectar quality offered by plant, attracting a high-quality mutualist is more important than attracting more ant species. We observed a dominance hierarchy in the S and S+A treatments, with the five numerically and behaviorally dominant species using the resources, foraging, attacking termites and defending the plants. The other ant species arrived in smaller numbers and, while they fed from nectaries occupied by the dominant species, they never showed defensive behaviors. Our results are in agreement with Miller (2007), who showed that plants bearing EFNs that were visited by multiple ant species benefited less than plants with an exclusive association with a single, high quality mutualist (see also Mody & Linsenmair 2004, Del-Claro & Marquis 2015). We have now included this explanation in the text (L286-L296).

"L226-228: In the results, there is no specific information about the increased aggressiveness of ants classified as dominant or subordinate when consuming different nectar types. I cannot see which results are supporting this assumption."

Response: This sentence was indeed confusing, and we have removed it.

"L228-230: Why only quantitative value? I believe as dominant ants promote a better plant defense, they can be also considered "mutualists of high qualitative value". There are several papers showing that dominant ants are better bodyguards to plants."

Response: We agree that dominant ants are better bodyguards for plants, and we believe that the term "mutualists of high quantitative value" is correct in this case. We have added further detail about the new conceptual framework proposed by Schupp *et al.* (2017) in the revised version of our manuscript (L94-108).

"L231: But if such ants behave more aggressively, nectar quality should also drive the qualitative component."

Response: Possibly, but since we did not measure any delayed outcomes of the interaction (i.e., an increase in fruit set per enemy removed) we cannot state this for sure.

"L234: As long as I know, there is no such thing as extrafloral nectar composed only of amino acids. This is an example of ant response that may not occur in nature, in plants bearing EFNs."

Response: Yes, we agree that there is no natural EFN composed only of amino acids. However, our results call attention to the importance of this nutrient for ant behavior. Although the sucrose treatment (S) recruited twice as many ants as the amino acid treatment (A), ants showed similar likelihood to encounter, attack, and exclude termites, and also similar exclusion efficiency between

treatments. Furthermore, it was important to evaluate ant behavior in the S and A treatments separately to conclude that behaviors elicited by the sucrose + amino acid treatment (S+A) were not simply the sum of the effects of the S-only and A-only treatments. As our results show, S+A effects are higher than the effects of S and A summed (see Fig. S1), suggesting a synergy between the main components of nectar, as proposed by Raubenheimer & Simpson (1997). We have now included this explanation in the text (L304-L311).

“L253: Interspecific competition is not only shaped by the resource relative value, but also by the availability of such resource in the environment. The availability of such high-quality resources should also be low, reinforcing the idea that ant competition may be driving such pattern. It should be mentioned here.”

Response: Yes, we agree. We have added this interesting point to the discussion (L321-L324).

“L264-265: As EFN secretion follow optimal defence theory (see Holland et al. 2009. Optimal defence theory predicts investment in extrafloral nectar resources in an ant-plant mutualism. Journal of Ecology), I would expect that plant would secrete expensive nectar in situations in which they depend more on their bodyguards. If such balanced nectar is more expensive to plants, natural selection would favour secretion of such nectar in situation in which benefits provided by ants are higher.”

Response: We have rephrased this sentence, taking this argument into account (L333-L339).

Reviewer: 2

“The abstract fails to show what was really tested in this very interesting study. Author must tell that sugar and amino acids were manipulated artificially and to present more detailed the results. The abstract introduction is too long, and few words were dedicated to the methodology and results.”

Response: We agree, and have tried to highlight these points in the revised version of our manuscript.

“Page five lines 12-16 “For plant protection mutualism, the quantitative component mostly encloses interactions frequencies and rates of enemies removal, while the qualitative component is based on the post interaction delayed outcomes, such as plant fitness (Schupp et al. 2017).” I suggest, see that it is a suggestion – that the paper “ LANGE, D., CALIXTO, E. S & DEL-CLARO, K. (2017) Variation in Extrafloral Nectary Productivity Influences the Ant Foraging. PLoS ONE12(1): e0169492. doi:10.1371/journal.pone.0169492” could be important to the comprehension of the qualitative aspects and its implications in results. Perhaps, this paper could contribute to improve the strong of the main hypothesis. It is a suggestion.”

Response: We have added Lange et al. (2013) and further detail about the new conceptual framework proposed by Schupp et al. (2017) in the revised version of our manuscript (L94-L108).

“Page 9, line 20 -21 – the ants you found “Brachymyrmex sp1, Camponotus blandus, C. crassus, C. rufipes, C. renggeri, C. vittatus, Ectatomma tuberculatum, and Pseudomyrmex gracillis” – are described as very aggressive in other studies did in the same or similar biomes. It is good to call

attention to this in the discussion, due the fact that ant species behaviour is a very relevant question in this type of interactions.”

Response: Yes, now we call attention to the importance of ant dominance behavior in the revised version of our manuscript (L286-L296).

Reviewer: 3

Minor Comments

“Page 2, Line 13. Authors need to introduce the theory of context-dependence of interactions presented in the second paragraph for better understanding of the argument presented.”

Response: Thank you. We have added further information on the context-dependence of interactions in the revised version of our manuscript (L67-L77).

“Page 2, Line38. For confirming the argument presented, be current, and related to the ecosystem under study, consider the insertion of reference: F, R, Dáttilo, w., Rajan, s. p., Rico-Gray, v., Jordanian, p., Del-of course, & k. (2017). Differences among ant species in plant protection are related to production of extrafloral nectar and degree of leaf herbivory. *Biological Journal of the Linnean Society* 122 (1), 71-83.”

Response: We now cite Fagundes *et al.* (2017) in L62.

“Page 3, Line 32. replace ' meal 'for ' intake '.”

Response: Replaced (L84).

“Page 7, Line 56. Consider quote: Individual-based ant-plant networks: diurnal-nocturnal structure and species-area relationship. W Dáttilo, R F, CAQ Gurka, MSA Silva, MCL Vieira, TJ Izzo, ... *PLoS One* 9 (6), e99838.”

Response: We now cite Dáttilo *et al.* (2014) in L220.

“Page 7, Line 28. The termites were put in plants at the time the first Ant ARRIVE?”

Response: After installing the artificial EFNs, we filled and monitored them daily for 12 days (20 - 31 January 2015), until the content of each Eppendorf vial was consumed or evaporated. During this period, we recorded ant richness and abundance, as well as occurrence and frequency of interactions. After this period, we noticed a stabilization in the frequencies of ant visitation and their abundances and a “ownership” behavior near the food source. We only began the behavioral experiment with termites after this pilot test. We have added further detail about the timing of termite release (L192-L196).

“Page8, Line 30. In addition to the treatments, the prior amount of ants in the plant, the taxonomic identity and behavior (attacked, ignored) OF ANT SPECIES that interacted with the termite can affect the time and the rate of removal of termites. The authors should considere to test these

cofactors to isolate the effect of the treatments or use them to explain the mechanisms of action of the treatments. For example, the defense is better when you have amino acids and sugar because it has more Ant patrolling, because there's a particular species of Ant, because ants are more aggressive, or a combination of these factors? The author has data for these tests, as their results suggest, that would make the study more complete and self-explanatory."

Response: Thank you for your suggestion. However, our main hypothesis does not involve the taxonomic identity of the ants as a covariate. We would like to clarify that if we put the variables "taxonomic identity" and "initial abundance" as covariates in our model, we would change our data matrix and reduce our sample N, because we would need to consider in the data matrix only those observations in which there were ants. This would lead to the loss of over 100 observations. In the model presented in the manuscript, all field observations (320), including those in which there were no ants at the moment of observation, generated "zeros" for our dependent variables (table 1 in the manuscript). Those zeros are biologically important for our analysis, because the very fact that there were no ants in the plant means that that resource was not able to attract them, and, consequently, the plant would not be defended. To expand upon our argument: in the W treatment, in 80 possible observations, we found no ants in 60 observations, while in treatment A we did not find ants in 28 observations, and in treatment S we did not find ants in only nine observations. However, in the S+A treatment always there were foraging ants, which corroborates that this treatment was better at attracting ants. In the data matrix "taxonomic identity" the new model would have 80 observations for S + A and only 20 for W, which obviously biases the results. We believe that whenever we build a model for a statistical test, above all we have to fine-tune the variables used with our biological hypothesis. In addition, the model should always be as simple as possible, because each additional variable implies a loss of degrees of freedom and error inflation. Therefore, we would like to keep the analysis and the results as they are.

"Page 10, Line 36. For confirming the argument presented, be current, and related to the ecosystem under study, consider the insertion of reference: F, R, Dáttilo, w., Rajan, s. p., Rico-Gray, v., Jordanian, p., Del-of course, & k. (2017). Differences among ant species in plant protection are related to production of extrafloral nectar and degree of leaf herbivory. *Biological Journal of the Linnean Society* 122 (1), 71-83."

Response: Thank you for the suggested reference. We highlight that the main author of this article is Fagundes and not Dáttilo (see <https://doi.org/10.1093/biolinnean/blx059>). We would prefer not to cite Fagundes et al. (2017) in the sentence mentioned, because their paper was already cited twice in our manuscript in other parts that seem more suitable.

"Page 11, Line 2. Discuss the plausibility of claiming that different foods can attract different species, or the same species in different frequency, resulting in variations in the efficiency of Defense. Since ' S + A ' attracts the species that 'S' and ' A ' would attract, the defense is increased."

Response: Table S1 shows that dominant species presented similar frequency of occurrence in all treatments and that the same ant species were observed defending the S+A, S, and A treatments. Therefore, we cannot say that an increased in efficiency of defense was due to species co-occurrences.

Supporting information

"Table S1. I suggest to present total abundance and the number of plants that the ants occurred, rather than average and deviation. Is more informative."

Response: We modified Table S1 as suggested, and have added the total number of interactions between ant workers and artificial nectaries.

“Table S2. Redundant with the Table S1, and little informative because of the colors. I suggest to present the amount of termites removed, instead of abundance already present in Table S1.”

Response: We modified Table S2 as suggested, and have added some parameters used to calculate the protection effectiveness of the eight ants species that either excluded or consumed the termites.

References cited in this response letter:

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Nectar quality affects ant aggressiveness and biotic defense provided to plants

FÁBIO T. PACELHE*¹, FERNANDA V. COSTA², FREDERICO S. NEVES^{1,3}, JUDITH BRONSTEIN⁴ & MARCO A. R. MELLO⁵

¹ Graduate School in Ecology, Conservation and Wildlife Management, Federal University of Minas Gerais, Belo Horizonte, Brazil.

² Department of Biodiversity Evolution and Environment, Federal University of Ouro Preto, Ouro Preto, Brazil.

³ Department of General Biology, Federal University of Minas Gerais, Belo Horizonte, Brazil.

⁴ Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ, USA.

⁵ Department of Ecology, University of São Paulo, São Paulo, Brazil.

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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¹ Corresponding author; e-mail: fabiotulio.bio@gmail.com

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

patrulhamento 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

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

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

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

Here, we report on an experiment in which we manipulated the two most important chemical components of extrafloral nectars: sugar and amino acids. Specifically, we tested whether the nutritional value of nectar, defined based on the levels of those two main nutrients, influences the quantity component of the plant protection mutualism (Schupp *et al.* 2017). We carried out a field experiment using treatments with artificial extrafloral nectar of different qualities and termites as model herbivores. We considered a nectar rich in sugar and amino acids to be more nutritious and balanced from the ants’ perspective than a nectar with only sugar or amino acids. We expected that, on plants offering artificial extrafloral nectar enriched with sugar plus amino acids, ants would: (1) exhibit higher recruitment and patrolling; (2) attack termites more frequently and remove them in larger 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 extrafloral nectar 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 extrafloral nectars 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 extrafloral nectar. 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 extrafloral nectar 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 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. Additionally, even similar and simpler formulas were already efficiently used to explore the role of extrafloral nectar to structure arboreal ant community in a similar approach in the Cerrado (see Camarota *et al.* 2015). 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 this period, we recorded ant richness and abundance, as well as occurrence and frequency of interactions. After this period, we noticed a stabilization in the frequencies of ant visitation and their abundances and “ownership” behavior near the food source. We only began the behavioral experiment with termites after this preliminary test. As stated above, the artificial nectar offered in our experiment was a simplification of natural extrafloral nectar, and was composed of the main nutrients observed in natural extrafloral nectar. Nevertheless, there is evidence that our experimental conditions induced natural behaviors. Even using a relatively simple food, we obtained similar results as other studies that measured the behavior of ants while interacting with natural extrafloral nectars or hemipteran honeydew (Katayama & Suzuki 2005, Campos & Camacho 2014).

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

We considered an ant-termite interaction to be an *attack* when the ant injured or consumed the termite, and a *touch* when the ant touched the termite with its antennae without injuring it. The outcome was considered

neutral when the ant did not interact with the termite (following Katayama and Suzuki 2005). We also recorded the number of attacks after first contact and whether these attacks led to termite exclusion from the plant.

We stopped observing the ants after 30 min or when the termite was consumed, left the plant, or fell from the plant due to vibrations caused by wind or ant attacks. The observations were made for 26 d (2-27 February 2015). We carried out a total of eight observations on each plant, four diurnal (from 0600 to 1200 h) and four nocturnal (from 1800 to 2400 h), totaling 320 observation events and 88 h of observations in field. For nocturnal observations, we used a headlamp with a red plastic filter to avoid disturbing the ants. We sampled the spectrum of ants over 24 h, because the composition of ant species interacting with extrafloral nectary-bearing plants is known to differ between day and night (Dáttilo *et al.* 2014, Anjos *et al.* 2016).

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

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

To test prediction 3, that ants should remove termites faster on plants whose extrafloral nectar was enriched with sugar and amino acids, we used a survival analysis with Weibull distribution (Weibull 1951), inside package *survival* (Therneau 2015) for R. For this analysis, we used termite exclusion from host plant (binary

variable: 1 = exclusion and 0 = no exclusion) and the time spent for exclusion as response variables, and treatment as explanatory factor.

For all GLMM models, when significant differences were observed among treatments, the models were submitted to contrast analysis by aggregating different levels of treatment variable and testing whether the model 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_(5,315) = 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 treatments (Deviance_(4,316) = 163.3, $R^2 = 0.05$, $\chi^2 = 9.55$, $P = 0.001$; Fig. 1). Supporting prediction 2, ants attacked termites more frequently on plants assigned the S+A treatment. Attack rate was twice as high in the S+A treatment than in either the S or A treatment and 7.6 times higher than in the W treatment (Deviance_(5,315) = 165, $R^2 = 0.19$, $\chi^2 = 11.25$, $P = 0.003$; Fig. 1). Termite removal from the plant was also higher in the S+A treatment, with an exclusion success rate 2.5 times higher than in the S or A treatment and 16 times higher than in the W treatment (Deviance_(5,315) = 254.9, $R^2 = 0.17$, $\chi^2 = 30.93$, $P < 0.001$; Fig. 1).

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

DISCUSSION

Our results are consistent with the hypothesis that biotic defense depends on the quality of the reward offered by plants. Specifically, our results suggest that plants that offer more nutritious extrafloral nectar receive better biotic

defense in return, due to increased aggressiveness. Consumption of high-quality nectar composed of sugar + amino acids was associated with higher ant recruitment, higher ant aggressiveness, and higher exclusion efficiency of a surrogate herbivore compared to nectar containing only sugar or amino acids.

Five ant species were dominant in all treatments and are considered as "core" in the studied environment (see Ribeiro *et al.* 2018). These ants have traits that define dominance, such as massive recruitment and high aggressiveness, suggesting that they can be considered "mutualists of high quantitative value", one of the components of the plant protection effectiveness framework (Schupp *et al.* 2017). We observed a marked dominance hierarchy in the S and S+A treatments, with the five numerically and behaviorally dominant species using the resources, foraging, attacking termites and defending the plants. The other ant species arrived in smaller numbers and fed from nectaries occupied by dominant species, but never showed defensive behaviors. In sum, our observations show that in this system, attracting more high-quality mutualists is more important than attracting more species. These results are in agreement with Miller (2007), who showed that plants with extrafloral nectar visited by multiple ant species received reduced benefits relative to those associated with a single, high quality mutualist species (see also Mody & Linsenmair 2004, Del-Claro & Marquis 2015).

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

Although in nature, extrafloral nectar is never composed of amino acids alone, our results call attention to the importance of this nutrient in eliciting mutualistic ant behaviors. Nectars composed by a single nutrient (S or A) produced similar levels of defense (same capacity to encounter, attack, and exclude termites), while the

treatment with both nutrients led to improved biotic defense. This suggests that the effects of those two nutrients might be synergistic (as proposed by Raubenheimer & Simpson 1997). In fact, S+A effects are higher than the effects of S and A summed (see Fig. S1). This synergistic response may be related to the nutritional requirements to sustain brood growth, growth rate, body mass, survival, and indicators of colony fitness (Byk & Del-Claro 2011). Moreover, at the colony level, energy requirements differ among individuals. While worker ants require 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, at 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:

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

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

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 encounters 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.

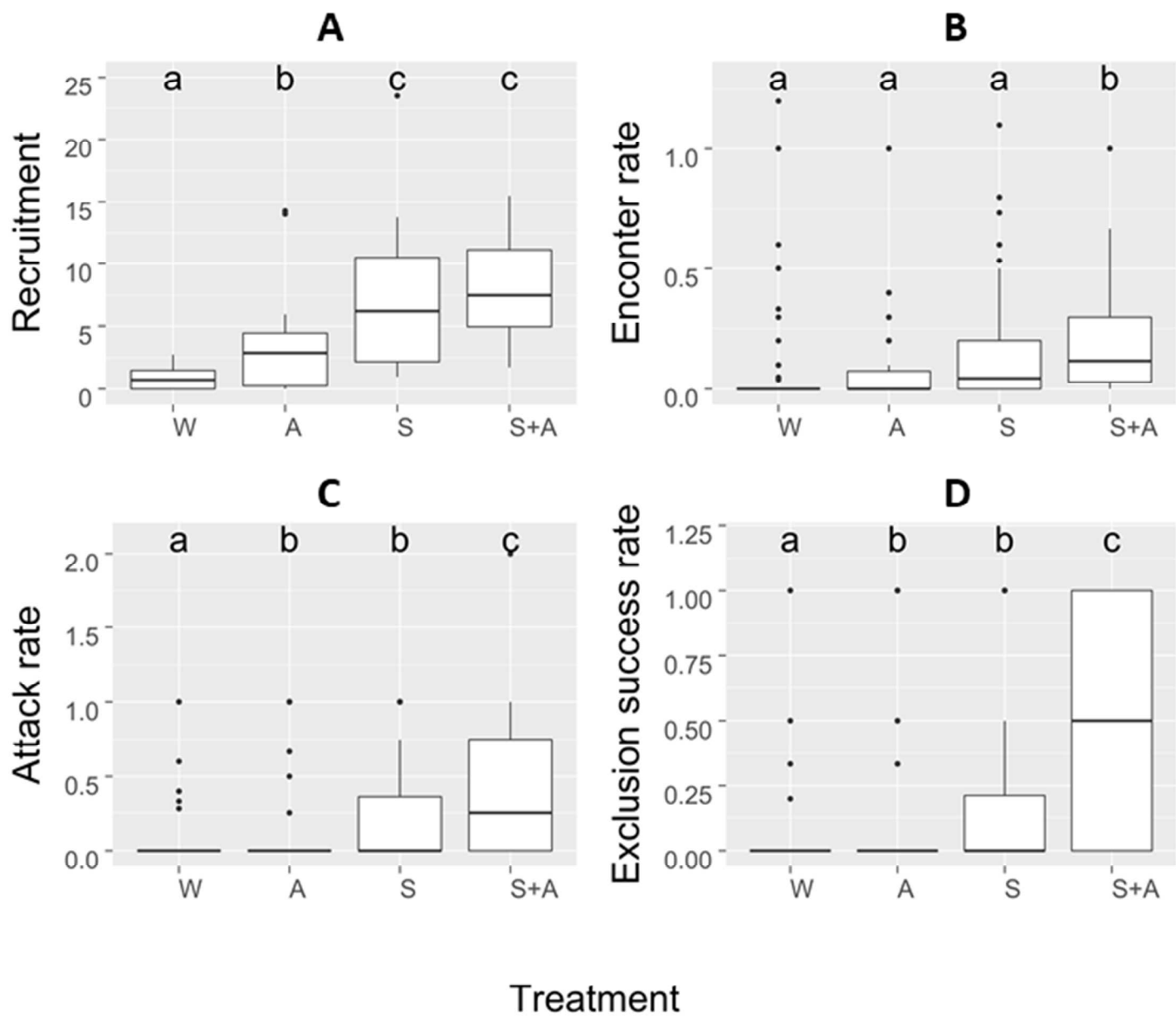
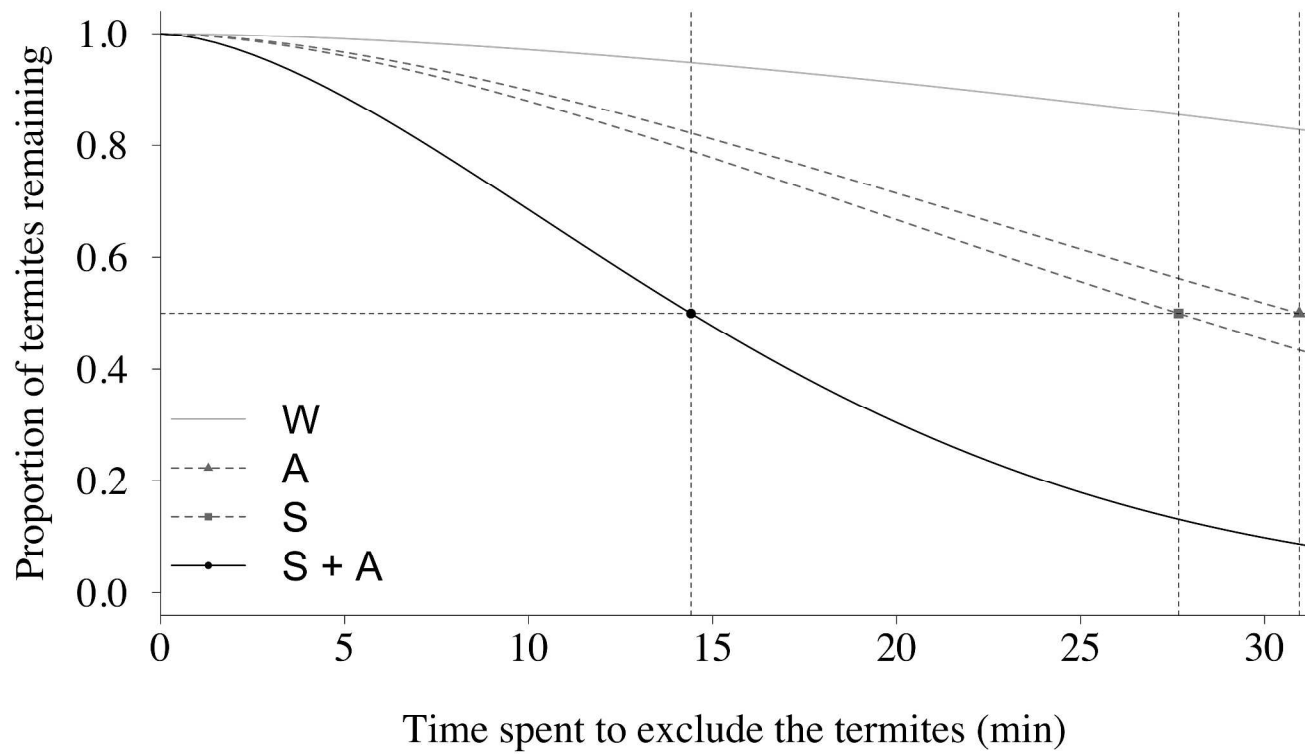


FIGURE 2.



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	Occurrence of ants						
	Treatments				T-Occur.	T-Abun.	Abun-Int.
	W	A	S	S + A			
Subfamily Dolichoderinae							
<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
Subfamily Ecitoninae							
<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			
Subfamily Ectatomminae							
<i>Ectatomma tuberculatum</i>	5/10	2/10	4/10	4/10	51/320	288	143
Subfamily Formicinae							
<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			
Subfamily Formicinae							
<i>Myrmelachista sp2</i>	-	-	1/10	-	2/320	4	4
Subfamily Myrmicinae							
<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			
Subfamily Pseudomyrmecinae							
<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
Total	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.

Species	Recruitment of workers	Observation time (min)	Encounter with Termites	Termites attacked	Termites removed	Exclusion efficiency
<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
Total	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.

