RESEARCH ARTICLE

Variation in the production of plant tissues bearing extrafloral nectaries explains temporal patterns of ant attendance in Amazonian understorey plants

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Abstract

1. Information on direct and indirect drivers of temporal variation in ant–plant interactions is scarce, compromising our ability to predict the functioning of these ecologically important interactions.

2. We investigated the roles of precipitation, ant activity, abundance of young plant tissues bearing extrafloral nectaries (EFNs) and EFN phenotypes in the establishment of EFN-mediated ant–plant interactions throughout the year in Amazonia, Brazil. We hypothesized that the frequency of ant–plant interactions follows a predictable seasonal pattern, being higher in wetter periods, during which plants invest more in the production of new plant tissues bearing EFNs, ultimately promoting ant attendance. We surveyed and tagged every understorey Bignonieae plant rooted inside 28,500-m^2 plots, and recorded ant–EFN interactions on each plant five times throughout the year. We also sampled ants with honey baits to estimate temporal variation in general ant activity.

3. Contrary to our hypothesis, the proportion of plants tended by ants in each plot was higher in drier, not wetter, months. Ant attendance was indirectly and negatively related to precipitation, which was attributed to a decrease in the proportion of plants producing new EFN-bearing plant tissues during the wetter period. Additionally, seasonal variation in an ant activity did not explain temporal patterns of plant attendance. At the plant level, ant attendance increased strongly with the number of recently formed shoot nodes, and ants almost never attended plants with limited or no young tissue. Among the 12 most abundant Bignonieae species, the amount of young tissue was the most important predictor of ant attendance, secondarily explained by the EFN secretory area.

4. *Synthesis*. Our results suggest that seasonal variation in the production of new plant tissues bearing EFNs is the primary driver of the temporal patterns of EFN–plant

attendance by ants in this system. Contrary to our expectations, production of new plant tissue is higher in the drier months of the year, which in turn boosts the frequency of interactions between ants and EFN-bearing plants in the dry season. These results highlight the role of plant phenology in the remarkable variation encountered in ant visitation to EFN-bearing plants in both space and time.

KEYWORDS

ant–plant interactions, ecological interactions, herbivory, indirect defence, mutualism, plant–herbivore interactions, tropical rainforest

1 | INTRODUCTION

In recent decades, it has become increasingly clear that mutualistic interactions are abundant in all ecosystems on Earth, yet highly variable across time and space (Bronstein, 2015). Several studies have highlighted remarkable variation in the frequency of interactions between species pairs in different habitats, both within and across years (e.g. Burns, 2004; Carnicer, Jordano, & Melián, 2009; Di Giusto, Marie-Charlotte, Dounias, & McKey, 2001; Heath, Stock, & Stinchcombe, 2010; Horvitz & Schemske, 1990; Olesen, Bascompte, Elberling, & Jordano, 2008). The sign and magnitude of the outcome of such interactions also vary widely over spatial and temporal gradients (Chamberlain, Bronstein, & Rudgers, 2014; Hoeksema & Bruna, 2015). Identifying factors driving such variation is a fundamental step towards an improved understanding of the ecological and evolutionary processes that shape mutualisms. However, most studies have focused on the spatial drivers of such variability while relatively neglecting the role of the temporal drivers, compromising our general understanding of how these interactions function at different scales.

Interactions between ants and plants bearing extrafloral nectaries (EFNs) are good examples of mutualistic interactions that vary across both time and space (Bronstein, 1998; Rico-Gray & Oliveira, 2007). In such interactions, plants secrete nectar from EFNs, glands located outside of flowers that are highly attractive to ants. These ants forage on the plants, repelling or consuming potential herbivores (Marazzi, Bronstein, & Koptur, 2013). The most active EFNs are found on younger plant tissues (Bentley, 1977; Heil, Fiala, Baumann, & Linsenmair, 2000; Rico-Gray & Oliveira, 2007). Plant investment in the production of extrafloral nectar generally decreases as EFNs age (Bixenmann, Coley, & Kursar, 2013; Calixto, Lange, & Del-Claro, 2015). Extrafloral nectaries eventually cease to secrete as they senesce (Dreisig, 2000), with toughness playing a key role in plant protection in older and mature tissues (Calixto et al., 2015; Coley & Barone, 1996). As the production of new plant tissues occurs in brief intervals throughout the year (Fenner, 1998), we should expect ant–plant interactions to vary over time in response to plant phenology (Vilela, Torezan-Silingardi, & Del-Claro, 2014). Indeed, ecologists have long used the age of plant parts as a criterion for when to study ant–EFN interactions (e.g. Di Giusto et al., 2001; Nogueira, Rey, Alcántara, Feitosa, & Lohmann, 2015).

In tropical forests world-wide, temporal patterns of water uptake determine biological cycles of many organisms, including plants (Chaves et al., 2002; Zeppel, Wilks, & Lewis, 2014). Temporal patterns of water uptake may also predict temporal patterns of interaction between ants and plants bearing EFNs, since water availability could lead to temporal variation in the production of new plant tissues, including the initiation of EFNs on the youngest plant parts (Wäckers & Bonifay, 2004). The frequency of ant–plant interactions mediated by EFNs has in fact been reported to be higher in wet than dry seasons (Belchior, Sendoya, & Del-Claro, 2016; Días-Castelazo & Rico Gray, 2004; Rico-Gray, 1993). Although seasonal variation in water uptake may be responsible for the higher frequency of EFN mediated ant–plant interactions, other proximate causes are also possible. In particular, the establishment of ant–plant interactions appears to be related to variation in plant resource availability and ant densities (Holland, Ness, Boyle, & Bronstein, 2005). Therefore, it is possible that the production of new EFN-bearing plant tissue, and, complementarily, local ant densities (Dáttilo et al., 2015), may be regulating temporal patterns of ant–plant–herbivore interactions in tropical ecosystems, rather than water availability per se.

Here, we investigated temporal patterns of ant–plant interactions and their direct and indirect drivers at a site within the Amazon rainforest. We hypothesized that ant attendance on EFNs would follow a predictable temporal pattern, being higher during wetter periods when plants invest more resources in the production of new tissues. At the community level, such an increase in ant visitation to EFNs might be driven either by an increase in EFN availability or by an increase in the local ant activity over time. Hence, we explicitly evaluated these alternatives. If the availability of young EFN-bearing tissues predicts patterns of ant–plant interactions at the community level, we also hypothesized that at the plant level, the probability of an individual plant being attended by ants would also be explained by the abundance of young tissues bearing active EFNs. To evaluate our hypotheses, we used as a model the most abundant and diverse clade of Bignoniaceae, the Neotropical lianas belonging to the tribe Bignonieae (Lohmann, 2006). Bignonieae bear nectar-secreting trichomes on younger plant tissues that function as EFNs (Nogueira, Guimarães, Machado, & Lohmann, 2012; Nogueira, Rey & Lohmann, 2012) and that are highly variable in abundance across species (Nogueira, Ottra, Guimarães,

Machado, & Lohmann, 2013). The high frequency of Bignonieae plants in Neotropical rainforests and the large variation in number and distribution of its EFNs make this plant group an ideal system for exploring potential mechanisms driving the functioning of ant–EFN plant interactions as a whole. Because of the quantitative variation in EFNs across Bignonieae species, we also explicitly evaluated if species bearing a higher secretory area of EFNs have a higher probability of being tended by ants, especially the dominant ant species that rapidly recruit workers on plant resources (Parr, 2008). Most studies have focused on the spatial drivers rather than the temporal drivers determining ant–plant interactions. Consequently, this work advances our understanding of the temporal variation in these interactions, explicitly considering plant phenology and local ant availability over time.

2 | MATERIALS AND METHODS

2.1 | Study site

We carried out this study at the Reserva Florestal Adolpho Ducke (Ducke Reserve), located 26 Km northwest of Manaus, Amazonas State (Brazil). This site is characterized by a mean annual temperature of ~26°C and an annual rainfall of 2,400 mm (Ribeiro et al., 1999). The peak of the wet season is January–March, when rainfall averages >300 mm per month; the peak of the dry season is July–September, when rainfall averages <100 mm per month (Marques-Filho, Ribeiro, Santos, & Santos, 1981). The Ducke Reserve is covered by a *terra-firme* tropical rainforest with vegetation characterized by a closed canopy and diverse understorey species with abundant subterranean-stemmed palms. The soils are a continuum from clayey latosols on the ridges becoming sandier as the altitude decreases (Chauvel, Lucas, & Boulet, 1987).

2.2 | Plant system and young tissues bearing secretory extrafloral nectaries

We choose the Bignonieae, a species-rich lineage of neotropical lianas that bear EFNs on vegetative plant parts (Lohmann, 2006), as our focal taxon. EFNs of Bignoniae are known to attract ants capable of excluding herbivores on plants (Nogueira, Guimarães, et al., 2012; Nogueira et al., 2015). Their EFNs are nectar-secreting trichomes derived from the differentiation of epidermal cells after primary growth. They occur in clusters on the interpetiolar region of stems, in the prophylls of axillary buds, and leaflet bases (Nogueira, Rey, et al., 2012; Seibert, 1948; Figure 1). In the field, clusters of active EFNs can be recognized as yellowish-green and turgid structures secreting crystalline and viscous nectar (Nogueira et al., 2013; Nogueira, Rey, et al., 2012).

Although EFN structure varies among vegetative plant parts across Bignonieae species, EFNs are generally organized into subunits or modules composed of a recently formed shoot node with four tiny prophylls and two opposite leaves (Nogueira, Rey, et al., 2012). The number of new modules can easily be determined in the field, and is

a good descriptor of the amount of young above-ground plant tissue within each individual. The young tissues are bright green, recently formed shoots that lack secondary tissues and that have incompletely expanded leaves (Nogueira, Guimarães, et al., 2012; Nogueira et al., 2015; Nogueira, Rey, et al., 2012; Figure 1). Therefore, individual plants can be described as having zero to multiple new modules of young tissues, depending on the phenological stage of the plant. Consequently, variation in EFN availability among plants can be generated by variation in the production of new modules per plant (the amount of young tissues) or by variation in the number/area of EFNs per module (see Nogueira et al., 2013; Nogueira, Rey, et al., 2012 for detailed morphological descriptions of EFNs of 108 Bignonieae species). As our general hypothesis predicts that availability of EFNs is one of the main factors driving the seasonality of the ant–plant interactions, we explicitly explored both aspects of plant phenotype, investigating the impact of the amount of young tissues and the secretory area of EFNs in each recently formed shoot node on ant–plant interactions over time.

2.3 | Sampling design and plant descriptors

We systematically established 28 plots over a 9 km^2 grid, formed by long trails crossing north-south and west-east within the Ducke Reserve. Plots were 500 m^2 (50 × 10 m) and located at least 500 m from each other. We surveyed and tagged individual plants of Bignonieae rooted inside the plot whose diameter at 30 cm from the rooting point (D_{30cm}) was ≥0.5 cm and whose vertical height was ≤3 m. Our sampling only included juvenile, non-reproductive, understorey individuals. We focused on this life stage because this is when plants tend to be most visited by herbivores (A. Nogueira, pers. obs.) and least tolerant of herbivore damage (Boege, Dirzo, Siemens, & Brown, 2007; Ochoa-López, Villamil, Zedillo-Avelleyra, & Boege, 2015), and when they exhibit the strongest responses to counter herbivory. Plants >3 m in height represented <5% of Bignonieae individuals per plot. These individuals were either reproductive lianas that access the canopy, or very large trees that extend well above the canopy.

We carried out five surveys on each tagged plant in each plot: October 2012 (Survey 1), November-December 2012 (Surveys 2 and 3), February 2013 (Survey 4) and May 2013 (Survey 5). In Survey 1, we recorded D_{30cm}, length of the main stem, number of leaves and number of recently formed shoot nodes of each tagged plant. The D_{30cm} of each plant and the length of the main plant stem were measured with callipers and a tape measure respectively. In subsequent Surveys 2, 3 and 4, we also measured the number of recently formed shoot nodes in each tagged plant.

2.4 | Ant sampling

Before characterizing the abundance of ants attending EFNs, we first checked whether diurnal observations alone could be a good

FIGURE 1 Ant–plant interactions on young EFN-bearing tissues in different Bignonieae species. Red and yellow arrows highlight ant attendance and nectar secretion on young tissues respectively. (a) Sprouting branch with young tissues of a typical Bignonieae liana in the Amazon rainforest with the first, second and third recently formed shoot node (plant modules described in the Methods). (b) Third recently formed shoot node with *Camponotus* ants on active EFNs. (c) Typical EFN cluster on young tissues accumulating nectar on the interpetiolar region of the stem (one EFN cluster in each plant side totalling two clusters per shoot node). (d) *Pachyptera aromatica* with active EFNs clustered in the interpetiolar region on recently formed shoot nodes attended by *Crematogaster* ants. (e, f) *Anemopaegma robustum* with active EFNs clustered in prophylls and young leaflets on recently formed shoot attended by *Camponotus* and *Crematogaster* ants respectively. (g, h) *Adenocalymma moringifolium* with EFNs clustered in the prophylls on recently formed shoot attended by *Solenopsis* ants. (i) *Bignonia prieurii* with active EFNs clustered in the prophylls on recently formed shoot nodes attended by *Crematogaster* ants

proxy of 24-hr ant visitation patterns. On a subset of 20 randomly selected plants, we performed ant censuses between 08:00 a.m. to 10:00 p.m. Approximately 80% of the plants had the same ant species attending the EFNs during the day and night (Table S1). Therefore, we performed our censuses on all tagged plants from each plot between 8:00 a.m. and 5:00 p.m. on each of the five censuses.

During each of the five surveys, we inspected each tagged plant for 10 min to identify whether ants were visiting the active EFNs. On very small plants (≤10 leaves) we reduced the period of observation by 5 min, as that was sufficient to count all ants visiting the EFNs on the entire plant. We counted the total number of ants patrolling the entire plant in Surveys 2–5, but not in Survey 1, in which only presence–absence data were collected. Due to differences in size, recruitment and identity of ants, we used only presence–absence data to establish differences in the patterns of ant attendance among plants (Baker-Méio & Marquis, 2012). As this binary variable has low sensitivity to variations in nectar production (e.g. induced response of nectar production to herbivory) or to ant recruitment behaviour, it provided us a more standardized way of measuring patterns of ant attendance over time among Bignonieae species.

In Surveys 2–5, we used honey baits to estimate ant activity on each plot over time. To accomplish this, we added ~1 ml of 50% water-diluted honey in baits available in 2 ml Eppendorf tubes positioned on the most apical portion of the stem, near the leaves, at a height of 0.6–1.5 m, depending on the plant height. This procedure allowed us to estimate local ant activity with a standardized resource independent of the natural availability of extrafloral nectar. We systematically distributed 10 honey baits (one per plant) within each plot. We placed one bait on the surface of five randomly chosen Bignonieae plants lacking young tissues with active EFNs, and on the surface of the closest five non-Bignonieae species lacking EFNs. We did not observe differences in the patterns of ant attendance of baits located on Bignonieae and non-Bignonieae plants (Figure S1). Hence, we did not consider these baits separately in our analyses. We also inspected the baited plants to make sure that there were no other sugar resources that could interfere with ant activity (e.g. honeydew-secreting insects). We collected all ants visiting the baits after 80–90 min of observation. Subsequently, all ants from surveys and baits were counted and identified in the laboratory. Ant species were classified as dominant or subordinate following Baccaro, Ketelhut, and Morais (2010) in order to assess the potential quality of anti-herbivore defence provided by the ants. Dominant ant species rapidly recruit workers that can displace other ant species from resources, allowing them to monopolize food sources (Parr, 2008).

These species tend to be more aggressive towards herbivores (Xu & Cheng, 2010).

2.5 | Statistical analyses

To determine the period of the year in which understorey ant–plant interactions are most frequent, we performed a GLMM with the proportion of tagged plants attended by ants in each plot as our response variable and the survey month as the fixed categorical factor. We also explored the temporal pattern of young tissue production and of ant activity using additional GLMMs. Response variables in those cases were the proportion of plants with young tissues and the proportion of honey baits occupied by ants in each survey respectively. In all analyses, we used the binomial error distribution in which the response variables were treated as the number of failures (i.e. plants not attended by ants) and successes (i.e. plants attended by ants) as a two-vector response variable, as suggested by Crawley (2007). Plot identity was included in the models as a random variable. Temporal patterns would be consistent with our hypothesis if periods of higher ant attendance to EFNs matched wetter periods, when plants invest more in the production of new tissues bearing EFN.

Since survey date cannot be considered the causal factor explaining variation in ant–plant interactions throughout the year, we also constructed a structural model expressed as a direct acyclic graph to relate our three variables (proportion of plants with young tissues, proportion of honey baits occupied by ants, and proportion of plants tended by ants per plot at each survey date) to seasonal variation in precipitation. The multivariate causal model consistent with our hypothesis incorporates precipitation, potentially determining local ant availability, and/or the number of plants producing new tissues but does not directly incorporate ant–plant interactions (Figure S2, upper panel). Precipitation was expressed as the proportion of days with rain in the month before each survey. We used this measure of water availability because it more accurately describes how rainfall is distributed over the days before each sampling than measures of total accumulation of rainfall. Therefore, in our model the effect of precipitation on ant attendance across the plots was mediated by the variation in the proportion of plants with young tissues, variation in the proportion of baits occupied by ants, or both.

Our structural model was an alternative confirmatory path analysis consisting of a generalization of Shipley's d-sep test that could incorporate hierarchical structures (e.g. resampling throughout time) and

variables with different sampling distributions (Shipley, 2009). Path analyses were performed in five steps, as suggested by Shipley (2009): (a) we constructed hypothetical causal relationships between variables in the form of a directed acyclic graph; (b) we listed each pair of variables without a direct link on the acyclic graph; (c) we defined a full set of the k independence claims [i.e. (Xi, Xj)|{Z}] that formed the basis set B_U; (d) for each element in this basic set, we obtained the probability, p_k, that the pair (Xi, Xj) is statistically independent conditional on the variables {Z}; finally, (e) we combined the k probabilities (p_i) using the C equation. The C value was compared to a chi-squared distribution with $2k$ df (Figure S2). We rejected the causal models whenever the C value was unlikely to have occurred by chance. After confirming or rejecting the hypothetical causal model, the path coefficients were obtained by regressing each variable within each of its direct causes, as detailed in Shipley (2009).

Considering each plant individually, we also asked if plants with a higher number of recently formed shoot nodes (and thus more young tissues per plant) had a higher probability of ant attendance. We performed a GLMM with a binomial distribution (logit link function) including all individual plants together. We modelled the presence/absence of ants on EFNs as the response variable, including the number of recently formed shoot nodes per plant and surveys as fixed factors. Given that the interaction between the two fixed factors did not explain the probability of ant attendance, we only maintained the additive effect of each predictive factor in this analysis. We also included the identity of the species and 'individual plants within plots' as random terms in this model. The latter variable explicitly described two aspects of our sampling design: the spatial aggregation of plants per plot and the repeated measurements of plants across surveys. Pseudo-R^2 (Maddala, 1983) was used as a global measure of fit of all logistic models (reviewed in Hu, Shao, & Pauta, 2006).

In the second set of analyses, to test whether the probability of ant attendance as a function of the number of recently formed shoot nodes varies across Bignonieae species, we applied a similar GLMM model, adding the categorical variable plant species as fixed factors jointly with the number of recently formed shoot nodes. After 10,000 iterations the model did not converge when the interaction term between the two fixed factors was included. Therefore, we only maintained the additive effect of each predictive factor in our model. In addition, we included the individual plants within plots as a random variable in the model. In these analyses, we only included the 12 species with more than 10 individuals across all 28 plots. Alternatively, we replaced the identity of the plant species in the GLMM by a mean descriptor of the EFN phenotype of each species as a fixed factor. In this case, we quantitatively described differences in EFNs per shoot node among species characterizing the total secretory area of EFNs (mm^2) in at least five individual plants per Bignonieae species. First, we estimated the number and size of EFNs in each cluster and the number of clusters per shoot node (see Nogueira, Rey, et al., 2012 for details about clustered EFNs in Bignonieae species). Then, we multiplied the total area occupied by each EFN by the number of EFNs in one cluster and added up the total secretory area by the number of clusters per shoot node (Table S2).

To explicitly evaluate how the young tissue availability is related to ant attendance in each species, we also performed individual analyses for each of the 12 most abundant Bignonieae species, using the same GLMM models described above (binomial distribution and logit link function). Among them, four species produced only a single recently formed shoot node at a time, showing low variation in the abundance of young tissues among individuals, which compromised the fitting of the GLM models in these cases.

All statistical analyses were performed in R 3.4 (R Development Core Team, 2009) with standard and additional packages, as follows: GLMMADMB (Bolker, Skaug, Magnusson, & Nielsen, 2012), LME4 (Bates et al., 2016), NLME (Pinheiro et al., 2016), VEGAN (Oksanen et al., 2016), MASS (Ripley et al., 2015) and GLMMML (Broström, 2018).

3 | RESULTS

We tagged 431 understorey plants from 22 species of Bignonieae across 28 plots (3–31 individuals per plot) (Table S3). Averaging across surveys, 10 ± 5% (mean ± SD) of the tagged plants were attended by ants in each plot, reaching a maximum of 14% in the October survey. A total of 33 species of ants distributed among 19 genera were recorded. Overall, 46.5% (range 39%–61% across surveys) of Bignonieae plants and 33% (29%–38% across surveys) of the ant-occupied honey baits were monopolized by six dominant ant species: *Crematogaster brasiliensis*, *Crematogaster limata*, *Crematogaster tenuicula*, *Ochetomyrmex semipolitus*, *Pheidole biconstricta* and *Wasmannia auropunctata*.

3.1 | Seasonal patterns of ant–plant interactions and the role of young plant tissues bearing EFNs

The proportion of plants attended by ants changed over time (N_{plots} = 28; pseudo-R^2 = .15; F = 22.6; p < .001), showing a sharp decline during the peak of the wet season (Figure 2). Similarly, the proportion of plants with recently formed shoot nodes (young tissue) was different between surveys (N_{plots} = 28; pseudo-R^2 = .07; F = 13.1; p < .001), lower during the peak of the wet season (February: 17 ± 8%) compared to all drier months (42 ± 20%; Table 1). The proportion of baits occupied by ants over the survey period exhibited a similar pattern (N_{plots} = 28; pseudo-R^2 = .12; F = 13.4; p < .001), with the proportion of baits occupied by ants being lower at the peak of the wet season (February: 30 ± 15%) compared to drier months (52 ± 8%; Table 1).

In the confirmatory path analysis, we did not reject our general causal model (C statistic = 7.46; p = .12; Figure S2): precipitation had an indirect and negative relationship with ant–plant interactions at the plot level, mediated by the decrease in the proportion of plants with young tissues in wetter periods (Figure 3; Table S5). Precipitation before each survey was negatively related to the proportion of plants with young tissues (N_{plots} = 28; Z = −6.96; p < .001)
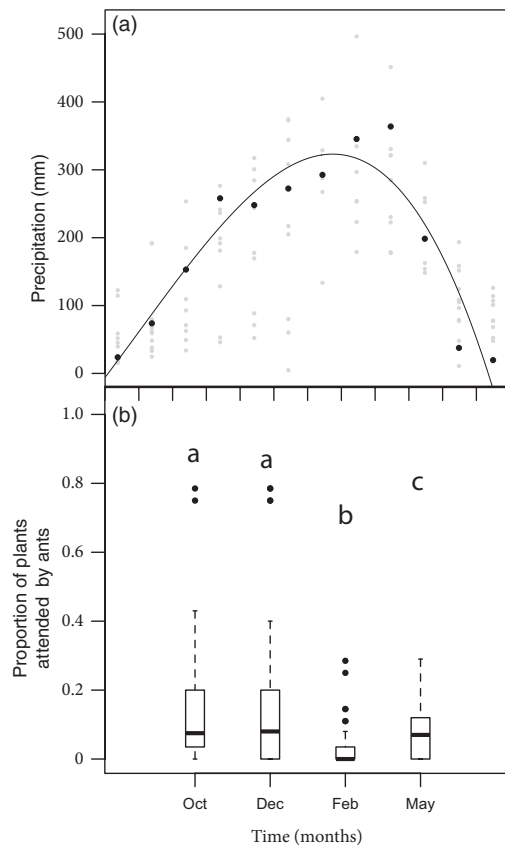
FIGURE 2 Temporal variation in precipitation (mm) and ant–plant interactions per plot from August 2012 to July 2013. (a) Monthly precipitation; dots in grey represent the monthly precipitation between 2008 and 2011 (data from INMET); in black, the third order polynomial describing the precipitation tendency of the current year under study. (b) Proportion of plants attended by ants showing higher average values in the dry season and smaller values in the wet season. Statistical details of the second graph are available in the last line of Table 1

and to the proportion of baits occupied by ants (N_{plots} = 28; Z = −4.18; p < .001; Figure 3; Tables S4 and S5). On average, plots with a higher proportion of plants with young tissues had a higher proportion of plants attended by ants (Z = 8.73; p < .001). In contrast, the proportion of baits occupied by ants was unrelated to the proportion of plants attended by ants in each plot (N_{plots} = 28; Z = 1.89; p = .064; Figure 4).

3.2 | Variation in the amount of young plant tissues bearing EFNs explaining ant attendance

The probability of a plant being attended by ants was positively related to its quantity of young tissues (solid line in Figure 5; N_{plants} = 454; pseudo-R^2 = .43; Z = 7.4; p < .001). This relationship varied marginally across surveys (different dashed lines in Figure 5; Survey effect: Z = −0.6; p = .318). In general, plants with two or more recently formed shoot nodes were more likely to be attended by ants than were plants bearing only one recently formed shoot node. Plants with shoots at the initial stage of elongation (i.e. possessing very little young tissue) exhibited more variable patterns of ant attendance, whereas plants lacking young tissues bearing EFNs were almost never occupied by ants (<1%; Figure 5).

Although ant attendance was positively related to the number of recently formed shoot nodes, this relationship differed across Bignonieae species (N_{plants} = 398; pseudo-R^2 = .48—Table 2, first model; Figure S4). Species with higher EFN secretory area per node had a higher probability of being attended by ants than did species with smaller secretory areas (N_{plants} = 398; pseudo-R^2 = .45—Table 2, second model; Figure S3). However, more of the variation in the occurrence of ant–plant interactions across Bignonieae species was explained by the quantity of young tissues than by the EFN secretory area per shoot node (Figure S3; Table 2). In six species (four species

TABLE 1 Summary of the multiple comparisons for the GLMM models evaluating the relationship between the proportion of plants with young tissues, ant-occupied baits, and plants attended by ants over time. We performed the GLMMs using binomial error distribution and plots identity as a random variable. Response variables were treated as the number of failures (e.g. plants without) and successes (e.g. plants with young tissues) in each logistic model. Values in bold were considered statistically significant
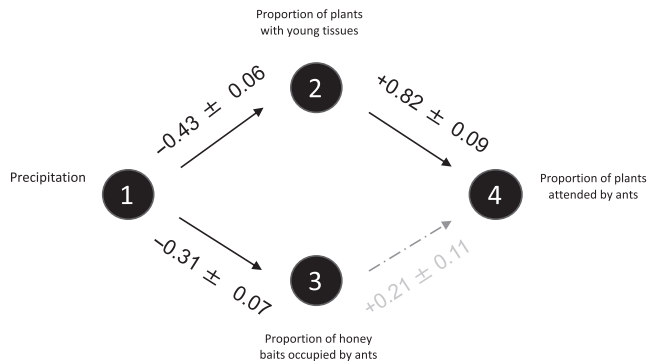
| Response variable (error distribution)	Fixed categorical factor in GLMM		Estimated coefficient ± SE	z	p
Proportion of plants with young tissues (binomial)	Time	October–December	−0.21 ± 0.14	−1.50	.426
		October–February	−1.70 ± 0.22	−7.72	**<.001**
		October–May	−0.64 ± 0.17	−3.78	**<.001**
		December–February	−1.49 ± 0.21	−7.22	**<.001**
		December–May	−0.44 ± 0.15	−2.85	**.021**
		February–May	−1.05 ± 0.23	−4.57	**<.001**
Proportion of ant baits occupied (binomial)	Time	December–February	−0.93 ± 0.19	−5.00	**<.001**
		December–May	−0.54 ± 0.18	−3.01	**.007**
		February–May	−0.39 ± 0.19	−2.07	.09
Proportion of plants attended by ants (binomial)	Time	October–December	0.15 ± 0.18	0.80	.89
		October–February	−1.73 ± 0.34	−5.09	**<.001**
		October–May	−0.61 ± 0.24	−2.51	.053
		December–February	−1.88 ± 0.32	−5.83	**<.001**
		December–May	−0.75 ± 0.21	−3.51	**.002**
		February–May	−1.13 ± 0.39	−3.14	**.008**

FIGURE 3 Confirmatory path analysis testing the direct and indirect effect of precipitation on the proportion of plants with young tissues, ants on honey baits and plants attended by ants. Precipitation directly decreases the number of plants with young tissues and the number of honey baits occupied by ants (ant availability). Furthermore, precipitation indirectly decreases ant attendance on EFNs mediated by the local change in the availability of plants with young tissues. Solid lines indicate standardized coefficients different than zero with $p \leq .05$. Dashed lines indicate standardized coefficients with $p \leq .10$. The complete statistics of each path coefficient are detailed in Table S4 and the causal hypothesis expressed as a directed acyclic graph is available in Figure S2

of *Adenocalymma*, one species of *Fridericia*, and *Callichlamys latifolia*), ant attendance was not predicted by the number of recently formed shoot nodes on the plants (Figure S4). On average, these species had less young tissue and a smaller EFN secretory area per node, characterizing a group of plants that were almost never attended by ants. The exception was *Adenocalymma tanaeciicarpum*, which had a large secretory area, but few recently formed shoot nodes. In contrast, in the other six species (three species of *Adenocalymma*, one *Anemopaegma*, one *Fridericia* and one *Pachyptera*), an ant attendance was positively related to the number of recently formed shoot nodes (Figure S4; Table 2). On average, these species had the highest number of recently formed shoot nodes per plant and a higher EFN secretory area in each shoot node. We detected no correlation between the amount of young tissue per plant and the secretory area of EFNs in each shoot node estimated per species ($r = .012$; $t = 0.47$; $p = .64$).

4 | DISCUSSION

Our results suggest that the proportion of plants attended by ants in the understorey at one tropical rainforest site is indirectly and negatively related to the precipitation over time. This pattern is the opposite of our expectation and was primarily explained by seasonal differences in the availability of young EFN-bearing tissues, rather than by variation in the local activity of ants at EFNs. Likewise, differences in the number of recently formed shoot nodes (young tissues) at the plant level were the primary driver of variation in the probability of ant attendance among individual plants and species. Variation
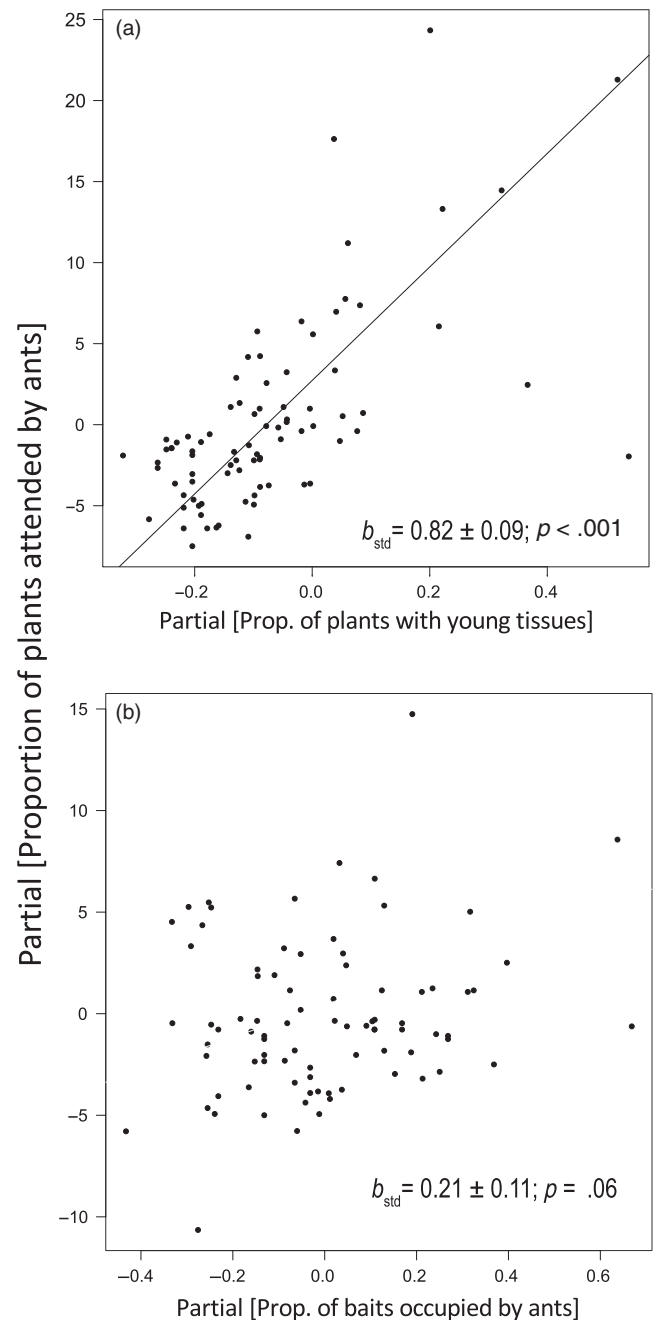
FIGURE 4 Partial regression describing the relationship between the proportion of plants attended by ants and either (a) the proportion of plants with young tissues and (b) the proportion of honey baits occupied by ants. Plots with a higher number of plants with young tissues had more plants attended by ants, while ant availability, assessed here with the experimental addition of honey baits in the field, was unrelated to plant attendance. Partial regression plots show the expected effect of a variable when the other variable in the model are statistically held constant. The model applied here is detailed in the Table S5 (third line)

in the EFN secretory area per plant node secondarily explained patterns of ant attendance across Bignonieae species. Our results reinforce the greater importance of plant phenology and the availability of new plant tissues than the EFN phenotype per se. Below, we discuss the importance of young EFN-bearing tissues for the temporal
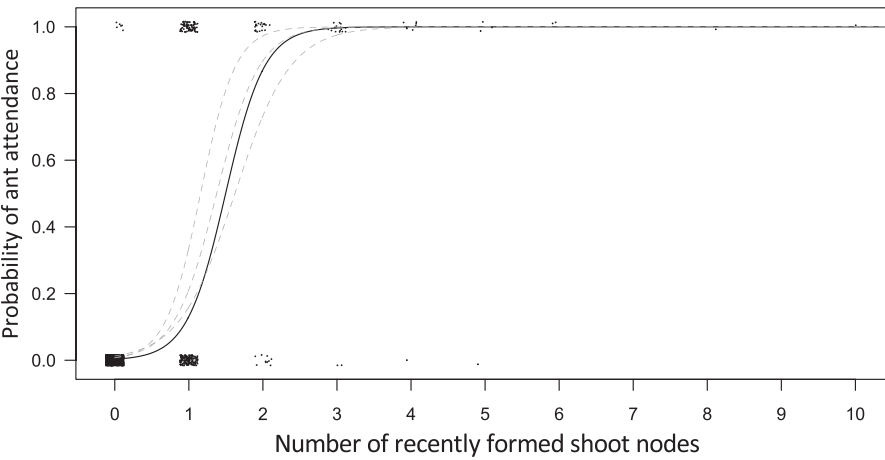
FIGURE 5 Predicted probabilities of ant attendance and the number of recently formed shoot nodes (the amount of young tissues) per plant. Continuous black line describes the general positive relationship between both variables without explicitly considering each sampling period or plant species. Dashed lines describe marginal variations in this relationship between surveys. Statistics are detailed in Table 2

TABLE 2 Summary of the GLMM testing the relationship between the number of recently formed shoot nodes (the amount of young tissues) in the plants and the probability of plant attendance by ants in Bignonieae. The two first models considered all individual plants and surveys, including the number of recently formed shoot nodes and plant species as fixed factors, and the occurrence of ants on plants as the binary response variable. We had five species not represented below in which GLMM was prohibited because the excess of zeros in the response variable (*A. adenophorum, A. bracteosum, A. flaviflorum, C. latifolia* and *F. nigrescens*). N = number of observations (multiple measurements per plant). Graphical representation of the relationship between number of recently formed shoot nodes and the probability of plant attendance by ants per Bignonieae species can be viewed in Figure S4. Values in bold were considered statistically significant

Plant species	Random variables	Fixed factors	Estimated coefficient ± *SE*	*z*	*N*	*p*
All dataset (first model)	Plot: individuals	Number of recently formed shoot nodes	2.7 ± 0.3	9.7	1,592	**<.001**
		Species	–	–	1,592	**<.001**
All dataset (second model)	Plot: individuals	Number of recently formed shoot nodes	4.6 ± 0.6	8.2	1,592	**<.001**
		Secretory area of EFNs	0.1 ± 0.1	2.9	1,592	**.004**
Adenocalymma longilineum	Plot: individuals	Number of recently formed shoot nodes	5.9 ± 1.3	4.6	357	**<.001**
Adenocalymma moringifolium	Plot: individuals	Number of recently formed shoot nodes	4.1 ± 1.3	3.2	436	**.002**
Adenocalymma tanaeciicarpum	Plot: individuals	Number of recently formed shoot nodes	8.3 ± 6.9	1.2	55	.231
Adenocalymma validum	Plot: individuals	Number of recently formed shoot nodes	6.6 ± 2.0	3.2	436	**.001**
Anemopaegma robustum	Plot: individuals	Number of recently formed shoot nodes	17.9 ± 8.6	2.1	277	**.038**
Fridericia prancei	Plot: individuals	Number of recently formed shoot nodes	2.3 ± 1.2	1.9	68	**.049**
Pachyptera aromatica	Plot: individuals	Number of recently formed shoot nodes	14.9 ± 8.2	2.0	120	**.044**

dynamics of ant–plant protective mutualisms in understorey tropical rainforests at both the community and individual plant scales.

4.1 | Temporal variation in ant attendance on EFNs throughout the year

Our results clearly show that ant attendance at EFN-bearing plants was higher in the drier months of the year at the Ducke Reserve. This finding contrasts with the temporal patterns of ant–plant interactions described in other, mostly dry, ecosystems, in which ant attendance is more frequent in the wet season in tropical (Belchior et al., 2016; Dias-Castelazo & Rico Gray, 2004; Dutra, Freitas, & Oliveira, 2006; Kersch & Fonseca, 2005; Lange, Dáttilo, & Del-Claro, 2013; Rico-Gray, 1993) and subtropical sites (Aranda-Rickert, Diez,

& Marazzi, 2014). It is tempting to conclude that water availability is the main abiotic factor-driving temporal patterns at previously studied sites, whereas ant–plant interactions at our study site are less dependent on this driver. However, our results also showed that drier months had the highest proportion of plants producing EFN-bearing tissues, as well as the highest local ant activity. Therefore, it is possible that the mismatch between the seasonal patterns of ant–plant interactions described here compared to what has been reported elsewhere primarily reflect ecosystem-specific variation in temporal patterns of bud activation and plant phenology.

Water supply is weakly seasonal in most wet tropical forests, including the Ducke Reserve, and covaries temporally with other abiotic factors that exhibit more marked variation over the year and that directly influence plant phenology. Light irradiance (Saleska et al., 2016), for example is a limiting factor for bud activation and shoot

elongation in Amazonian rainforest plants (Fenner, 1998), especially for those in the light-limited understorey. In the transition between wet and dry season, tree species from forest canopies replace many of their leaves (Wright & Van Schaik, 1994), creating a gradient of light availability in the forest understorey. Furthermore, drier seasons generally have a lower cloud cover. Thus, in drier periods of the year, more sunlight reaches the forest understorey, favouring leaf production in recently formed shoot nodes (Aide, 1988). This increase in light availability during the driest period of the year can act as a signal for the activation of shoot apical meristems (Fenner, 1998), increasing photosynthetic plant rates (Wu et al., 2016) and starting biochemical pathways controlling nectar secretion in EFN-bearing plant species (e.g. Radhika, Kost, Mithöfer, & Boland, 2010). Cumulatively, these phenomena might explain the higher proportion of plants with young tissues bearing active EFNs during the driest months in our study site, creating a favourable temporal window in which associations between ants and EFN-bearing plants can develop.

Although water and light availability are major factors regulating plant phenology in tropical rainforests, herbivory pressure can be an additional factor associated with the production of new leaves in plants (van Schaik, Terborgh, & Wrigth, 1993). At our study site, we did not quantify herbivory rates over time. However, there are several lines of evidence in the literature indicating that herbivory pressure varies little across the year in wet forests (Aide, 1988; Coley & Barone, 1996, 2001; Wolda, 1988). If so, the increased production of new tissues bearing active EFNs by Bignonieae plants in drier periods is more likely to have evolved as a response to the variation in the light availability than as a strategy to avoid herbivores.

However, even though it is unlikely, it is important to consider some implications of our results if herbivory pressure is in fact seasonal, and thus could be associated with the observed patterns of ant attendance on EFNs. We can envisage two alternative hypothetical scenarios in which herbivory might interact with the seasonal patterns of rainfall, irradiance, leaf and EFN nectar production at our study site. First, if herbivory pressure is in fact lower in the driest months, it is possible that Bignonieae plants are exhibiting a 'seasonal escape strategy' (Aide, 1992, 1993; Coley & Barone, 2001). Under this scenario, the production of young tissues bearing EFNs would be favoured in the driest season, when plants encounter less harmful herbivores and experience higher light availability. In this situation, plants would have more active EFNs during the season when they benefit less from ants' anti-herbivore services. However, the cost of EFNs for plants would be relatively lower in these driest months, due to higher light availability. Second, if herbivory pressure is in fact higher in the driest months, the production of young tissues would be well-matched to the periods of heavy herbivore attack. In this scenario, the presence of active EFNs on young tissues would play an important role ensuring plant defence during the period in which the plant tissues are most susceptible to herbivore attack.

In this second hypothetical scenario (higher herbivore pressure in the driest months), it is also possible that the higher frequency of ant attendance in our plants is an induced response to herbivory. Certain plant species are known to respond to herbivory in this way, increasing nectar production when attacked (Heil & Bueno, 2007; Huang, Siemann, Carrillo, & Ding, 2015; Yamawo & Suzuki, 2018), consequently attracting more ant defenders (Wäckers, Zuber, Wunderlin, & Keller, 2001). However, for our study system, we consider this unlikely. Induced nectar production by herbivory has been commonly described in fast-growing annuals from resource-rich sites and in tree species within second-growth forests (reviewed in Heil, 2008). The factors triggering the growth of plants belonging to these groups and the temporal dynamic of these ecosystems are very different from our study system. Indeed, it has been shown that secretion of extrafloral nectar in species from wet forest understorey varies strongly in response to light irradiance, and secondarily to ant presence, rather than to herbivore damage (e.g. Bixenmann, Coley, & Kursar 2011). Therefore, temporal variation in the abiotic factors triggering the production of new plant tissues should play a more critical role driving the pattern of ant attendance to EFNs than should the temporal dynamic of herbivory, at least in weakly seasonal habitats like in the wet tropical rainforests.

4.2 | Abundance of young EFN-bearing tissues determine patterns of ant attendance

In our study, both the number of recently formed shoot nodes and local ant activity increased in the dry season. Theoretically, any increases in these two factors could lead to an increase in the probability of EFN-bearing plants attendance by ants. However, our confirmatory path analyses showed that the proportions of EFN-bearing plants attended by ants over time were only explained by the proportion of plants with young tissues per plot. The abundance of young tissues not only explained temporal patterns of ant–plant interaction at the community level, but also the probability of ant attendance at the individual plant level. Individual plants with a higher number of recently formed shoot nodes showed a higher probability of ant attendance. Therefore, ant attendance to EFN-bearing plants was consistent with our hypothesis at the plant level, reinforcing the importance of the phenological stage of plants tended by ants (Bentley, 1977; Rico-Gray & Oliveira, 2007).

Many studies have shown that secretion of extrafloral nectar is greatest during periods of rapid vegetative growth, in which new leaves bearing EFNs are produced in flushes (Elias, 1983; Koptur, 1992; Rico-Gray & Oliveira, 2007). This phenological pattern was explicitly stressed by Bentley (1977). Active EFNs secreting sugar-rich resources are found on younger portions of the plant, usually above the first, most recently formed shoot nodes below an actively growing meristem. Therefore, during periods in which EFN-bearing plants are investing more in the production of new tissues, ants will be supplied with more extrafloral nectar. This increase in the uptake of extrafloral nectar is expected to fuel ant activities (Aranda-Rickert et al., 2014), boosting both the number of ants patrolling on the plant surface and the number of local EFN-bearing plants that those ants explore (Ness, Morris, & Bronstein, 2009). Therefore, it

is possible that the elevated ant activity observed during the driest months can be explained by the increased availability of extrafloral nectar produced on the young plant tissues at this period of the year.

Plant species are commonly categorized according to the presence or absence of EFNs (e.g. Dattilo, Marquitti, Guimarães, & Izzo, 2014). However, our results highlight that much of the time, EFN-bearing plant species behave like plants without EFNs when they lack young tissues, depending more upon the phenological status of plants than on the EFN phenotype per se for establishing interactions with ants. EFN-bearing plants producing young tissues not only become predictable sources of food to ants but sources of energy that increase foraging success and constancy of an ant attendance (Nogueira et al., 2015). Consequently, EFN-bearing plants supporting more recently formed shoot nodes simultaneously are more attractive to ants, and therefore likely to be tended by ants. In fact, the six Bignonieae species for which ant attendance probabilities were unrelated to the amount of young tissues were the same species that had the fewest recently formed shoot nodes over our five surveys. In these species, the amount of extrafloral nectar offered to ants should be low, compromising the quality of the plant as partners to ants.

In our study, using a conservative estimate of dominant ant species based on Baccaro et al. (2010), six dominant aggressive ant species occupied about half of the plants producing young EFN-bearing tissues, independent of the sampling period. As dominant ants are more aggressive towards herbivores (Buckley & Gullan, 1991; Xu & Chen, 2010), most Bignonieae plants at our study site are interacting with high-quality ant partners. This association has the potential to intensify biotic defence on young tissues that are preferred by herbivores (Coley, 1980), even though the herbivory pressure is likely to be slightly lower in the dry season (Aide, 1988; Coley & Barone, 1996; Wolda, 1988). In such interactions, dominant ant species recruit more workers to plants (Cerdá, Arnan, & Retana, 2013), which could impose high costs to the plant partner except when plants are under high herbivore pressure (Melati & Leal, 2018). Therefore, the role of such association over time should be interpreted with caution, mainly because the direction and magnitude of ant effect on plant fitness depend on herbivore pressure.

5 | CONCLUSIONS

Temporal patterns of ant attendance at EFNs coincide with the seasonal appearance of young EFN-bearing tissues, on which EFNs appear to be more active. Therefore, ant attendance at both the community and individual levels over time seems to be driven by the amount of young tissues bearing EFNs. Contrasting temporal patterns of ant–plant interactions across seasonal and aseasonal tropical sites can be explained by variation in factors triggering the changes into the phenological stages of EFN-bearing plants. In tropical seasonal environments, most plant species produce new tissues at the onset of the rainy season (Levings, 1983), when water availability for plant growth is higher. However, in the understorey of tropical

rainforest, plants have available a relatively high supply of water all over the year. As a consequence, plant growth in less seasonal environments should be triggered by other environmental factors, such as light that is more abundant in the understorey of tropical forests in the dry season. Additionally, differences in ant attendance among species in the understorey were only poorly explained by the secretory area of EFNs per shoot node, highlighting the stronger effect of plant phenology and the availability of young plant tissue in explaining the establishment of ant–plant interactions. A potential limitation in our study is the lack of herbivore data over time; herbivory, jointly with environmental factors, could explain variations in plant phenology and, ultimately, in ant–plant protective mutualism. Plant phenology could be especially important and even critical in seasonally drier areas where growing seasons are shorter, and where EFN-bearing plants are more common than in wet environments (Leal & Peixoto, 2017). In such environments, EFN-bearing plants should experience not only a more marked temporal pattern of ant attendance, but also a narrower window in which ants can more efficiently protect plants.

AUTHORS' CONTRIBUTIONS

A.N. and F.B.B. conceived the ideas, experimental design and collected the data; A.N. analysed the data and led the writing of the manuscript. A.N., F.B.B., L.C.L., P.J.R., L.G.L. & J.L.B. contributed to the writing and gave final approval for publication.

DATA AVAILABILITY STATEMENT

Data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.18931zcsc (Nogueira et al., 2019).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.