Nitrogen-fixing and non-fixing trees differ in leaf chemistry and defence but not herbivory in a lowland Costa Rican rain forest

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Abstract

Nitrogen-fixing plants provide critical nitrogen inputs that support the high productivity of tropical forests, but our understanding of the ecology of nitrogen fixers – and especially their interactions with herbivores – remains incomplete. Herbivores may interact differently with nitrogen fixers vs. non-fixers due to differences in leaf nitrogen content and herbivore defence strategies. To examine these potential differences, our study compared leaf carbon, nitrogen, toughness, chemical defence and herbivory for four nitrogen-fixing tree species (*Inga oerstediana*, *Inga sapindoides*, *Inga thibaudiana* and *Pentaclethra macroloba*) and three non-fixing species (*Anaxagorea crassipetala*, *Casearia arborea* and *Dipteryx panamensis*) in a lowland tropical rain forest. Leaf chemical defence, not nutritional content, was the primary driver of herbivore damage among our species. Even though nitrogen fixers exhibited 21.1% higher leaf nitrogen content, 20.1% lower C:N ratios and 15.4% lower leaf toughness than non-fixers, we found no differences in herbivory or chemical defence between these two plant groups. Our results do not support the common hypotheses that nitrogen fixers experience preferential herbivory or that they produce more nitrogen-rich defensive compounds than non-fixers. Rather, these findings suggest strong species-specific differences in plant–herbivore relationships among both nitrogen-fixing and non-fixing tropical trees.

Introduction

Nitrogen (N)-fixing plants (hereafter N fixers) play critical roles in the ecosystems they inhabit due to their ability to convert atmospheric N_2 into bio-available N (Vitousek & Howarth 1991). These N fixers can supply over 100 kg N ha^{-1} y^{-1} into terrestrial ecosystems (Binkley *et al.* 1994, Binkley & Giardina 1997), potentially relieving N limitation and fuelling primary production (Schlesinger & Bernhardt 2013). The potential influence of N fixers is especially large in tropical forests due to their relatively high abundances, highlighted by the 10-fold greater abundance of N-fixing trees in Neotropical forests compared with their temperate counterparts (Menge *et al.* 2014, ter Steege *et al.* 2006). Because N fixers supply important N resources that fuel tropical forest productivity (Batterman *et al.* 2013*a*), identifying the ecological interactions of N fixers, non-fixing trees, and the organisms that consume them is critical for our broader understanding of tropical forest ecosystem processes as a whole.

Fundamental ecological differences between N fixers and non-fixers determine the competitive success of N fixers relative to their non-fixing neighbours in various environments (Vitousek *et al.* 2002). Differences between N fixers and non-fixers include different reliance on the availability of light (Gutschick 1981, Myster 2006, Taylor & Menge 2018) and soil resources (Batterman *et al.* 2013*b*, Nasto *et al.* 2014, Taylor & Menge 2018, Vitousek & Howarth 1991), resource use efficiency (Menge *et al.* 2008, Wolf *et al.* 2017), responses to changes in temperature (Houlton *et al.* 2008), and changes in atmospheric CO_2 (Hungate *et al.* 2004). In addition to these environmental factors, differential herbivory pressure on N fixers and non-fixers might also drive important ecological differences between these plant groups (Menge *et al.* 2008, Vitousek & Field 1999, Vitousek & Howarth 1991).

Competing alternative hypotheses have been proposed for how herbivores might differentially interact with N fixers relative to neighbouring non-fixers. Both hypotheses depend on N fixers having higher foliar N concentrations than non-fixers, which has been demonstrated in many ecosystems (Adams *et al.* 2016, McKey 1994, Wolf *et al.* 2017) including tropical forests (Fyllas *et al.* 2009, Nasto *et al.* 2014, Townsend *et al.* 2007). One hypothesis poses that this N-rich leaf material may attract herbivores (Coley & Barone 1996, Mattson 1980), creating more intense herbivory pressure on N fixers than their non-fixing neighbours (Menge *et al.* 2008, Vitousek & Field 1999, Vitousek & Howarth 1991). The alternative hypothesis states that if N fixers use their high foliar N concentrations to create N-rich secondary defensive compounds, these chemical defences may mitigate herbivore preferences or even make herbivores avoid

N fixers relative to neighbouring non-fixers (Mattson 1980, Menge *et al.* 2008). Limited experimental evidence does suggest that herbivores prefer N fixers in some systems (Hulme 2008, Knops *et al.* 2000, Ritchie & Tilman 1995, Ritchie *et al.* 1998) but this has not been explicitly tested for tropical forest trees and differences in herbivore palatability between N fixers and non-fixers have never been tested in any ecosystem to our knowledge.

The effects of herbivory on N fixers may be particularly important in tropical forests. Leaf damage from herbivores is, on average, 56% greater in tropical vs. temperate forests (Coley & Barone 1996), and herbivory has been shown to be especially important for carbon and N cycling (Metcalfe *et al.* 2014) and plant trait selection in tropical forests (Coley & Barone 1996, Sedio *et al.* 2018). For N fixers, herbivory has been shown to offset the benefits of harbouring mutualistic N-fixing bacteria, which means that herbivory pressure may also play a role in N fixation rates of an individual N fixer (Simonsen & Stinchcombe 2014). Thus, the relationship between tropical forest N fixers and the herbivores that consume them may have important effects on both N fixers themselves and the amount of N that N fixers bring into the surrounding ecosystem.

Given the importance of herbivory on N fixers to multiple ecosystem processes and the paucity of data on the drivers of this relationship in tropical forests, we designed a study to assess if N fixers exhibit different foliar elemental composition and leaf defence, and if differences in these traits lead herbivores to preferentially consume or avoid tropical N-fixing trees relative to neighbouring non-fixers. Based on available theory and previous data, we hypothesized that N fixers would have higher leaf N concentrations than non-fixers, use this high leaf N to produce and rely more heavily on chemical defences than non-fixers, and that there would be a trade-off (negative relationship) between physical and chemical defensive strategies among our study species. From the few studies that have assessed N-fixer herbivory in other ecosystems, we also hypothesized that N fixers would suffer greater herbivory damage than non-fixers.

Materials and methods

Study site and species

Our study was conducted at La Selva Biological Station in the Caribbean lowlands of north-eastern Costa Rica (10°25′N, 84°00′W). La Selva comprises 1615 hectares of tropical rain forest and averages 3962 mm of rainfall a year, with a mean annual temperature of 25.8°C (McDade & Hartshorn 1994). The elevation ranges from 30 to 135 m above sea level, and the vegetation predominately consists of evergreen, broad-leaved trees (McDade & Hartshorn 1994). Data were collected during the rainy season in June and July 2016 in old-growth forests located on alluvial and semi-alluvial soils.

We studied seedlings of seven broad-leaved evergreen species, including four species of N fixers (*Pentaclethra macroloba*, *Inga sapindoides*, *Inga oerstediana* and *Inga thibaudiana*) and three species of non-fixers (*Dipteryx panamensis*, *Anaxagorea crassipetala* and *Casearia arborea*). Seedlings of these species are all abundant along forest edges and light gaps in old-growth forests at La Selva. To minimize the phylogenetic signal inherent when comparing N-fixers (all of which are Fabaceae at this site) to non-fixers (a highly diverse group), we included N fixers from two different tribes within the Fabaceae (Mimoseae for *P. macroloba* and Ingeae for *Inga* spp.) and included a non-fixing Fabaceae in our set of

non-fixers (*Dipteryx panamensis*; Sprent 2009). All seedlings studied were under 1.5 m in height and were spaced at least 10 m apart.

Elemental analyses

Leaves were collected from 10 individual seedlings of each of the seven study species. For elemental analyses, a leaf was selected randomly from each seedling excluding the oldest and the youngest leaves. The 10 leaves from each species were dried to constant mass and ground to a fine powder. We measured the per cent foliar carbon (C), per cent foliar N and C:N mass ratio for each sample using an EC 1112 flash elemental analyzer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Chemical defence bioassay

We conducted a field bioassay experiment (methodology described in Dyer *et al.* 2003*a*) to determine the presence and strength of leaf chemical defensive compounds by assessing palatability of leaf chemical extracts using a common omnivorous ant species at La Selva, *Paraponera clavata*. Palatability of leaf extracts by *P. clavata* has been established as a reliable indicator of secondary metabolites known to deter a wide range of insect herbivores, and the efficacy of this bioassay has been thoroughly evaluated at our study site (Dyer *et al.* 2003*b*). Thus, we assessed feeding preferences of *P. clavata* for our leaf chemical extracts to gain a broad predictor of insect antifeedant compounds, not to infer herbivory defence against *P. clavata*, specifically, for our seven tree species. We prepared extracts of leaves from each study species (described below) and presented the extracts to the ants in sugar water solutions to test palatability of leaf compounds.

Fifteen grams of dried leaf material from each species was soaked in methanol overnight to extract foliar chemical compounds. Control and extract vials were then presented to colonies of *P. clavata* ants in the field. The extract vial contained 2.25 mL of a 20% sucrose solution and 0.1 mL of leaf extract. The control vial contained 2.25 mL of the sucrose solution and 0.1 mL of methanol void of leaf extract. Nests of *P. clavata* are located at the bases of trees, and the ants typically ascend the trunk of their nest tree to forage in the canopy. One control and one extract vial were attached side by side to trees above *P. clavata* nests (not necessarily trees of our seven study species) for 1 hour to allow the ants to choose between liquid in the control and extraction vials. We compared the mass of the vials before and after the trial to determine the amount of liquid consumed by the ants. Trials were conducted in this manner for each of our study species on seven different ant nests, which were at least 50 m apart, for a total of 49 bioassay trials (7 study species × 7 ant nests) and only one bioassay trial was conducted on a single ant nest per day.

We then calculated leaf chemical defence as the adjusted consumption difference (ACD) for each species according to the method in Dyer (1995). ACD was calculated as:

$$ACD = \frac{mc_c - mc_e}{mc_c + mc_e}$$

where mc_c and mc_e represent the mass consumed by *P. clavata* of control and extract sample vials, respectively. ACD values are unitless and range from −1 to 1. Values from 0 to 1 indicate unpalatability of leaf compounds, with higher values being increasingly unpalatable. Values from −1 to 0 indicate palatable leaf compounds. Thus, our bioassay does not assess specific secondary

defensive compounds in the leaf but provides a broad metric of the chemical palatability of extracts from each species' leaves.

Leaf toughness

To assess leaf physical defence, we tested the leaf toughness of three leaves on 10 seedlings of each species using a Medio-Line 40300 penetrometer (Pesola, Switzerland). The penetrometer measures the mass in grams required for a surface of known area to puncture a leaf. Because penetrometers do not measure the work required to fracture a leaf in units of energy, they are not a true measure of 'fracture toughness' (Lucas & Pereira 1990). However, the force that penetrometers measure can be a strong indicator of leaf physical defence against herbivory (Coley 1983), and we refer to this measure as leaf toughness based on convention in the ecological literature. For each leaf tested, we averaged toughness values for 3 puncture holes on each leaf while the leaf was still attached to the plant. This yielded 90 toughness values for each species. We were unable to measure leaf toughness of *Pentaclethra macroloba* in the field due to the small size of leaflets of the bipinnately compound leaves of this species. We performed the toughness measurements on this species in the lab by placing individual leaflets over a hole the size of the penetrometer tip to stabilize the device.

Herbivory and leaf area consumed

We measured the per cent leaf area consumed by herbivores on the same leaves used in elemental analyses. Although some herbivory studies focus specifically on the youngest leaves, we sampled random leaves to capture a fuller range of herbivory through leaf life-span while maintaining the ability to detect species differences by sampling in the same manner across species. We digitally scanned each leaf and used ImageJ software (NIH) to determine the per cent of the leaf area consumed (% LAC) by herbivores. Per cent LAC was calculated as:

$$\%LAC = \frac{LA_t - LA_h}{LA_t}$$

where LA_t and LA_h are the total leaf area in the absence of herbivory (estimated by manually filling in consumed segments of the leaf using ImageJ) and the leaf area remaining after herbivory, respectively.

Our assessment of % LAC focuses on partial-leaf herbivory and is not well suited to assessing herbivory of entire leaves. Thus, our data predominantly reflect herbivory by insects, which are more likely than vertebrate herbivores to remove sections of a leaf. Because the abscission of leaves or leaflets can be an induced response to herbivory (Karban & Baldwin 1997, Williams & Whitham 1986), we accounted for the absence of entire leaflets in our measure of % LAC for compound-leaf species. For *P. macroloba*, which has very small leaflets, we manually filled in missing leaflets using ImageJ. For the four study species that exhibit compound leaves with larger leaflets (*Inga* species and *D. panamensis*), we used the closest leaflet opposite the rachis as an estimate of the area of the missing leaflet. This method accounts for the loss of leaf area due to leaflet abscission in response to partial herbivory but may over-estimate herbivory if leaflets were abscised for reasons other than herbivory. Herbivory data were log-transformed for statistical analyses to meet assumptions of normality but are presented in tables and figures on linear scales.

Statistical analyses

To test for differences in foliar elemental composition, chemical defences, physical defences, and herbivory between N fixers and non-fixers and between our seven study species, we used a set of one-way Analysis of Variance (ANOVA) models with fixer type and species as independent variables. Models testing for differences in chemical defence and effects of chemical defences on herbivory included a covariate for ant nest, and models testing for differences in leaf physical defence and the effect of physical defences on herbivory included a covariate for leaf due to multiple penetrometer measurements on each individual leaf. Tukey HSD post-hoc tests were conducted to identify differences between pairs of species using the glht function in the multcomp package of R statistical software (Hothorn *et al.* 2008).

To determine if herbivory was significantly correlated with foliar elemental composition, chemical defences, or physical defences, we used a series of linear regression models with herbivory (% LAC) as the dependent variable and either foliar elemental composition or defensive traits as the independent variable. Models assessing the relationship between herbivory and foliar elemental composition each included a species covariate. To assess the relationship between physical and chemical defences between species, we used a linear regression model with leaf toughness as the dependent variable, ACD as the independent variable, and a covariate for individual leaf similar to our other models testing leaf toughness. Because a large number of leaves were pooled to create the extraction for our chemical-defence bioassay, models comparing chemical defences to other leaf traits used the mean ACD value for each species tested. All statistical analyses were done using the base and multcomp packages in R statistical software v.3.2.2.

Results

Leaf elemental composition

Leaf elemental composition differed significantly between N fixers and non-fixers. Foliar N concentrations in nitrogen fixers were 1.21 times those of non-fixers (F = 30.64, P <0.0001; Figure 1a, Table 1). We also found significant differences in foliar N between our seven study species (F = 7.89, P <0.0001; Figure 1b), but no significant difference between our two genera of N fixers, *Pentaclethra* and *Inga* (F = 0.13, P = 0.72). When assessing the five species in our study from the family *Fabaceae*, N fixers (*Inga* and *Pentaclethra* species) had significantly higher foliar N concentrations than our non-fixing Fabaceae, *D. panamensis* (F = 8.025, P = 0.007). As a group, N fixers did not have different foliar C concentrations than non-fixers (F = 3.96, P = 0.0512; Figure 1c). We did find significant differences in foliar C between our seven species (F = 25.04, P <0.0001, Figure 1d), and within our N-fixers, *P. macroloba* had significantly higher leaf C than the *Inga* species (F = 61.84, P <0.0001). The substantially higher N concentrations in N-fixer leaves drove significantly lower foliar C:N ratios in N fixers (F = 20.78, P <0.0001; Figure 1e). Species differed significantly in their C:N ratios (F = 5.21, P = 0.0002; Figure 1f), and *Inga* leaves had significantly lower C:N ratios than those of *Pentaclethra* (F = 5.44, P = 0.0258).

Leaf defence strategies

We found no differences in our palatability bioassay of leaf chemical defence (adjusted consumption difference, ACD) between N
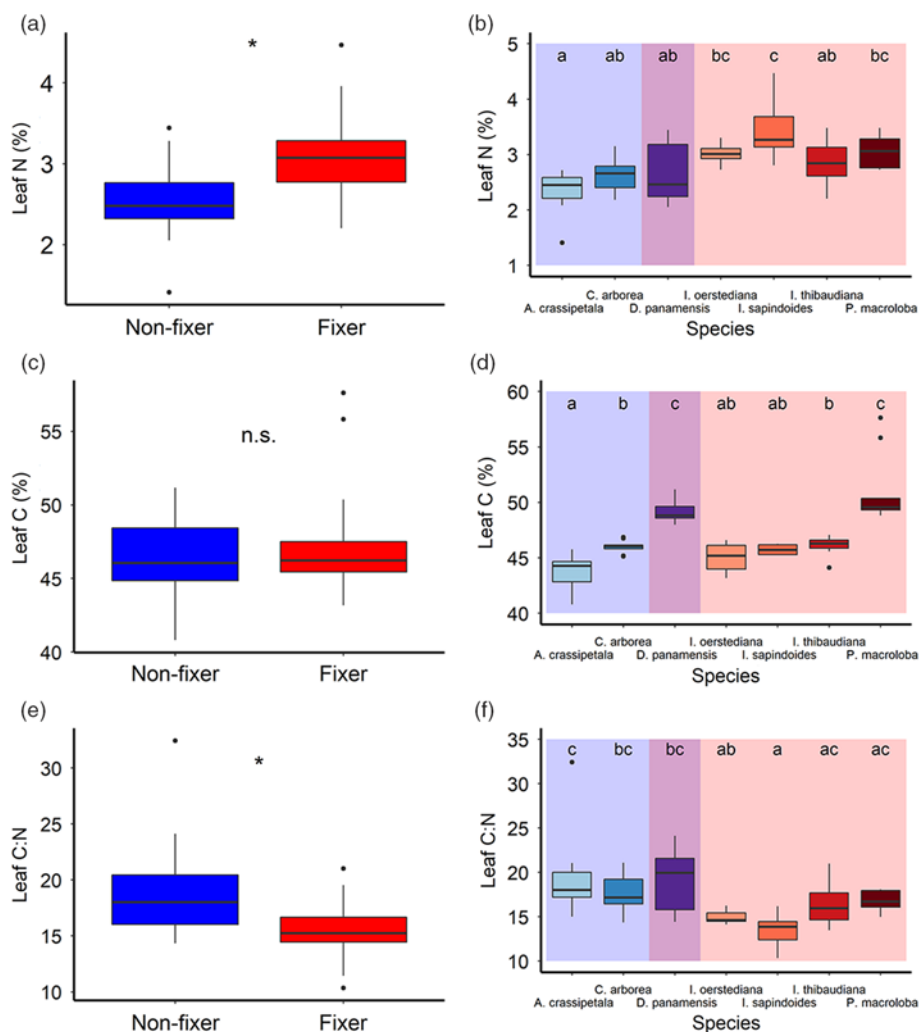
Figure 1. Foliar elemental composition of N fixers and non-fixers. Foliar % N (a), % C (c), and C:N mass ratio (e) are shown for N fixers (red) and non-fixers (blue). The same elemental composition data are shown for our seven study species individually in (b) (d) and (f). Species on blue backgrounds in (b) (d) and (f) are non-Fabaceae non-fixers, species on red backgrounds are N fixers, and the species on purple backgrounds (*D. panamensis*) is a non-fixing species in the same family, Fabaceae, as our N fixers. Asterisks in (a) (c) and (e) indicate significant differences between N fixers and non-fixers. Different letters indicate significant differences between species in (b) (d) and (f).

fixers and non-fixers (F = 2.94, P = 0.095; Figure 2a) or between species (F = 2.30, P = 0.0556; Figure 2b). However, we did find significant differences in leaf physical defence (leaf toughness) between N fixers and non-fixers (F = 5.61, P = 0.0188; Figure 2c), with non-fixers having, on average, tougher leaves than N fixers. Tukey post-hoc analyses determined that the difference in leaf toughness between N fixers and non-fixers was driven by the low leaf toughness of *P. macroloba*, which was significantly lower than *Inga* N fixers and non-fixers (F = 78.61, P <0.0001), which had similar leaf toughness. Leaf toughness also varied significantly by species (F = 48.72, P <0.0001; Figure 2d).

There was a significant trade-off between chemical and physical leaf defences across our seven species. ACD was significantly negatively correlated with leaf toughness (t = −8.48, Adjusted R² = 0.26, P <0.0001; Figure 3), indicating that species tended to predominantly employ either physical or chemical defences, but not both. However, N-fixing and non-fixing species did not tend to occupy separate ends of this trade-off spectrum (Figure 3). The defence strategy trade-off was correlated most strongly with each species' leaf C concentration, with higher foliar C being associated with higher chemical defence production, but lower leaf toughness.

Chemical defence was significantly positively correlated with foliar C concentrations across our study species (t = 4.53, Adjusted R² = 0.23, P < 0.001; Figure 4b) but, contrary to our predictions, not significantly correlated with foliar N concentrations (t = −0.38, P = 0.706; Figure 4a). Surprisingly, leaf toughness was negatively correlated with foliar C concentrations (t = 10.23, Adjusted R² = 0.29, P < 0.0001; Figure 4e) but was not significantly related to foliar N concentrations (t = −1.17, P = 0.242; Figure 4d). C:N ratios were not significantly related to either leaf chemical defence (t = 1.31, P = 0.19) or physical defence (t = 0.206, P = 0.84; Figure 4c, f).

Herbivory

Contrary to our expectation, the per cent of leaf area consumed (% LAC) by herbivores did not differ significantly between N fixers and non-fixers (F = 3.12, P = 0.0834; Figure 5a) nor did % LAC differ between Fabaceae and non-Fabaceae species (F = 0.684, P = 0.412). Herbivory did vary significantly between our seven study species (F = 3.35, P = 0.0069; Figure 5b), with two of our *Inga* N-fixers (*I. oerstediana* and *I. sapindoides*) experiencing
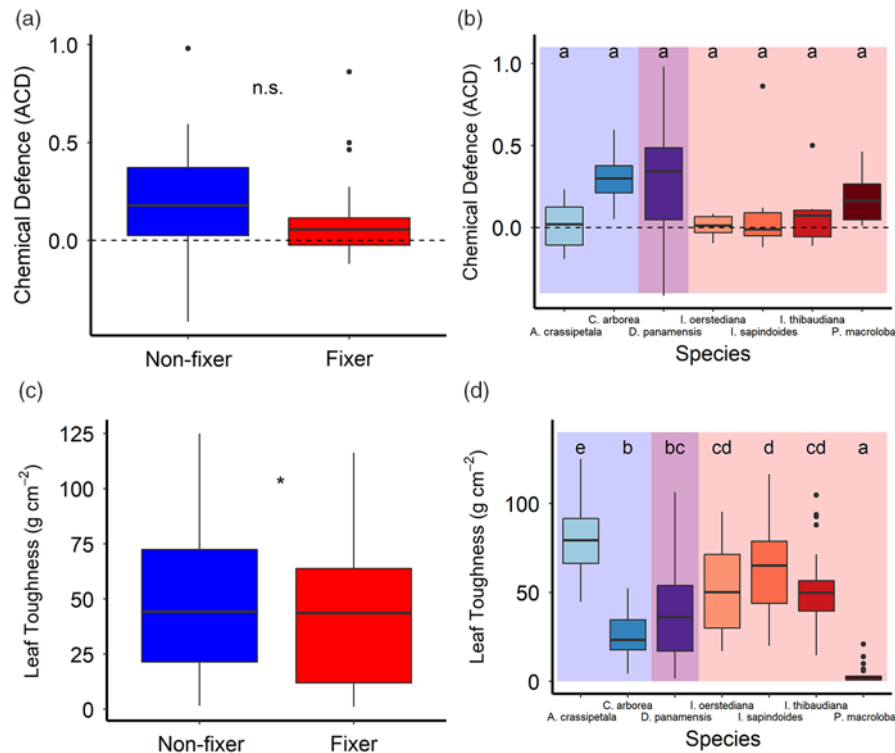
Figure 2. Leaf defence strategies of N fixers and non-fixers. Our chemical defence assay (Adjusted Consumption Difference) did not differ between N fixers and non-fixers (a) or by species (b). Leaf toughness significantly differed by both fixer type (c) and species (d). Letters, colour schemes and indications of significance are as in Figure 1.
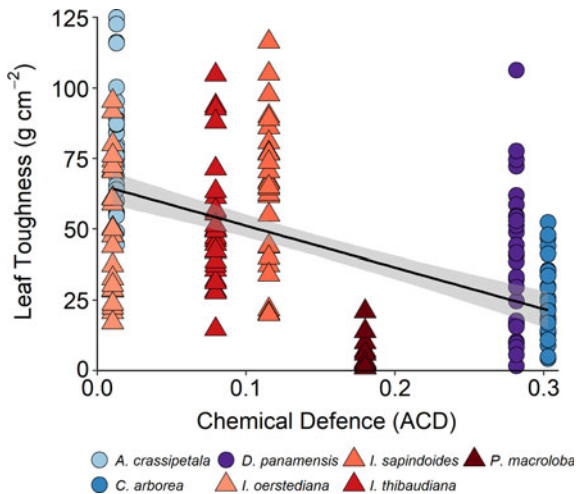

Figure 3. The trade-off between physical and chemical leaf defences. Leaf physical defence (leaf toughness) was negatively correlated to chemical defence (ACD). Colours of individual points follow the species-level colour scheme in Figure 1. Circles represent non-fixing species and triangles represent N-fixing species.

the most herbivore damage and the other two N fixers and our non-fixing legume (*I. thibaudiana*, *P. macroloba* and *D. panamensis*) experiencing the lowest herbivory damage (Figure 5b, Table 1).

Herbivory was not significantly correlated with foliar N concentrations ($t = 0.697$, $P = 0.489$; Figure 4g), foliar C concentrations ($t = 1.253$, $P = 0.215$, Figure 4h), or foliar C:N ratio ($t = -0.288$, $P = 0.774$; Figure 4i). Leaf defences, however, did have a significant effect on herbivory. As expected, chemical defence (ACD) was

significantly negatively correlated with herbivory across our study species ($t = -3.16$, Adjusted $R^2 = 0.13$, $P < 0.001$; Figure 6a). Leaf toughness did have a significant effect on % LAC, but in the opposite direction from our expectation: % LAC was greater for tougher leaves ($t = 4.74$, Adjusted $R^2 = 0.10$, $P < 0.001$; Figure 6b). Because *P. macroloba* leaves had anomalously low leaf toughness (Figure 2d), we also analysed the relationship between herbivory and leaf toughness excluding this species to ensure that it was not driving the overall pattern in our data. We found a significant positive relationship between herbivory and leaf toughness even when excluding data for *P. macroloba* ($t = 3.72$, Adjusted $R^2 = 0.07$, $P < 0.001$).

Discussion

The results of this study demonstrate a large, species-specific range in foliar elemental composition, anti-herbivore defence strategies and herbivory damage. Supporting our first hypothesis, N fixers had higher foliar N concentrations than non-fixers. Also in line with our expectations, defence strategies ranged across a trade-off spectrum – species with tougher leaves had lower chemical defences, and vice versa; but contrary to our third hypothesis, N fixers were not more likely to employ either chemical or physical defences than non-fixers. Despite significantly higher foliar N concentrations, N fixers did not incur more herbivory damage than non-fixers, contradicting our final hypothesis. Overall, we found that leaf chemical defence was an effective anti-herbivore deterrent and that chemical defences, not leaf nutritional content, drove herbivory damage in the species we studied.
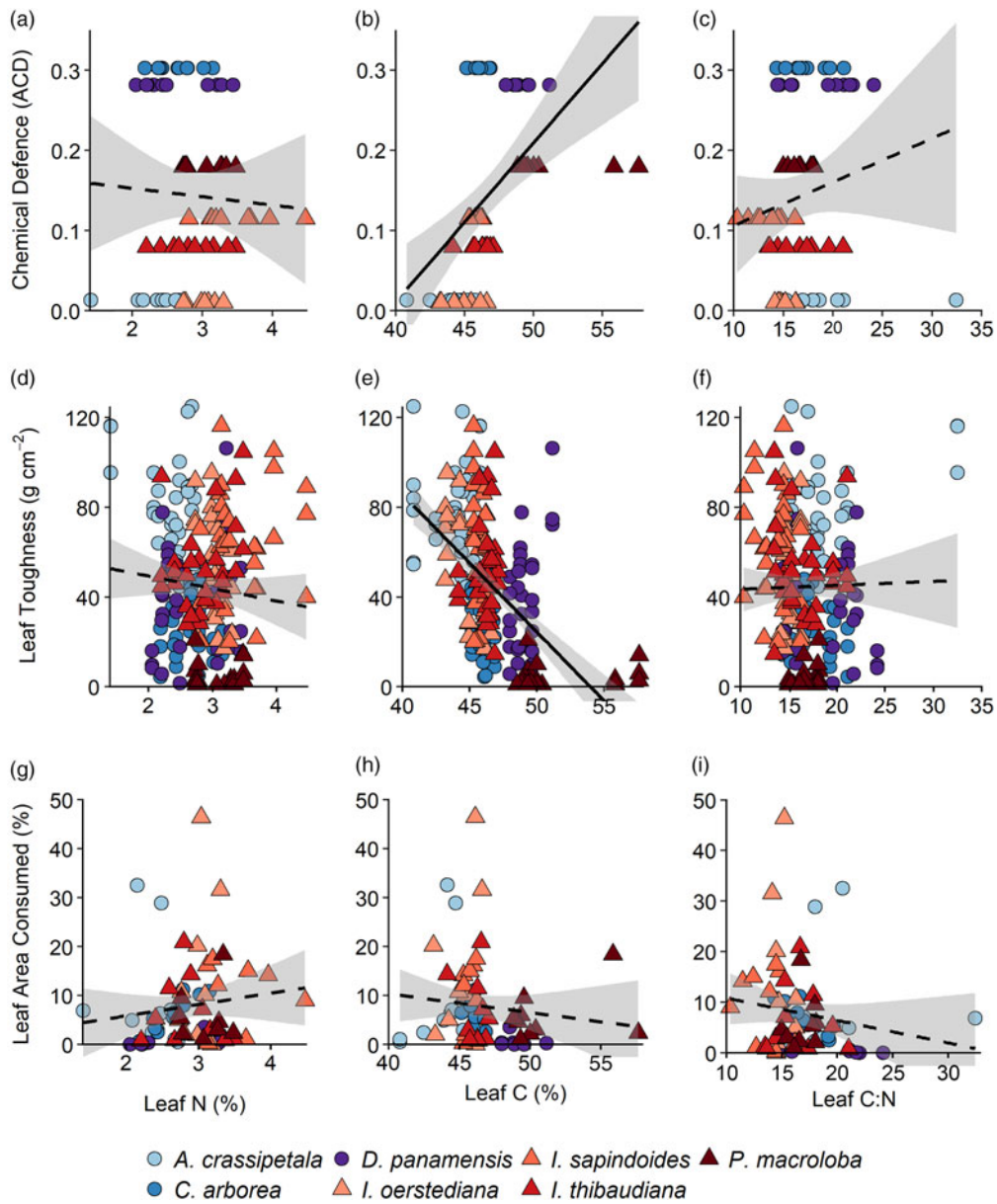
Figure 4. Relationships between chemical defence, physical defence, herbivory and foliar elemental composition. Chemical defence (ACD) (a-c) was positively related to foliar % C, but not % N or C:N. Leaf toughness (g cm^{-2}) (d–f) was negatively related to foliar % C, but not % N or C:N. Herbivory (% LAC) (g–i) was not significantly correlated to foliar % N, % C or C:N. Colours and shapes of individual points follow the colour and shape scheme in Figure 3. Solid lines indicate significant relationships and dashed lines indicate non-significant trends.
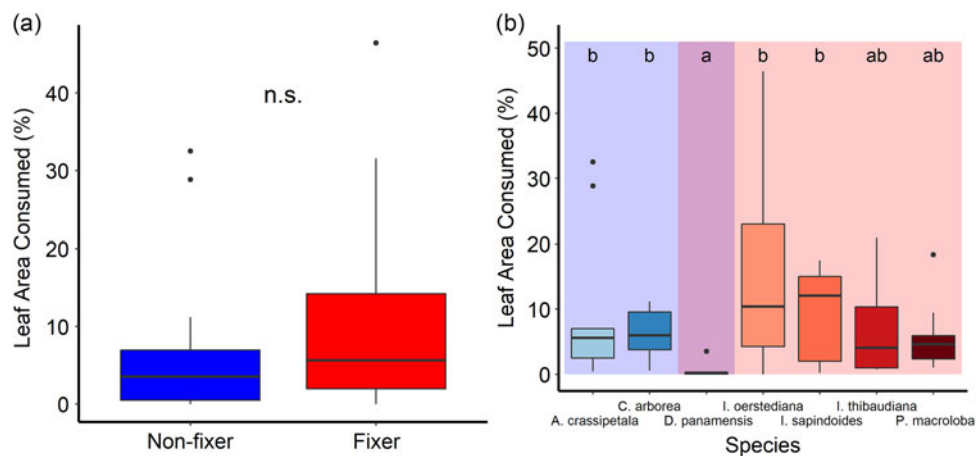

Figure 5. Average herbivory of N fixers and non-fixers. Mean (± SE) herbivory damage (% leaf area consumed) is shown for N fixers and non-fixers (a), and for each of our study species (b). Letters, colour schemes and indications of significance are as in Figure 1.

Table 1. Foliar traits for our three non-fixer (NF) and four N fixer (F) study species sampled at La Selva Biological Station, Heredia, Costa Rica. Mean (± SE) values for foliar %N, %C, C:N mass ratio, herbivory (% Leaf Area Consumed, LAC), leaf toughness and chemical defences (adjusted consumption difference, ACD) are presented

| Species	Fixer type	%N(± SE)	%C(± SE)	C:N(± SE)	% Leaf area consumed(± SE)	Toughness(g cm^{-2} ± SE)	ACD(± SE)
Anaxagorea crassipetala	NF	2.34 ± 0.12	43.7 ± 0.55	19.37 ± 1.57	9.32 ± 3.65	81.13 ± 3.86	0.013 ± 0.061
Casearia arborea	NF	2.64 ± 0.09	46.0 ± 0.18	17.61 ± 0.67	6.37 ± 1.15	25.39 ± 2.32	0.303 ± 0.065
Dipteryx panamensis	NF	2.67 ± 0.17	49.1 ± 0.30	18.96 ± 1.10	0.64 ± 0.40	38.11 ± 4.61	0.282 ± 0.169
Inga oerstediana	F	3.0 ± 0.07	45.0 ± 0.47	15.00 ± 0.29	15.74 ± 5.70	51.86 ± 4.81	0.010 ± 0.026
Inga sapindoides	F	3.48 ± 0.17	45.7 ± 0.14	13.39 ± 0.61	9.69 ± 2.29	63.98 ± 4.90	0.115 ± 0.128
Inga thibaudiana	F	2.86 ± 0.12	46.1 ± 0.27	16.44 ± 0.80	6.56 ± 2.19	52.33 ± 3.79	0.079 ± 0.078
Pentaclethra macroloba	F	3.05 ± 0.10	51.1 ± 1.08	16.84 ± 0.39	5.80 ± 1.79	3.19 ± 0.82	0.180 ± 0.062
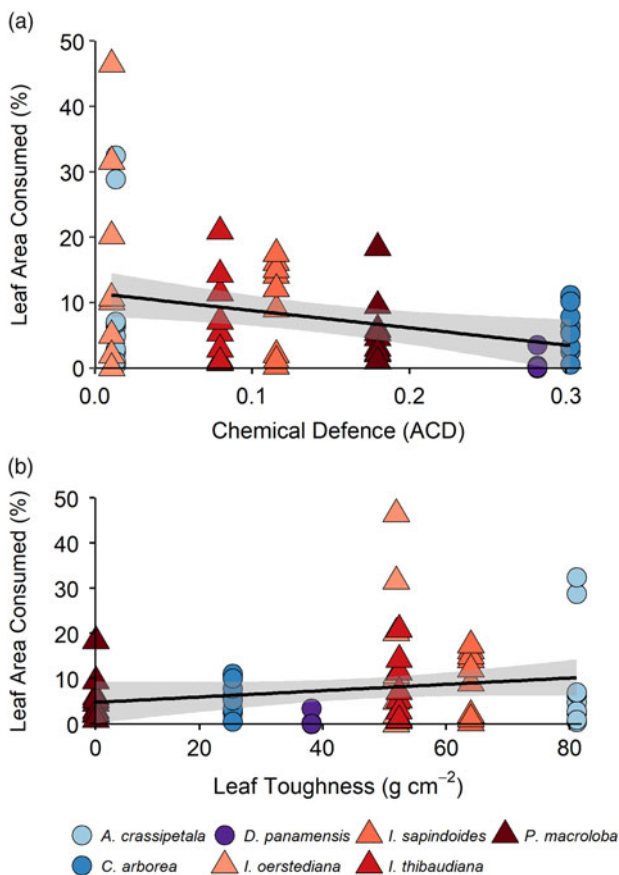
Figure 6. The relationship between herbivory and leaf defences. Herbivory (% LAC) was significantly negatively correlated to chemical leaf defences (ACD) (a), but positively correlated to physical leaf defences (leaf toughness) (b). Colours and shapes of individual points follow the colour and shape scheme in Figure 3.

Phylogenetic considerations

Potential confounding effects of phylogeny are inherent when comparing N fixers and non-fixers because N fixers belong exclusively to the Rosid I clade (Sprent 2009) and N fixers at our study site all belong to a single family, *Fabaceae* (Menge & Chazdon 2016), while non-fixers belong to a diverse set of plant families. This raises the possibility that any differences between N fixers and non-fixers reported in this study could simply be characteristics of the family *Fabaceae* and not necessarily characteristics of species that can fix N. However, for both sets of traits that varied

significantly between N fixers and non-fixers in our study (elemental composition and leaf toughness), models comparing *Fabaceae* to non-*Fabaceae* species provided worse fits for our data than models comparing N fixers with non-fixers. This strongly suggests that the differences between N fixers and non-fixers found in our study are not simply due to traits of the family *Fabaceae*, but are characteristics of species that can actively fix N.

Leaf elemental composition

Our data on foliar N concentrations support ample evidence from the literature that N fixers have higher average leaf N concentrations than non-fixers (Adams *et al.* 2016, Fyllas *et al.* 2009, McKey 1994, Townsend *et al.* 2007, Wolf *et al.* 2017). Less well resolved in the literature is whether high foliar N concentrations are a trait associated with the ability to fix N (e.g. Wolf *et al.* 2017), or primarily a trait of the family *Fabaceae*, which contains most N-fixing species (e.g. McKey 1994). Within our five *Fabaceae* species, significantly higher foliar N concentrations in our N fixers than our non-fixing species, *D. panamensis*, suggest that leaf N is a trait associated with N fixation rather than a trait of the *Fabaceae* family itself.

Leaf defence

Despite having higher foliar N concentrations, N fixers did not exhibit stronger chemical defences than non-fixers according to our palatability bioassay, contrary to our prediction. When assessing leaf chemical defence, we opted to use a bioassay to directly assess the chemical palatability of our leaves rather than isolating specific secondary compounds in the leaf tissue. Although this approach does not allow us to identify the specific chemicals that the leaves of our species produce, it has the distinct advantage of directly assessing the effect of the leaves' chemicals on insect palatability, which was our primary interest. Our findings that N fixers do not exhibit stronger chemical unpalatability (Figure 2a) and that chemical unpalatability was positively correlated with %C but not %N (Figure 4a, b) suggest that the N-fixing species in this study do not primarily use their high leaf N concentrations to produce N-rich secondary compounds, as has been hypothesized (Menge *et al.* 2008, Vitousek & Field 1999).

One of the clearest relationships in our dataset was the trade-off between chemical and physical defence strategies employed by our seven study species. Those species with the toughest leaves tended to rate lowest in our assay of chemical defence. This trade-off in defensive strategies supports the hypotheses that either the efficacies of different defensive strategies are ecologically context-dependent, that physiological constraints inhibit single

plant species from exhibiting multiple defence strategies, or both (Coley & Kursor 1996). N fixers and non-fixers were somewhat mixed along this trade-off spectrum. *Pentaclethra macroloba* exhibited the lowest leaf toughness but relatively high chemical defences, our three non-fixing species varied widely in both chemical and physical defences, and the three *Inga* N fixers had relatively tough leaves and low chemical defence scores.

Efficacy of leaf defences

Although both of our metrics of leaf defence were correlated with herbivory, leaf chemical defence reduced herbivory damage while leaf physical defence was associated with higher herbivory damage (Figure 6). The positive association between leaf toughness and herbivory damage contradicts previous work on anti-herbivore defences in tropical forests (Coley 1983). One species in our study, *P. macroloba*, had extremely low leaf toughness values, due primarily to its highly subdivided bipinnately compound leaves. However, we found the same patterns of herbivory and leaf toughness after removing *P. macroloba* from the dataset (data not shown) indicating that this outlier species did not entirely drive the discrepancy between our findings and previous work.

In contrast to leaf toughness, our palatability bioassay suggested that leaf chemical defence was an effective anti-herbivore defence strategy, supporting a wide body of literature demonstrating the efficacy of secondary chemical compounds as herbivore deterrents in tropical forests (e.g. Agrawal & Weber 2015, Coley 1986, Dyer *et al.* 2003a, Swain 1977). The lack of relationship between foliar N concentration and chemical defence in our dataset suggests that either N-based defensive alkaloids are not the primary chemicals used by our study species to deter herbivores, that the methanol extractions we used for our bioassay did not adequately extract many N-based leaf alkaloids, or that foliar N concentration is simply a poor predictor of leaf alkaloid concentrations. Previous work linking the palatability bioassay that we use to specific leaf chemical defensive compounds suggests that our methanol extraction does effectively isolate defensive alkaloids that have strong anti-herbivore properties (Dyer *et al.* 2003b). Rather, it seems more likely that foliar elemental composition is simply a poor predictor of leaf chemical defence, as has been suggested elsewhere (Hamilton *et al.* 2001). Alternatively, the methanol extractions used in our bioassay may have also extracted some non-defence, N-rich compounds that are palatable to the ants used in our bioassay. This has been seen in previous studies of this bioassay (Dyer *et al.* 2003b), and could influence our ability to detect a relationship between leaf N and chemical herbivore deterrence.

Tropical trees can employ a wide variety of additional anti-herbivore defences that were not assessed in our study. One additional defence strategy that may be of particular importance for this study is the use of extrafloral nectaries to attract ant species that deter insect herbivores because extrafloral nectaries are a common trait in the *Fabaceae* family (e.g. Bentley 1977). The N-fixing species in our study and the non-fixing *Fabaceae* species *D. panamensis* all produce extrafloral nectaries (Weber *et al.* 2015), which could be significant deterrents to herbivores for these species. However, the fact that species in our study with extrafloral nectaries span the entire range of % LAC measured (Figure 5b) indicates that these extrafloral nectaries are not creating a strong bias driving species- or fixer-level comparisons of herbivory damage in our study.

Herbivory

While we found support for high N concentrations in N-fixer leaves, this high foliar nutrient content neither increased nor decreased herbivory in our study species (Figure 4g) and did not drive significant differences in herbivory between N fixers and non-fixers (Figure 5a). This contrasts with the pattern that is often hypothesized in the literature (Mattson 1980, Menge *et al.* 2008, Vitousek & Field 1999, Vitousek & Howarth 1991) and with the limited experimental evidence available from other ecosystems (Hulme 2008, Knops *et al.* 2000, Ritchie *et al.* 1998, Ritchie & Tilman 1995, Simonsen & Stinchcombe 2014). The lack of a relationship between foliar N concentrations and herbivory also contradicts ecological stoichiometry theory, which posits that leaf nutritional content shapes herbivore choice (Elser *et al.* 2000). Instead, our results agree with recent work suggesting that leaf defences, not leaf nutritional content, are the dominant driver of herbivory for tropical trees (Agrawal & Weber 2015).

Discrepancies between our findings and previous results may be due to site differences (grasslands and savannahs vs. tropical rain forests), taxonomic differences among N-fixing species, or that variation in N fixation rates among our N fixers created sufficient variation to preclude statistical differences in some foliar traits between our qualitative N fixer and non-fixer groups. Ample data suggest that *P. macroloba* exhibits high N fixation rates in these forests (Taylor *et al.* 2019, Taylor & Menge 2018), but no data currently exist for fixation rates of the three *Inga* N-fixers at our study site. However, the fact that we did find significant differences in foliar chemistry and leaf toughness between N fixers and non-fixers suggests that type II error did not preclude us from identifying differences between these plant groups when present.

When contextualizing our results on herbivore damage, it is important to consider exactly what our measurement, % LAC, can assess. Because we did not track herbivory damage through time, these data should not be interpreted as herbivory rates, but rather as the average per cent of leaf area that a plant has invested in but that was subsequently damaged by herbivores. We focused on this metric because it provides a good assessment of the reduction in total photosynthetic surface area of a plant due to herbivory (assuming plant-level herbivory is at relative steady state), but it is important to recognize the potential limitations of % LAC – particularly the influences of leaf lifespan and leaf abscission.

Because leaf lifespan can vary substantially between species, for a given % LAC, a species with relatively short leaf lifespan would indicate higher rates of herbivory than a species with longer leaf lifespan (Poorter *et al.* 2004). The lack of leaf lifespan data in the literature for six of our seven study species (King & Maindonald 1999) precluded us from incorporating this into our analyses, but evidence that leaf lifespan is negatively related to leaf N concentrations (Reich *et al.* 1992) suggests that this should be an important consideration for studies assessing differences in herbivory rates (rather than % LAC) between N fixers and non-fixers. Leaf or leaflet abscission in response to partial herbivory may also influence the interpretation of % LAC if species differ in their propensity to shed leaf tissue in response to partial herbivory. The fact that our assessment of % LAC accounted for abscised leaflets but not fully abscised leaves, raises the possibility that compound-leaf species with small leaflets in our dataset could abscise these small leaflets more readily in response to partial herbivory, inflating % LAC for these species. However, the fact that the two species with the smallest leaflets (*P. macroloba* and to a lesser extent *D. panamensis*) exhibited the

lowest % LAC (Figure 5b) indicates that the effect of leaflet abscission was not strong in our study but does raise the possibility that leaflet abscission in response to partial herbivory is an effective anti-herbivore strategy.

Effects of species-level taxonomic variation

Overall, our results indicate substantial species-level differences in leaf chemistry, defence and herbivory between our seven study species. We found a ~3-fold difference in herbivory among our N-fixing species and more than a 14-fold difference in herbivory among our non-fixing species. We did not, however, find evidence that herbivory was phylogenetically structured among our N-fixing species, as we found no significant differences in herbivory between the two N-fixing genera used in this study, *Inga* and *Pentaclethra*.

Similar to herbivory damage, we found that species differ markedly in their reliance on chemical defence. Our metric of leaf chemical defence, ACD, varied 24-fold among our species, and varied by an order of magnitude even among our congeneric N-fixing *Inga* species (Table 1). This supports recent findings that even closely related species in tropical forests can employ highly diverse chemical defensive compounds (Sedio *et al.* 2018). Our findings highlight that tropical N fixers represent a diverse group (Doyle & Luckow 2003) that has presumably evolved a variety of strategies to deal with herbivore pressure.

Our finding that herbivory does not differ between our N-fixing and non-fixing species groups (Figure 5a) provides important insight on the interaction between herbivory and tropical N-fixer ecology. Among our study species, the N fixer *P. macroloba* exhibited one of the highest foliar N concentrations but among the lowest levels of herbivory. This is particularly interesting given the notably high relative abundance of *P. macroloba* in these forests (McDade & Hartshorn 1994, Menge & Chazdon 2016). It is possible that high foliar N, which drives high photosynthetic rates (e.g. Wright *et al.* 2004), combined with an ability to avoid herbivory are key components to the high survival rates that drive the dominance of *P. macroloba* in this region (Menge & Chazdon 2016). Furthermore, among our four N-fixing species, those with the highest relative abundances (*P. macroloba* and *I. thibaudiana*) exhibited the lowest herbivory damage (Figure 5b), but those that sustained high herbivory damage (*I. oerstediana* and *I. sapindoides*) are relatively rare in these forests (Table 2 in Menge & Chazdon 2016). Thus, we conclude that while herbivory may be an important factor constraining the relative abundances of some N-fixing species, other N fixers seem able to avoid this ecological constraint.

Conclusion

Given the importance of both N fixers and herbivory in the C and nutrient cycling of tropical forests (Metcalfe *et al.* 2014, Vitousek *et al.* 2013), understanding how herbivores influence N-fixer ecology is critical to our broader knowledge of the ecosystem processes of tropical forests. The current paucity of data on the herbivory of tropical N fixers leaves a large gap in this understanding. Overall, our results suggest that herbivory pressure and anti-herbivore defence strategies are species-specific in tropical forests, even within N-fixing and non-fixing plant groups. These species-specific responses to herbivores may help determine the distribution and abundance of N-fixing species within tropical forest landscapes. The range of herbivory pressure and anti-herbivore defence strategies among N fixers that this study demonstrates suggests

that many more studies are needed on a wider set of N-fixing and non-fixing tree species to fully understand the dynamics between N fixers, herbivores and element cycling in tropical forests.

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