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Beyond the gardens: The extended mutualism from ant-garden ants to nectary-bearing plants growing in Amazon tree-fall gaps

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Abstract

The regime of frequent small disturbances, such as the formation of tree-fall gaps, is an important mechanism maintaining the diversity in tropical forests. Occurring primarily in forest gaps, or in riparian vegetation, ant gardens (hereafter AGs) are mutualistic associations involving several epiphytic plant species and ants. In the Amazon rainforest, a widespread AGs association is assembled by two aggressive species of parabiotic ants (*Camponotus femoratus* and *Crematogaster levior*). Working together, these ants aggressively monopolize food resources and attack any intruder on the AGs. Here, we demonstrated the extended protective effect of parabiotic ants on the leaf herbivory of neighboring plants, contrasting plants with and without extrafloral nectaries (hereafter EFNs). We performed the fieldwork in South Amazonia where we found 33 forest gaps, 16 with and 17 without AGs. Independently of the presence of AGs, plants possessing EFNs have less area removed by herbivores and fewer leaves with herbivore marks than those without EFNs. This decrease in herbivory was remarkable on EFN-plants growing in gaps that also harbor AGs. Additional manipulative experiments confirmed this pattern: The AG-ants were more efficient than no-AG ants in removing live termite baits placed on EFN-plants, but few attacks were observed on plants without EFNs. Thus, AG-ants can extend their role in defending ant gardens from other plants growing in the same area, particularly those with EFNs. This extension of the mutualism benefits can affect the dynamics of the ecological succession and coevolutionary process in the Amazon forest.

Abstract in Portuguese is available with online material

KEYWORDS

coevolutionary hotspot, extrafloral nectaries, forest gaps, herbivory, indirect biotic defenses, Insect–plant interaction, mutualism

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1 | INTRODUCTION

In tropical forests, small and frequent disturbances, such as the formation of tree-fall gaps, are one of the main factors which promotes and maintains species diversity (Brokaw, 1985; Denslow, 1987; Hartshorn, 1980; Schnitzer & Carson, 2001; Wright, 2002). These disturbances are important by making resource-rich environments (for example in luminosity and nutrients) continuously available for the colonizing plants and animals (Brokaw & Busing, 2000). The success of colonization will depend on several factors, such as (i) period of gap occurrence, (ii) distance from a propagules fount, (iii) gap size, (iv) substrate conditions and (v) plant–herbivore interactions (Hartshorn, 1980; Hubbell, 2001). In fact, herbivory in young plants imposes a barrier to their establishment and may alter the structure of the local population and future diversity (Coley, 1983; Schupp et al., 1989; Sullivan, 2003). Also, herbivores can be vectors of several plant diseases (Nault, 1997). Therefore, the investment in defense against herbivores can reduce the loss of photosynthetically active tissue as well as decreasing the probability of contracting an infection, bringing competitive advantages to plants growing in the gaps (Coley et al., 1985).

Different types of anti-herbivory defense strategies have evolved in response to the selective pressure of herbivores, such as chemical defenses through compounds of secondary metabolism and/or physical defenses as spines and trichomes (Bixenmann, Coley, & Kursar, 2011, 2013; Coley & Barone, 2001). A remarkable kind of anti-herbivore defense is the indirect biotic defense, which involves interactions with herbivore natural enemies (Ballaré, 2014; Coley & Barone, 2001; Heil, 2008; Nascimento & Del-Claro, 2010). Ants are pervasive on terrestrial ecosystems and one of the most common and widespread adaptation related to biotic defense in tropical forest plants are extrafloral nectaries (EFNs). These secretory glands are usually located in vegetative parts of plants and produce energetic-rich rewards, as nectar (Heil, 2015). The main function of EFNs is to attract ants that actively decrease the presence of herbivores and herbivory on the plants (Nascimento & Del-Claro, 2010; Rico-Gray & Oliveira, 2007; Rosumeck et al., 2009). Plants with EFNs are found in abundance in tree-fall gaps (Bentley, 1976) where, due to the high incidence of light, the production of extrafloral nectar becomes less expensive (Coley & Barone, 1996).

The simple presence of ants on plants with EFNs may change the herbivores behavior (*e.g.*, Sendoya et al., 2009). Usually, these interactions are facultative and conditional and may be mutualistic or not (Dáttilo et al., 2013). The result of the interaction may depend on abiotic factors, such as temperature and nutrient availability, or biotic, such as ant species and behavior (Fiala & Linsemair, 1995). However, there are other types of more specialized associations where the plants have structures that permit the maintenance of an entire ant colony (Bronstein et al., 2006), minimizing behavioral variation among ant species, such as the forage period. A rather peculiar type of ant–plant interactions is the "Ant Gardens" (thereafter the AGs) which are known for the tropical regions of East Asia, and Central and South America (Davidson et al., 1990; Orivel & Leroy, 2011; Blüthgen et al., 2001; Kaufmann, Weissflog, Hashim & Maschwitz, 2001; Kaufmann & Maschwitz, 2006; Vicente et al., 2014; Morales-Linares et al., 2016). They can be roughly defined as aggregates of epiphytes assembled by ants, but in fact, are sophisticated ant–plant mutualistic associations involving both protective and dispersal mutualisms, and myrmecotrophy (reviewed in Orivel & Leroy, 2011).

In the Neotropical region, several genera and species of ants are associated with AGs. The most common and widespread AG is built by both, *Camponotus femoratus* (Fabricius, 1804) (Formicinae) and *Crematogaster levior* Longino, 2003 (Myrmicinae) (Figure 1a), which are found living together in a relationship known as parabiosis, sharing their nests and foraging trails in forest gaps and riverside areas (Davidson, 1988; Longino, 2003; Vicente et al., 2014). When living in the AGs, ant colonies benefit from the availability of nesting sites, structural stability of the nest, microclimatic conditions and food rewards such as EFNs, elaiosomes and fruit pulp (Kaufmann, 2002; Leroy et al., 2009). In contrast, the epiphytes are favored with a nutrient-rich substrate, seed dispersal and most notably defense against herbivorous attack (Orivel & Leroy, 2011; Vicente et al., 2014). Eventually,

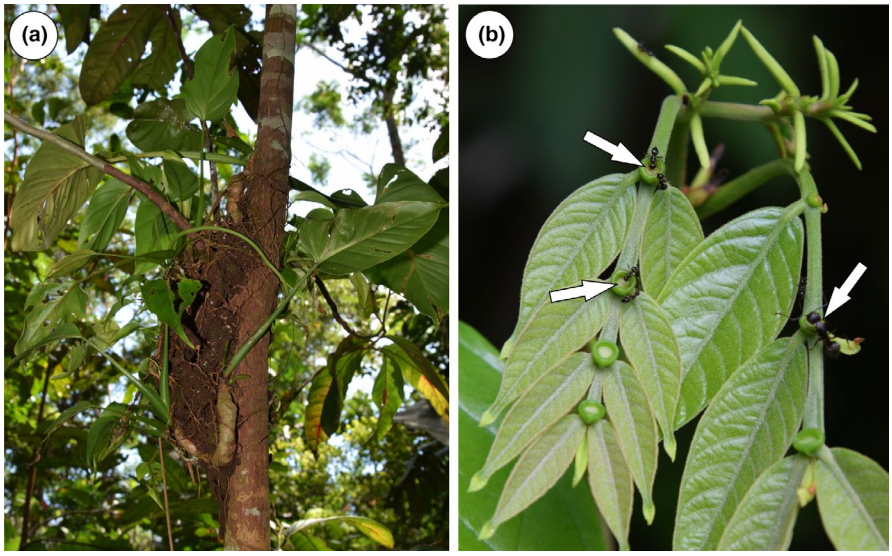


FIGURE 1 Ant–plant interactions between parabiotic ants *Camponotus femoratus* and *Crematogaster levior*. (a) typical ant-garden (AG) with predominance of Araceae and Orchidaceae epiphytes; (b) both parabiotic ant species foraging on *Inga* sp. (Fabaceae) extrafloral nectaries (EFNs) (arrows)

the ant protection against herbivory is also expected for the plant that sustains the AGs (Kaufmann, 2002). However, the foraging of *C. femoratus* and *Cr. levior* is not restricted to the AGs or to the tree harboring the AG (Davidson, 1988; Vicente & Izzo, 2017). Instead, parabiotic ants actively forage through the gap, in both soil and lower vegetation using lianas and epiphytic roots as bridges and monopolizing food resources on the foliage as extrafloral nectary-bearing plants (Figure 1b) and honeydew-producing hemipterans (Davidson, 1988; authors, pers. obs.). Therefore, a hypothesis raised by this Natural History information is whether the protective mutualistic role of AG-ants can be extrapolated to other neighbor plants in the gap. For this statement to be true, plants from forest gaps with AG-ants (*C. femoratus* and *Cr. levior*) should have a lower herbivory rate and herbivory incidence when compared to those without AGs. Once AG-ants forage on EFN-bearing plants for food, the herbivory rates and herbivory incidence on plants with EFNs should be even lower when growing in forest gap harboring AGs. And, finally, the efficacy of ants in remove the herbivores should be higher on forest gaps with than without AGs, being even higher on plants with, than those without EFNs. To address these questions, we conducted a series of standardized samplings and field experiments.

2 | MATERIALS AND METHODS

2.1 | Study site and sampling protocol

The sampling protocol of this study was carried out between 27 April to 15 May 2015, in two forested sites of the Biodiversity Research Program (PPBio) in southern Amazonia. Site I (11°34'S, 55°17'W) and site II (11°35'S, 55°17'W) are distanced about 15 Km apart but both located on the Continental farm, located in the municipality of Cláudia, Mato Grosso, Brazil. The climate for the region is tropical, warm and humid, type Am in the Köppen classification (Alvares et al., 2013). Average annual temperature is 24°C, and average rainfall is 2,200 mm (Gonçalves, 2012). Vegetal composition of the region is dominated by perennial trees, typical of the Amazonian forest (Priante-Filho et al., 2004).

The tree-fall gaps studied here were located along a 12 km trail system in each area (24 km total). We searched for gaps that where part of this area and within 10 meters of each side of the trail (20 meters wide). Later, for each tree-fall gap harboring AGs, we also took the nearest gap similar in size (with less of 10% difference in area), where there were no AGs, totaling 33 selected clearings, of which 16 had AGs. In each gap, we estimated the area (m^2) using the length and width of the gap. By assuming that the gaps had an elliptical shape, we calculated the area as follows:

$$A = \pi \cdot B \cdot C.$$

where A = area of the ellipse, $\pi = 3.1416$; B = length and C = width of the gap.

In each studied gap, we tried to choose all plant species with EFN between 1.0 and 3.0 m of height. To each EFN plant marked, we took another plant without EFN, with approximately the same height (no more than 15 cm of difference). We took all plants till the number of 20 individuals of each category, however, in some few gaps, we did not reach 20 individuals. For each plant, we measured the proportion of leaf area removed by herbivores on five (5) apical leaves using a millimeter paper (see Vasconcelos, 1991). We took these measurements for 6,530 leaves on 1,306 plants. We chose totally expanded apical leaves to minimize the difference in lifespan measured leaves. Also, the major part of herbivory was usually performed during the expansion period of the leaf (Aide, 1993; Coley & Aide, 1991).

We collected plant parts and made exsiccates for each plant. We measured the herbivory for later identification in laboratory. Plant species were identified with the help of the botanists from UFMT herbarium, the Dr. Adarilda Petini-Benelli and Dr. Márcia Cleia Vilela dos Santos and through identification guides (for example, Ribeiro et al., 2002). However, as expected, the major part of plants in gaps are young growing trees, the major part of the material was infertile. So, a large part of the collected plants was not identified but separated in morphospecies. These species/morphospecies were used to compare the composition of plants growing on gaps with and without AGs.

2.2 | Experiment of intruder survival

In August 2019 we did a bioassay where we used isopteran workers as a model of intruder to evaluate the efficiency of ant protection. In this experiment, we gently pasted live termite baits on leaves of plants growing on a tree-fall gap close to an abandoned road harboring about 75 AGs. As control, we used a neighbor area without AGs, also a gap closes to the same abandoned road, and with similar size. On both sites, we pasted the termites on their backs to the leaf surface by using white glue. In total, we used 122 plants in the bioassay: 64 plants in gaps with AGs (33 with EFNs and 31 without EFNs); 58 plants in gaps without AG (31 with EFNs and 27 without EFNs). In the experimental group, all plants were located in no more than 3 meters from an individual AG. In the control sites, we choose plants about 4m apart each other. In all plants, we looked for the most apical leaf without herbivory marks to paste the termites. In the EFN-bearing plant, we pasted the termite on the leaf surface, 5 cm apart from the EFN. In plants without EFN, we placed in a similar position, 5 cm apart from petiole. After pasting the termite, we measured the time interval to the first ant attack on the termite. We here considered as attack when the ant found and touched on the termite with the mandibles (probably biting). The maximum period of experiment was 15 min (900 s).

2.3 | Data analysis

Assuming that the identity of the plant may affect herbivory by inter-specific differences in chemical composition or physical defense mechanisms (Bixenmann, Coley, & Kursar, 2011, 2013; Coley

TABLE 1 Results of the general linear mixed models (GLMM) analyzing the effect of ant gardens (AGs) ants and presence of extrafloral nectaries (EFNs) on removed leaf area and presence of damage on leaves of plants growing on forest gaps

	Fixed effect coefficients				
Dependent variable	**Parameters**	**Estimate**	**Std. Error**	**Z-value**	***p***
Removed foliar damaged per plant (Negative binomial)	Intercept	1.51	0.11	−13.8	.001
	EFNs Presence	0.96	0.124	7.71	<.001
	AGs Presence	0.34	0.146	2.33	.02
	EFN * AGs presence	−0.41	0.169	−2.43	.015
Presence of foliar damage per leave (Binomial)	Intercept	0.506	0.171	2.95	<.001
	EFNs Presence	1.387	0.178	−7.75	<.001
	AGs Presence	0.33	0.2445	−1.34	.03
	EFNs * AGs presence	−0.287	0.263	−1.09	.274

& Barone, 2001), *a priory* we evaluated whether the composition of the plant community varied between gaps with and without AGs using the Permutational Multivariate Analysis of Variance (PERMANOVA, with 999 permutations). We did not observe difference in the composition of the plant community (PERMANOVA, $F = 0.5173$, $p = .874$, which led us to infer that AGs are not preferentially found associated with a given plant community and any difference in herbivory between those gaps should be a by-product of herbivore specificity.

The effect of the presence of the parabiotic AG-ants on the herbivory of the plants was evaluated with generalized linear mixed-effects models (GLMM). This approach is widely used for the analysis of non-normal data that have random factors (Bolker, 2008). Initially, the model was tested using Beta error distribution implemented in glmmTMB package, available in program R version 3.2.5 (R Core Team, 2016). As we detected over dispersion in the data, we used negative binomial error distribution in the final model using the MASS package (Venables & Ripley, 2002). This error distribution is one of the best when parametrizating results with overdispersed data (Hilbe, 2011, Linden & Mantinyemi, 2011). In these analyses, the response variable was the mean proportion of leaf area removed per plant and the explanatory variables were as follows: 1) presence of AGs in the gap; 2) presence of EFNs in the plant and 3) interaction between the two variables. As in our sampling protocol be paired (gaps with and without AGS), in this analysis, we used the gap nested within the pair of gaps (1|forest gap/pair of gaps) as random variable. Once an interaction among the explanatory variables was observed, we employed Bonferroni adjusted multiple contrast comparisons, in order to determine where are the differences, by using the emmeans package.

In addition, we also used GLMM to test whether the simple presence of any leaf damage made by herbivores differed between forest gaps with and without AGs, so as to build a model using binomial error distribution and the lme4 package (Bates et al., 2015). The response variable was the presence/absence of damage in each of the five leaves evaluated for each plant and the explanatory variables were the same as those of the previous model: 1) Presence of AGs in the gap; 2) presence of EFNs in the plant and 3) interaction between the two variables. Following the analysis described above, we grouped the gaps and plants as random effects, nested within the pair of gaps (1 plant/forest gap/pair of gaps; Bates et al., 2015).

We compare the ant efficiency in removal herbivores of plant´s foliage between plants with and without EFNs growing in gaps with and without AGs by using a Survival Analysis, the "survival" package, available in program R version 3.2.5 (R Core Team, 2016). As response variable, the survival model we employed the attack event as a binomial response (attack or not) conjointly to the time interval the ants took to attack the termite. Also, the explanatory variables were as follows: 1) presence of AGs in the gap; 2) presence of EFNs in the plant and 3) interaction between the two variables. In all cases, when it was not statistically significant, the interaction between the explanatory variables was discarded of the final model. All analyses were performed in program R, version 3.2.5 (R Core Team, 2016).

3 | RESULTS

3.1 | Herbivory

The foliar area removed by herbivores did differ among non-EFN-bearing plants growing in forest gaps with or without AGs. However, the area removed in EFN-bearing plants was onefold less than plants without EFNs (Table 1, Figure 2a, b). We found an interaction between the presence of AGs and the presence of the EFNs in the percentage of removed area by herbivores (GLMM, $Z = -2.43$; $p = .015$, Table 1). So, the presence of EFNs in deterring herbivory seems to be stronger on plants growing in gaps harboring AGs (4.86 ± 12.58) than in gaps without AGs (6.70 ± 15.28) (Figure 2b).

The incidence of herbivore damage on leaf tissues shows similar tendencies to the area removed by herbivores mentioned above: Plants growing in a gap that harbors AGs presented few leaves with herbivory marks. Also, plants with EFNs were less attacked (GLMM, $Z = 2.413$; $p = .0158$, Table 1, Figure 2c,d). In this case, there is no interaction between the factors (Figure 2d).

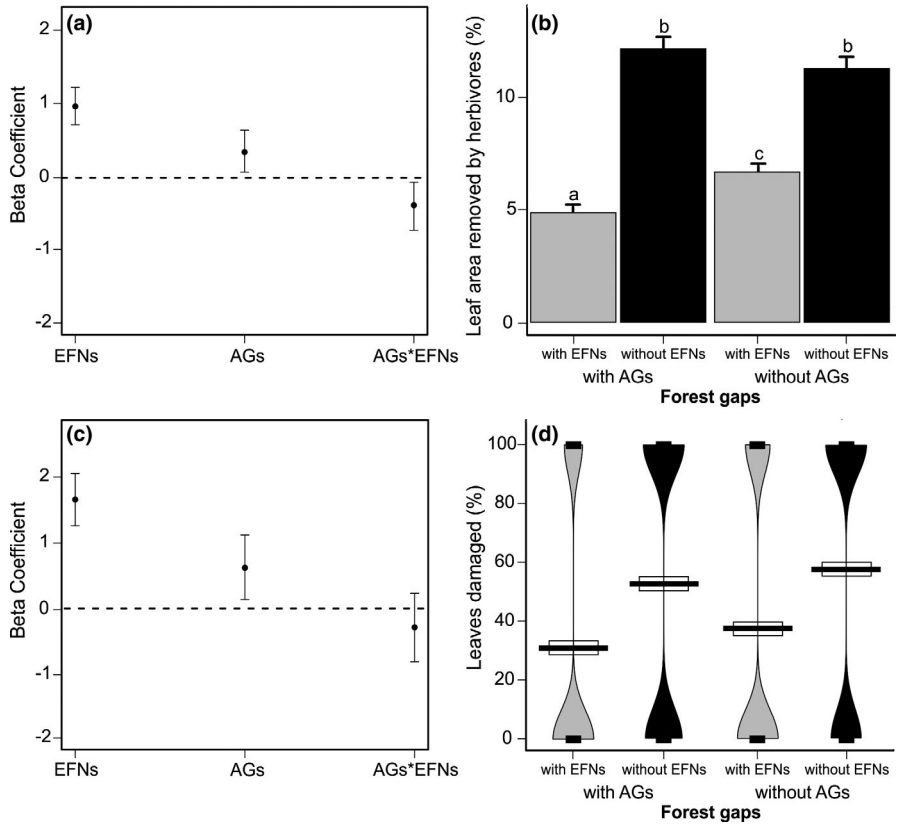
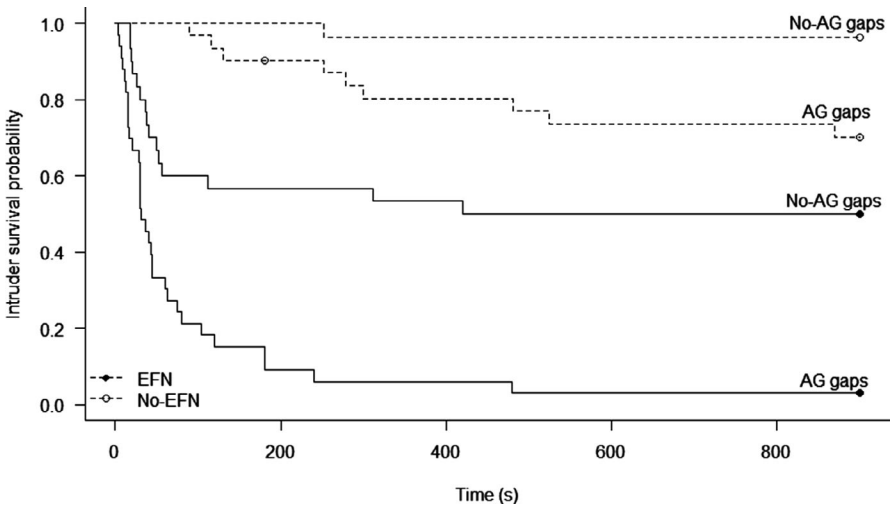


FIGURE 2 Effects of parabiotic ant gardens (AGs) and presence of extrafloral nectaries (EFNs) on leaf area removed by herbivores (a-b) and incidence of leaves damage (c-d) on plants growing in Amazon tree-fall gaps. The coefficients of GLMM for both, the (a) percentage of leaf area removed and; c) the probability of leaf damaged, modelled by the presence of EFNs in the plant, the presence of AGs in the gap the plant was growing and the interaction between these two factors (AGs*EFNs). The bars in the graph (b) represent the mean values (+ standard error) of percentage of area removed by herbivores. Different letters embedded in the figures (b) means statistical difference in a posteriori contrast analysis. In the graph (d), the points represents observed values of the incidence of damage to each leave and to each studied plant. The width of the shaded area in (d) corresponds to the frequency of points on presenting "1" (or not presenting "0") incidence of herbivore damage and the lateral bar brings the mean (black) and standard deviation (white) of frequency of leaf damage

FIGURE 3 Survival curves: The probability of an intruder (live termite bait pasted on the leaves) be attacked by ants in plants with extrafloral nectaries (EFNs) (solid line) or without EFNs (dashed line) when the plant is found in a gap with ant gardens (AGs) or without AGs. In a posteriori contrast analysis, all curves were different among each other



3.2 | Intruder survival

The bioassay results support the patterns observed in the herbivory evaluation: We observed that the proportion of non-attacked termites decreased faster and in a greater proportion in plants with EFNs (Deviance$_{1,118}$ = 55.5; p < .001, Figure 3). Also, the decrease was greater if such plant was growing close to AGs (Deviance$_{1,117}$ = 39.7; p < .001, Figure 3). There is no interaction between explanatory variables. In fact, the AG-ants were extremally aggressive and all experimental termite was found and attacked within 15 min when placed on

EFN plants growing close to AGs. In the only case where we did not observe any attack, the termite remained on the leaf for the 15 min. In plants of the site without AGs, just 50% of termites placed on plants with EFN were found and attacked by several different species of ants.

4 | DISCUSSION

Our findings demonstrate that the ants associated with AGs can extrapolate the well-known defense mechanisms against herbivores provided to AG-plants partners (*e.g.*, Davidson et al., 1990; Vicente et al., 2014) to the EFN-bearing plants growing in vicinities. On average, the composition of plant communities does not differ between forest gaps that have or do not have AGs, which suggests that AGs do not occur preferentially in gaps with specific vegetation and that herbivory patterns are not a product of plant composition. In the plants that grew in tree-fall gaps where ants (*C. femoratus* and *Cr. levior*) forage, a lower percentage of herbivory was observed when compared to plants in gaps where these parabiotic ants were absent. Also, our experiments demonstrated that both *C. femoratus* and *Cr. levior* are extremely efficient in detecting intruders from plants they are foraging for extrafloral nectar. In fact, the C/N balance in nectar composition (low in nitrogen and rich in carbon) makes ants more likely to prey upon other invertebrates and, consequently, to defend the plant against herbivores (Ness et al., 2009). Thus, as ants preferentially forage and defend plants that offer resources, herbivores that venture onto these plants may be found and predated upon by AGs ants.

Ant-Garden studies usually focused on the effect of occupant ants on the nest scale. Until now there are reports of the demonstration of the protective benefits against herbivores only for AG epiphytes, but not for plants outside the AGs. For instance, AG-ants, *C. femoratus* and *Cr. levior*, protect the epiphyte *Codonanthe calcarata* (Gesneriaceae) against herbivores, but apparently this benefit was not extended to their phorophytes (Vantaux et al., 2007). Additionally, Vicente et al., (2014) demonstrated the recruitment of *C. femoratus* when confronted with a chemical simulation of herbivory to some AG species, but not when chemicals from non-AG-plants were employed. In the tree-fall gaps studied here, the herbivory was lower in plants with EFNs, regardless of the presence of AGs nearby. This lower herbivory, even in the absence of AGs, was probably caused by other species of foraging ants, naturally associated with EFNs, as other *Crematogaster* and *Camponotus* species, or *Ectatomma* and *Azteca* species observed attacking termites on our bioassay. But both the observed herbivory and the time for ants to attack an intruder were even smaller in places where AGs were present. Both results together strongly suggest that AG-ants are better defenders of EFN-bearing plants than usual and the protection against herbivores could be a by-product of the abundance and the notorious aggressive behavior of these parabiotic ants (Davidson, 1988; Wilson, 1987).

The presence of ants on plants, attracted by EFNs, is widely recognized as a good defensive strategy to reduce the damage caused by herbivores (Barton, 1986; Bentley, 1976; Nascimento & Del Claro, 2010, Campos & Camacho, 2014). However, Zhang et al., (2015) compared the anti-herbivory strategies of ant-defended and chemically defended plants species and both obtained similar net results. Obviously, extrafloral nectar implies an energy cost for the plants that produce it (Heil, 2015). In this study, we did not have the energy expenditure in the production of nectar or in defensive chemical compounds. So, even though there was a significant drop from almost half, 12 to 6.7%, in the average leaf area consumed by herbivores between plants with and without EFN, this study does not have subsidies to support the effectiveness, in terms of costs and benefits, of production of extrafloral nectar. However, as herbivores can be vectors of infections (Nault, 1997), this decrease in both the percentage of area consumed and in the proportion of damaged leaves implies in a decrease in the probability of the plant be infected by some pathogen. This decrease should be more expressive when the plant meets both conditions: having EFN and growing in a gap hosting AGs.

When growing in a gap with AGs, we observed a mean drop of leaf area removed by herbivores from 6.7% to 4.8%, corresponding to mean reduction of about 2% in the herbivory. Even this value sounds apparently small, in comparative terms this reduction implies a relative decrease of about 28% in leaf area loss for plants with the same investment in defense, without any apparent increase in cost. The apparently low herbivory rates, usually caused by insects as observed in this study, are within the normal rates of the herbivory observed in plants growing in natural habitats (reviewed by Zhang, Zhang, & Mah, 2016). From the plant's point of view, the loss of photosynthetic active area may culminate in a decrease in fitness (Kozlov & Zvereva, 2017; Marquis, 1984). In fact, there are several examples in literature showing that even a small increase in herbivory rates implies in significant negative effects on reproduction and growth of plants (reviewed by Kozlov & Zvereva, 2017). For example, Lehndal & Âgren (2015) have shown that rates of herbivory of between 1% and 3% may affect the relative fecundity of *Lythrum salicaria* L. (Lythraceae) populations. The plants studied here are young trees, so the energy surplus obtained by the lower incident herbivory should be employed in increasing vegetative growth. In a transient environment, such as tree-fall gaps, where there is light competition for canopy establishment among treelets, rapid growth may be the difference between being suppressed by competitors or accessing the canopy (Dalgleish et al., 2015). It is particularly expressive for plants with EFN competing with non-EFN plants for light. Thus, forest gaps dominated by parabiotic ants can be considered a coevolutionary hotspot (Thompson, 1999), because the adaptive value of EFNs in ant-based mutualisms is markedly high in these sites.

5 | CONCLUSION

This study demonstrated that two species of parabiotic ants, *C. femoratus* and *Cr. levior*, which inhabit AGs in Amazonian gaps may offer benefits by attacking intruders and reducing the leaf area removed by herbivores, especially for plants that have EFNs. Considering that EFN-plants growing in AG-gaps will suffer lower herbivory over time, they may have a competitive advantage by growing faster and

shading the others. A slight increase in the speed of growth is an expressive advantage, mainly in a transient environment, as is the case of gaps. The effect of this possible key role of AG-ants in a selective modification on the community favoring the persistence of plants with EFN in the canopy still needs to be clarified in future studies. Since this ant–plant system studied here is widely geographically distributed in the Amazon its effect on the regeneration and survival of tree species growing in tree-fall gaps can be determinant on plant diversity in a broader scale.

6 | AUTHOR´S CONTRIBUTIONS

CBS and TJI delineated, analyzed the data and wrote the text. REV collected the data, wrote and reviewed the text. LAK delineated, wrote and reviewed the text.

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DATA AVAILABILITY STATEMENT

Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.h9w0vt4gc (da SilvaViana et al., 2020), and the PPBIO repository: https://ppbio.inpa.gov.br/repositorio/dados

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