Research

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Light fuels while nitrogen suppresses symbiotic nitrogen fixation hotspots in neotropical canopy gap seedlings

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Summary

- Mature neotropical lowland forests have relatively lower symbiotic nitrogen fixation (SNF) rates compared with secondary forests. Canopy gap formation may create transient SNF hotspots in mature forests that increase overall SNF rates in these ecosystems, as canopy gaps are pervasive across the landscape and increasing in frequency. However, what environmental conditions are driving SNF upregulation in canopy gaps is unknown.
- In a field experiment to test these potential environmental controls on SNF, we grew 540 neotropical nitrogen-fixing legume seedlings (*Pentaclethra macroloba*, *Zygia longifolia*, and *Stryphnodendron microstachyum*) under manipulated light and soil nitrogen availability in canopy gaps and intact forests at La Selva Biological Station, Costa Rica.
- Seedling biomass, nodule biomass, and SNF (g N seedling^{-1} h^{-1}) were 4-, 17- and 42-fold higher, respectively, in canopy gaps than in the intact forest. Nitrogen additions decreased SNF, but light had a stronger positive effect. Upregulation of SNF in canopy gaps was driven by increased plant growth and not a disproportionate increased SNF allocation.
- These data provide evidence that canopy gap SNF hotspots are driven, in part, by light availability, demonstrating a potential driver of SNF spatial heterogeneity. This further suggests that canopy gap dynamics are important for understanding the biogeochemistry of neotropical forests.

Introduction

Tropical forests play an outsized role in global carbon, water and nutrient cycling (Brown & Lugo, 1990; Carswell *et al.*, 2000; Chazdon *et al.*, 2009; Pan *et al.*, 2011, 2013). The high primary productivity of these forests may be fuelled, in part, by high legume abundance and symbiotic nitrogen fixation (SNF) rates (Cleveland *et al.*, 1999; Houlton *et al.*, 2008; Hedin *et al.*, 2009; Batterman *et al.*, 2013a; Davidson *et al.*, 2018; Taylor *et al.*, 2019). The high carbon cost of SNF suggests that the benefit of SNF should decrease when soil N availability is high, leading to SNF downregulation under these conditions (Menge *et al.*, 2009). This factor is one reason why high SNF rates in neotropical forests, despite generally high soil N availability, are thought to be paradoxical (Cleveland *et al.*, 1999; Hedin *et al.*, 2009; Menge & Levin, 2017). One proposed explanation of this paradox is a spatial disconnect between where SNF occurs and where soil N availability is high, such as within vertical layers of the soil and canopy (Hedin *et al.*, 2009) or horizontally across a landscape (Gehring *et al.*, 2005; Menge *et al.*, 2009; Barron *et al.*, 2011; Batterman *et al.*, 2013a; Bauters *et al.*, 2016; Menge & Levin, 2017; Osborne *et al.*, 2017; Taylor *et al.*, 2019). This spatial disconnect suggests that hotspots of SNF may be driven by factors that increase N demand, as well as differences in N supply. For example, a survey of root nodules (the plant tissue where SNF occurs) found higher nodulation in high light environments, including canopy gaps, relative to the mature intact forest (Barron *et al.*, 2011). SNF estimates increase five-fold when considering the potential canopy gap effect in mature neotropical forests (Sullivan *et al.*, 2014). However, whether, how much and why canopy gaps are hotspots remains poorly understood. Therefore, a better understanding of the drivers that could lead to SNF upregulation in canopy gaps awaits field-based manipulative experiments.

One hypothesis for SNF upregulation in canopy gaps is the increased availability of light in these microenvironments. Tree and/or branch falls create gaps in the dense forest canopy, leading to high light environments within the mature tropical forest matrix (Hartshorn, 1978; Lieberman *et al.*, 1985). This may allow legumes to increase photosynthesis and allocate more carbon (in either absolute or relative terms) to their N-fixing bacterial partners and/or increase the N demand of legumes regardless of soil N availability (Myster, 2006; Kaschuk *et al.*, 2009; Taylor & Menge, 2018). Indeed, this light effect has been assumed to drive observed increased nodulation in high light edge environments (Barron *et al.*, 2011).

Light availability is not the only potential driver of SNF upregulation that differs between canopy gaps and intact forests. The creation of canopy gaps alters soil nutrient cycling dynamics in several ways (Vitousek & Denslow, 1986; Denslow *et al.*, 1998;

Ostertag, 1998). For example, the combination of high N leaching and high N demand from rapid plant growth rates may lead to N limitation of seedling growth and increased benefit of SNF (Brokaw, 1985; Vitousek & Denslow, 1986; Denslow *et al.*, 1998; Ostertag, 1998; Chou *et al.*, 2018). Denitrification also contributes to N losses in tropical forests (Houlton *et al.*, 2006; Winbourne *et al.*, 2018a; Soper *et al.*, 2018b), but to our knowledge denitrification rates in relation to canopy gap dynamics has not been quantified. By contrast with mechanisms that create N limitation, canopy gap formation may also increase N availability through increases in litter inputs and mineralisation rates (Vitousek & Denslow, 1986; Denslow *et al.*, 1998; Ostertag, 1998). The relative importance of these various processes and the implications they have on overall N supply vs demand in canopy gaps and intact forests precludes a robust prediction of how N availability may differ between canopy gaps and adjacent forests.

Observations across a successional chronosequence, and a shadehouse experiment, both suggest stronger SNF regulation in seedlings by light compared with soil N for one neotropical legume species (*Pentaclethra macroloba*; Taylor & Menge, 2018). Based on this evidence, it is possible that either or both light and soil N availability influence SNF in legumes growing in canopy gaps. However, in addition to the lack of experimental evidence from field manipulations, it is likely that the response of different legume species to these abiotic drivers may be species specific. Neotropical legume species identity not only explains significant differences in SNF rates among species, but also can determine differences in SNF responses to abiotic variables (e.g. soil P and water availability; Wurzburger & Hedin, 2016; Batterman *et al.*, 2018; McCulloch *et al.*, 2021).

While the mechanisms are not well documented, the idea that canopy gaps can be SNF hotspots has some empirical support (Barron *et al.*, 2011; Wurzburger & Hedin, 2016). One study found 10-fold the number of root nodules around adult legume trees near canopy gaps and riverbanks compared with less disturbed sites (Barron *et al.*, 2011). Based on this finding, and the plausible hypothesis that higher light availability might lead to higher SNF, accounting for canopy gap dynamics may increase SNF estimates in mature neotropical forests. In fact, one estimate of SNF found a five-fold increase in SNF when including canopy gap dynamics in mature forests by aggregating SNF rates in secondary forests and applying these values to canopy gap turnover rates (Sullivan *et al.*, 2014). We do not currently understand the potentially large effects of canopy gaps on landscape-scale SNF estimates, and one of the only estimates of mature tropical forest SNF (Sullivan *et al.*, 2014) is based on a reasonable, but untested, assumption. As a result, a better understanding of the mechanistic controls on SNF in canopy gaps and their relative importance has the potential to greatly improve our understanding of SNF in neotropical forest landscapes. These processes may be increasingly more prevalent as tree morality, and therefore canopy gap formation, is increasing (McDowell *et al.*, 2018; Aleixo *et al.*, 2019).

To test if and why canopy gaps are SNF hotspots, and specifically how light and soil N availability alter SNF in field conditions, we planted seedlings from three neotropical N-fixing legume species directly into canopy gaps and in adjacent undisturbed understory sites under manipulated levels of light and soil N availability. We used this experimental design to ask:

(**1**) Is SNF in these seedlings higher in canopy gaps?

(**2**) If so, what abiotic factors drive this upregulation?

(**3**) Is SNF downregulated with N fertiliser addition?

(**4**) Are there species-specific responses to the experimental treatments?

We hypothesised that the increased light availability in canopy gaps would lead to higher SNF in these seedlings and therefore significantly upregulate SNF, whereas SNF would be downregulated under N fertiliser addition. We also hypothesised that the SNF responses of individual legume species might differ in the magnitude of the response, but not the direction (e.g. SNF in each species will respond positively to light, but some more strongly than others).

Materials and Methods

Study site

We planted 540 legume seedlings in canopy gaps formed during a windstorm in May 2018 that created a matrix of disturbed forest (*c.* 6 ha) at La Selva Biological Station (Costa Rica, 10°25′53.14″N, 84°0′10.51″W). The average daytime temperature at La Selva is 25°C, and remains relatively constant throughout the year. The annual precipitation is *c.* 4500 mm yr^{-1} with a dry season from January through April (>100 mm $month^{-1}$) and a less pronounced dry season in September and October (Chazdon & Fetcher, 1984; Loescher *et al.*, 2005). This study was conducted in a >70-yr-old secondary forest overlying primarily Inceptisol soils (Sancho & Mata, 1987; Sollins *et al.*, 1994). These soils are more fertile than adjacent upland Ultisols soils at La Selva Biological Station, and also than many other lowland tropical soils (Sollins *et al.*, 1994; Powers *et al.*, 2005a; Porder *et al.*, 2006).

Study species

We grew three tree species of known N fixers from the Fabaceae family that are native to La Selva Biological Station. These included: *Pentaclethra macroloba*, which is the most abundant canopy species at this site (Hartshorn, 1980; Lieberman & Lieberman, 1987), *Zygia longifolia*, a species commonly found in riparian zones in the region, and *Stryphnodendron microstachyum*. Most neotropical N-fixing legumes are thought to be facultative N fixers (Sheffer *et al.*, 2015) and while we have more information about some (*P. macroloba*) than others (*S. microstachyum*), all three species are relatively common as canopy trees at La Selva. For each species, we collected seeds from a parental tree in January 2019 and planted these seeds in individuals pots with native soil in a shadehouse at La Selva. While in the shadehouse the seedlings were watered daily and received no fertiliser. After 6 wk, they were transplanted into the field and each seedling was randomly assigned an experimental treatment.

Experimental design

In March 2019, we established five spatially separated gap sites and five sites in the adjacent understory. Each site was *c.* 10 m in diameter and a minimum of 30 m apart from the next closest site. We transplanted the seedlings into the field in March 2019, and allowed them to grow for 4 months under one of three light conditions: (1) in the gap under natural light intensities (gap-full light); (2) in the gap under a 50% light reducing shadecloth (gap-shade); or (3) in adjacent undisturbed understory (understory). These three light conditions were crossed with N fertiliser treatments (N addition ($20\,g\,N\,m^{-2}\,yr^{-1}$) or no N addition). Liquid N fertiliser (ammonium nitrate solution) made in the laboratory was dispensed at the beginning (March 2019) and midpoint (May 2019) of the experiment. This created an experimental design with six treatments: gap-full light + N, gap-full light, gap-shadecloth + N, gap-shadecloth, understory + N, and understory. We grew 30 individuals from each species in each of the six treatments. To hedge against losing an entire site to a treefall or large animal damage, we spread seedlings throughout the site in groups of six seedlings (two of each species).

The seedlings had no nodules at the time of planting and we did not inoculate the seedlings with N-fixing bacteria. Instead, we planted seedlings bare rooted in the forest soil and therefore exposed the seedlings to a native suite of N-fixing bacteria under field conditions.

Site characterisation

Light conditions (lux) were measured using Onset HOBO Pendant® Temperature/Light 8K data loggers at each of our 10 sites. Each of the five gap sites had three data loggers mounted above seedlings (but below the shadecloth) in the shadecloth treatment (15 total), and another three in each gap-full light treatment (15 total). As light in the understory was much less variable, we set out a total of five data loggers there, one at each of the five understory sites. All data loggers were placed in the middle of a group of seedlings at a height of 0.85 m. Light measurements were taken every hour for the 4-month duration of the field experiment. To calculate total daily photosynthetically active radiation, we calculated average daily area under the curve (AUC) values from the light amounts reported from each data logger. Two additional data loggers were placed in the laboratory clearing at La Selva Biological Station (*c.* 1.5 km away) for 'total light' values, and we report light in the experiment as a percentage of the light incident on the clearing at the laboratory.

In addition to light, we measured soil nitrate and phosphate availability, and soil moisture at each of the 10 sites to estimate site-level soil variation (Supporting information Table S1). We used anion-exchange resins membranes to determine nitrate and phosphate availability over two separate 2-wk periods (March and July 2019 to capture potential dry vs wet season variability). We cut 2 × 10 cm anion-exchange membrane strips from sheets of cross-linked copolymers of vinyl monomers (General Electric #AR204SZRA). We prepared the strips by shaking them for 24 h in $NaHCO_3$ (for phosphate) and 1 M NaCl (for nitrate). We placed 12 anion-exchange membranes (six phosphate, six nitrate) at a 45° angle into the top 10 cm of soil at each site in March and 20 (10 phosphate, 10 nitrate) at each site in May. After 14 d in the field, we collected the membranes and rinsed them with distilled water to remove residual soil. Strips were kept at 4°C and transported to Brown University, where resin strips were shaken for 4 h in either 30 ml of 0.5 M HCl (for phosphate) or 30 ml of 2 M KCl solution (for nitrate). We then filtered samples through Swinnex filters and concentrations of nitrate and phosphate extracted from samples were analysed colorimetrically on a Westco SmartChem 200 Discrete Element Analyser (Westco Instruments, Brookfield, CT, USA). While we did not measure soil ammonium, previous work in canopy gaps and intact forests at La Selva showed comparable ammonium and nitrate levels (Vitousek & Denslow, 1986). Based on this, we expect nitrate concentrations to broadly indicate patterns in soil N availability, as it does in other lowland Costa Rican rainforests (Osborne *et al.*, 2020). We took soil moisture measurements monthly using a soil moisture probe (FieldScout TDR 300 #6430FS; Spectrum Technologies Inc., Aurora, IL, USA) and soil moisture values are reported as % volumetric water content (VWC).

Seedling growth and data collection

Sites were visited weekly to note seedling mortality (*P. macroloba*, $n = 16$; *S. microstachyum*, $n = 21$; *Z. longifolia*, $n = 8$) and to remove encroaching vegetation from treatment plots. In July 2019, *c.* 6 months after seedling germination and 4 months after experimental treatments started, we harvested seedlings and measured SNF rates, root, shoot, foliar and nodule biomass. To prevent temporal sampling bias, we harvested six seedlings from each of the six experimental treatments each day, for a daily total of 36 harvested seedlings. During harvest, we dug the seedlings out, gently removed excess dirt, washed the root system in the field, and immediately began incubations to measure SNF (see section on *Symbiotic nitrogen fixation*). At the time of harvest, we also noted if seedlings had high levels of herbivory, which we determined to be when *c.* > 25% of the leaf area appeared damage. After excision of nodules for SNF incubations, seedlings were transported back the laboratory, separated into leaves, shoots and roots, and placed in a drying oven at 60°C for 72 h before weighing for biomass estimates.

Symbiotic nitrogen fixation

For each seedling with nodules, we randomly selected 20 nodules at the time of harvest and alternately placed 10 each into two glass vials to estimate SNF using $^{15}N_2$ enriched gas incubations. All incubations were performed in the morning in the field immediately after removing the seedling from the ground. If root systems had more than 20 nodules, we collected the excess nodules separately to include in the measurements of total nodule number and biomass. For each seedling with nodules, we injected vials containing up to 10 nodules with 20% headspace of isotopically $^{15}N_2$ enriched N_2 gas (99‰) and incubated the vials for 30 min (Batterman *et al.*, 2013a, 2018; Winbourne *et al.*,

2018a). Another jar with nodules from the same seedling was incubated with ambient air to control for natural ^{15}N abundance in each sample. When seedlings had fewer than 20 nodules, the nodules were evenly divided between enrichment and control incubations. After incubation, nodules were dried on silica gel for at least 72 h before weighing. We sent *c.* 2.5 mg of enriched and control nodule samples to the UC Davis Stable Isotope Facility for total nitrogen and $\delta^{15}N$ analysis using a PDZ Europa ANCA-GSL elemental analyser interfaced to a continuous flow isotope ratio mass spectrometer (PDZ Europa 20-20; Sercon Ltd, Cheshire, UK). We used the difference between $\%^{15}N$ in enriched nodules and $\%^{15}N$ in control nodules to calculate the N fixation that occurred during the incubation (Anderson *et al.*, 2004). When control nodules were not available (32 of 540 seedlings had one only nodule or negligible nodule biomass) we used a natural abundance atom $\%^{15}N$ of 0.3663 (Menge *et al.*, 2015; Taylor *et al.*, 2019), which was not significantly different from the mean nodule natural abundance in our samples (0.3686 $\pm$ 0.0070), to calculate SNF rates.

Statistical analysis

To test variability in light intensities (% total transmittance) among and within the three categorical light treatments (gap-full light, gap-shadecloth, and understory), we used Kruskal–Wallis rank sum test with Wilcox pairwise comparisons to test for differences between light treatments as assumptions for normality were not met. However, to explore the effects of light on seedling characteristics, we leveraged site-to-site variation within light treatments and assessed light as a continuous variable. We used Wilcox rank sum tests to test for differences in soil N and P availability and soil moisture between canopy gap and intact forest sites, as assumptions for normality and homogeneity were not met. We reported the arithmetic means and standard deviations of these values for the different light treatments or environment type (canopy gap vs understory).

We used generalised additive models for location, scale and shape (GAMLSS) to test for significant differences between environment type (canopy gap vs understory), while testing for light and N fertiliser treatments effects on the response variables (plant biomass (g), N fixed per g nodule (g N g^{-1} nodule h^{-1}, nodule biomass (g), nodule allocation (g nodule g^{-1} seedling), N fixed (g N seedling^{-1} h^{-1}), and N fixed per g seedling (g N g^{-1} seedling h^{-1})). GAMLSSs work similarly to a generalised linear model or a generalised additive model, but also to allow for data distributions such as those found in our response variables, which were zero-inflated with a strong positive skew (Rigby *et al.*, 2005; Stasinopoulos & Rigby, 2007). We used log-normal distribution for plant biomass and a zero adjusted gamma distribution for all SNF variables. Both distributions have a parameter that represents the mean of distribution (denoted as μ) and the coefficient of variation of the distribution (denoted as σ). The zero adjusted gamma distribution has a third parameter (denoted as ν) that models the probability of zeros occurring and the model outputs include an intercept for each distribution parameter (Tables 1, S2, S3).

Fixed effects in the GAMLSSs for SNF variables included: light, N fertiliser treatment, measurements of site characteristics (nitrate and phosphate availability, and soil moisture), species identity, and environment type (canopy gap vs understory). We used the same fixed effects for plant biomass with the addition of the presence or absence of heavy herbivory (*c.* >25% leaf area damage). We used site-specific light conditions (% light transmittance) as opposed to the three categorical light treatments in these analyses and only reported light and N fertiliser treatment interaction terms when they were significant (Tables 1, S2, S3). When species identity was significant, we then ran models separately for each species to capture potential species-specific responses (Table S2). The number of observations in each model sometimes varied among the SNF response variables because of how we defined missing values. For example, when there were no nodules present we denoted this as 0 g and we have 495 observations (all the seedlings that survived to harvest). For N fixed a zero signified that there was no N fixation detected from isotopic analysis but there were nodules present and NA (which are not run in the model) are seedlings that had no nodules present, which lead to a total of 396 observations in the model. All statistical analyses and figures were performed in R v.3.6.2, with TIDYVERSE (Wickham *et al.*, 2019), GGPLOT2 (Wickham, 2016), GAMLSS (Rigby *et al.*, 2005), and DESCTOOLS (Signorell, 2020) packages.

As no single measurement of SNF in the field can capture the amount of N fixed over a 4-month experiment (Unkovich *et al.*, 2008), we leveraged a wide range of SNF-related response variables to test how the experimental treatments influenced SNF. Nodule biomass (g seedling^{-1}) and N fixed (g N seedling^{-1} h^{-1}) estimate the SNF capacity of the seedlings, the former over the course of the experiment and the latter at the moment of harvest. However, high values of these variables in response to a particular treatment could indicate an indirect effect of more plant growth on SNF (bigger plants might have more nodules and therefore fix more N). Changes in SNF variables normalised by plant biomass, would indicate a direct effect on SNF (a treatment could drive plants to have more nodules, or more effective nodules, even if plant growth overall was not different). To explore these two possibilities, we normalised the SNF response by plant or nodule biomass and report: N fixed per gram nodule (g N g^{-1} nodule h^{-1}, derived from ^{15}N analysis), nodule allocation (g nodule g^{-1} seedling), and N fixed per gram seedling (g N g^{-1} seedling h^{-1}, again derived from ^{15}N analysis). Additionally, we used non-normalised variables (nodule biomass and N fixed) determine if the experimental treatments or site variables had an effect on the amount of SNF occurring overall in canopy gaps vs understories, whereas normalised variables were more indicative of whether the experimental treatments upregulated SNF within individual seedlings.

Results

Environment conditions differ between gaps and intact understory

Seedlings in the gap-full light received on average significantly more light (38.3 $\pm$ 9.7% total light transmittance of laboratory

Table 1 GAMLSS summary statistics for total biomass, nodule biomass, nodule allocation and N fixed.[1]

Predictors	Estimates	Std. error	Statistics	*P*-value
Total biomass (g)				
Intercept (μ)	0.11	0.33	0.34	0.731
Light (% light transmittance)	1.01	0.21	4.84	**<0.001**
Nitrogen fertiliser addition	0.16	0.05	2.85	**0.005**
Light × nitrogen fertiliser addition	NA	NA	NA	NA
Nitrate availability (mg N cm^{-2} d^{-1})	0.05	0.05	0.9	0.369
Phosphate availability (mg P cm^{-2} d^{-1})	2.38	5.49	0.43	0.664
Soil moisture (%VWC)	0.04	0.01	3.75	**<0.001**
Zygia longifolia	−1.4	0.11	−12.22	**<0.001**
Stryphnodendron microstachyum	−1.03	0.08	−12.46	**<0.001**
Environment type (understory)	−1.28	0.33	−3.82	**<0.001**
Herbivory (no herbivory)	0.19	0.07	2.57	**0.01**
Intercept (σ)	3.02	0.10	34.74	**<0.001**
Observations	495			
Nodule biomass (g)				
Intercept (μ)	−4.41	0.45	−9.9	**<0.001**
Light (% light transmittance)	1.66	0.46	3.61	**<0.001**
Nitrogen fertiliser addition	−0.39	0.1	−3.91	**<0.001**
Light × nitrogen fertiliser addition	NA	NA	NA	NA
Nitrate availability (mg N cm^{-2} d^{-1})	0.03	0.04	0.8	0.426
Phosphate availability (mg P cm^{-2} d^{-1})	−6.11	2.94	−2.08	**0.038**
Soil moisture (%VWC)	0.07	0.02	4.63	**<0.001**
Z. longifolia	−1.55	0.12	−12.49	**<0.001**
S. microstachyum	−2.08	0.13	−16.66	**<0.001**
Environment type (understory)	−1.98	0.19	−10.37	**<0.001**
Intercept (σ)	0.02	0.03	0.59	0.557
Intercept (ν)	−1.96	0.14	−14.35	**<0.001**
Observations	495			
Nodule allocation (g nodule g^{-1} seedling)				
Intercept (μ)	−5.47	0.39	−14.18	**<0.001**
Light (% light transmittance)	0.35	0.41	0.86	0.391
Nitrogen fertiliser addition	−0.39	0.09	−4.47	**<0.001**
Light × nitrogen fertiliser addition	NA	NA	NA	NA
Nitrate availability (mg N cm^{-2} d^{-1})	−0.04	0.03	−1.27	0.205
Phosphate availability (mg P cm^{-2} d^{-1})	−0.56	2.74	−0.2	0.839
Soil moisture (%VWC)	0.04	0.01	3.21	**0.001**
Z. longifolia	−0.14	0.11	−1.3	0.195
S. microstachyum	−0.6	0.11	−5.29	**<0.001**
Environment type (understory)	−0.66	0.18	−3.74	**<0.001**
Intercept (σ)	−0.09	0.03	−3.1	**0.002**
Intercept (ν)	−1.96	0.14	−14.35	**<0.001**
Observations	495			
N fixed (g N $seedling^{-1}$ h^{-1})				
Intercept (μ)	−5.55	0.85	−6.51	**<0.001**
Light (% light transmittance)	3.01	0.71	4.26	**<0.001**
Nitrogen fertiliser addition	−0.31	0.17	−1.79	0.074
Light × nitrogen fertiliser addition	NA	NA	NA	NA
Nitrate availability (mg N cm^{-2} d^{-1})	−0.02	0.09	−0.19	0.852
Phosphate availability (mg P cm^{-2} d^{-1})	−14.02	8.64	−1.62	0.106
Soil moisture (%VWC)	0.13	0.03	4.32	**<0.001**
Z. longifolia	−1.94	0.2	−9.85	**<0.001**
S. microstachyum	−2.2	0.22	−9.78	**<0.001**
Environment type (understory)	−3.79	0.47	−8.12	**<0.001**
Intercept (σ)	0.34	0.03	9.8	**<0.001**
Intercept (ν)	−0.97	0.11	−8.6	**<0.001**
Observations	396			

[1]*Pentaclethra macroloba* is used as the reference for species comparisons. For environment type and herbivory estimate values are based on the comparison of understory sites to gap sites and comparisons of no herbivory to herbivory factors, respectively. For example, a negative estimate suggests that understory or no herbivory is lower than gap or herbivory, respectively. There are intercept values for each of the distribution parameter values (μ, σ, and ν) of the GAMLSS distributions used. 'NA' values indicate not applicable, as treatment interaction terms were not included in the model if they were not significant. *P*-values less than 0.05 are emboldened.

clearing) compared with those in the gap-shade (15.3 ± 4.2%, $P = 0.024$) and understory (7.5 ± 2%, $P = 0.024$) treatments (Fig. S1). The gap-shade light treatment was slightly higher than the understory treatment ($P = 0.048$), with the lowest light treatment levels found in the understory (Fig. S1). We found that canopy gap sites had significantly lower soil moisture (29.6% VWC) compared with intact forests (35.3% VWC; $P < 0.001$; Fig. S2). Soil N availability in the 10 study sites was variable (3.04 ± 4.03 mg N cm^{-2} d^{-1}), and we found higher N availability in canopy gap sites ($P < 0.001$). This difference in site soil N availability was driven by one site (Site #2; $P < 0.001$; Table S1) and soil N availability did not vary significantly between our two sampling times ($P = 0.81$). Soil P availability was higher in understory sites (0.049 ± 0.087 mg P cm^{-2} d^{-1}) compared with canopy gap sites (0.039 ± 0.064 mg P cm^{-2} d^{-1}; $P < 0.001$; Table S1; Fig. S2) and was significantly lower in the wet season (0.036 ± 0.077 mg P cm^{-2} d^{-1}) compared with the dry season (0.058 ± 0.064 mg P cm^{-2} d^{-1}; $P < 0.001$).

We found a significant increase in all measured response variables (total biomass, nodule biomass, nodule allocation, N fixed per seedling, N fixed per gram seedling and N fixed per gram nodule) when seedlings were grown in canopy gaps (with gap-full light and gap-shadecloth combined) vs intact understory for all the species combined, and for *P. macroloba* and *Z. longifolia* individually (Table 1; Fig. 1). For *S. microstachyum*, total biomass and nodule biomass were significantly higher in the canopy gap sites than in the understory sites ($P < 0.001$, $P < 0.001$, respectively; Table 1). For all the species combined, the mean

(arithmetic) total plant biomass (g) was 3.6-fold higher in canopy gap sites than in understory sites ($P < 0.001$, Fig. 1a). There was over 17-fold higher nodule biomass and two-fold higher nodule allocation (g nodule g^{-1} seedling) in gap sites based on the arithmetic means ($P < 0.001$, $P < 0.001$, respectively; Fig. 1b,c). Nitrogen fixed (g N seedling^{-1} h^{-1}) was more than 42-fold higher in the seedlings grown in the gap sites compared with those grown in understory sites based on the arithmetic means of the environment types ($P < 0.001$; Fig. 1d).

Seedling biomass increased with light and soil N availability

There was a significant positive relationship between total plant biomass and site-specific light intensities (measured using data loggers under every light treatment at each site) when all three species were pooled ($P < 0.001$; Table 1; Fig. 2a). Nitrogen fertiliser addition also had a significant positive effect on total biomass ($P < 0.003$). However, GAMLSSs run for each individual species (Table S2) indicated that N fertiliser addition only had a marginally significant positive effect on *P. macroloba* biomass ($P = 0.057$; Fig. 2), and not on *S. microstachyum* ($P = 0.81$; Fig. 2) or *Z. longifolia* ($P = 0.54$; Fig. 2a). These two species did exhibit significant positive relationships between plant biomass and light intensities (*S. microstachyum*, $P < 0.012$; *Z. longifolia*, $P < 0.001$; Fig. 2a; Table S2). Additionally, plant biomass had significant relationships with herbivory (negative, $P = 0.01$) and soil moisture (positive, $P < 0.001$; Table 1).

Light availability increased N fixation

Overall, higher light availability increased nodule biomass ($P < 0.001$; Table 1; Fig. 3a), while N fertiliser addition significantly decreased nodule biomass ($P < 0.001$; Table 1; Fig. 3a). We also found significant relationships between nodule biomass and soil P availability (negative; $P = 0.038$; Table 1; Fig. S2a), as well as soil moisture (positive; $P < 0.001$; Table 1; Fig. S2b). Individually, two species exhibited significant increases in nodule biomass with increased light intensities (*P. macroloba*, $P = 0.003$; *Z. longifolia*, $P = 0.007$; Table S2; Fig. 2b). Nitrogen fertiliser addition significantly decreased nodule biomass for *P. macroloba* and *S. microstachyum* ($P < 0.001$, $P = 0.024$, respectively; Table S2; Fig. 2b). Nodule biomass differed significantly among species, as the arithmetic mean nodule biomass of *P. macroloba* was five-fold greater than that of *S. microstachyum* and *Z. longifolia* ($P < 0.001$; Fig. 2b).

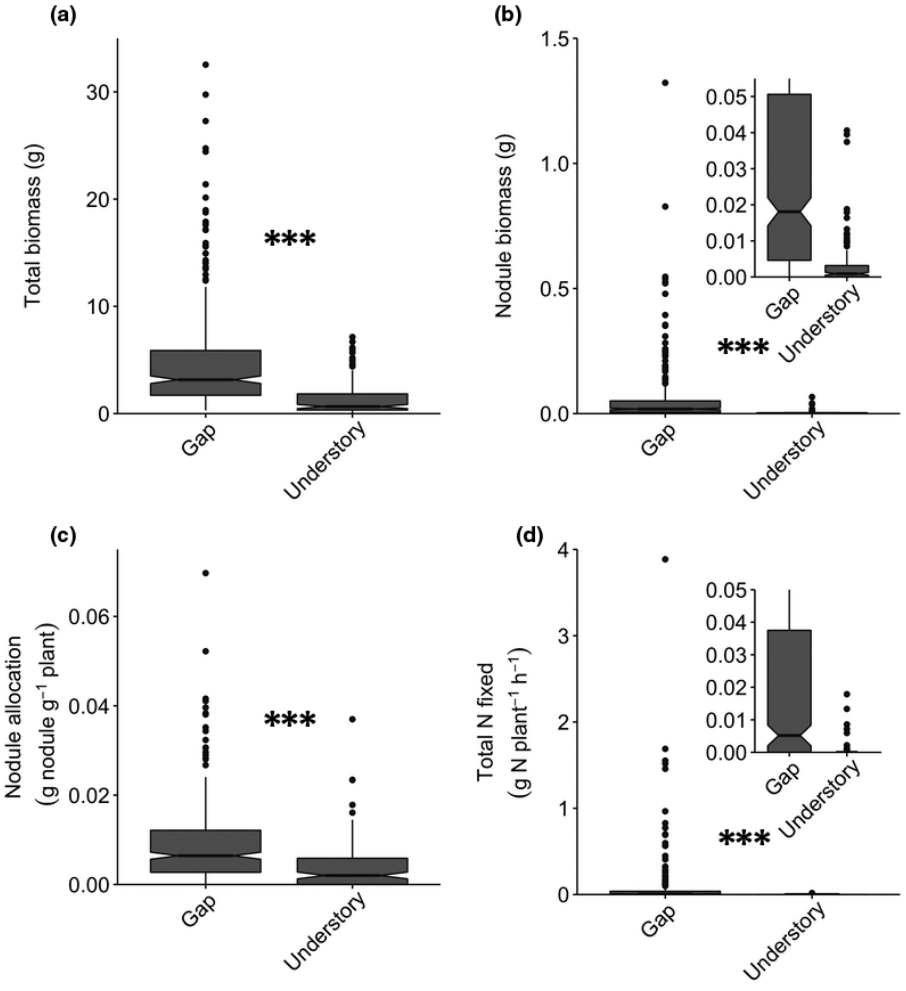

Fig. 1 Gap (gap-full light and gap-shade combined) and understory comparisons for (a) total plant biomass (g); (b) nodule biomass per seedling (g); (c) nodule allocation (g nodule g^{-1} seedling); and (d) total nitrogen (N) fixed (g N seedling^{-1} h^{-1}) for all seedlings combined. Boxes represent the interquartile range, the line is the median, and the whiskers are the lowest or highest value within 1.5-fold the interquartile range. All points are beyond 1.5-fold the interquartile range. Insets in (b) and (d) provide an expanded view of y-axes at lower values. Asterisks indicate $P < 0.05$.

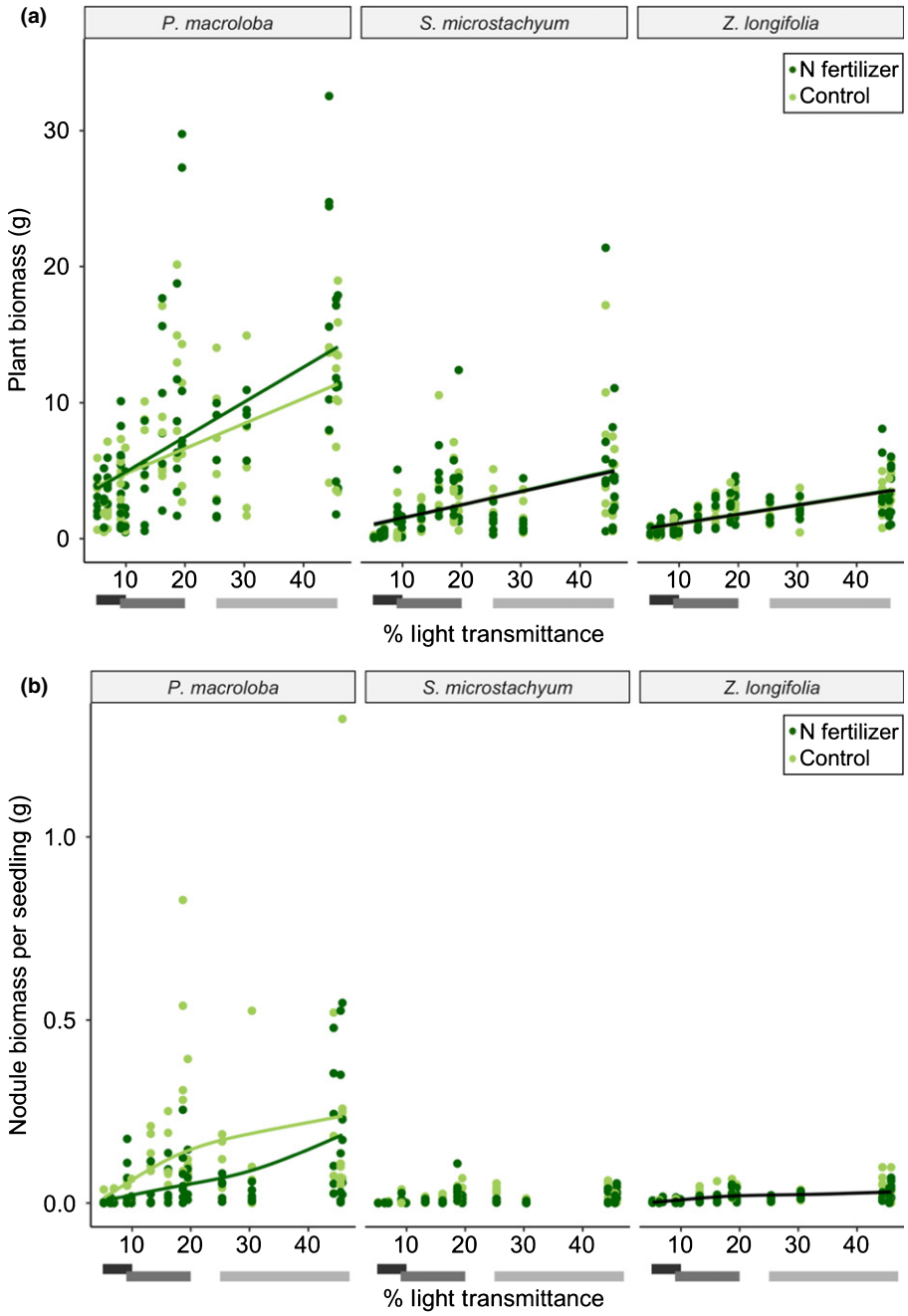

Fig. 2 Light intensity and nitrogen (N) fertilisation treatment effects on (a) total biomass and (b) nodule biomass per seedling, both expressed in grams for each species (*Pentaclethra macroloba*, *Stryphnodendron microstachyum* and *Zygia longifolia*). Horizontal grey bars below the x-axes indicate the light intensities (% Light transmittance) associated with each of the three light treatments (grey colour scale indicates understory, gap-shade and gap-full light treatments from dark to light, respectively). Dark green circles and lines denote seedlings that received N fertiliser and light green circles and lines denote seedlings that received no N fertiliser. Trendlines presented are significant ($P < 0.05$) with different trendlines produced for N fertiliser (dark green) vs no N fertiliser (light green) for *P. macroloba* as the N fertiliser treatment had a marginally significant ($P = 0.057$) effect on (a) total biomass and a significant effect on (b) nodule biomass. Only one trendline (black) was produced when N fertiliser had no significant effect on (a) total biomass or (b) nodule biomass.

With all species pooled, N fixed (g N seedling^{-1} h^{-1}) significantly increased with increased light availability ($P < 0.001$; Fig. 3b) and there was no significant effect of N fertiliser on N fixed ($P = 0.074$). As with nodule biomass, there were species-specific responses. *Z. longifolia* had higher N fixed at higher light availability ($P = 0.002$; Table S2), while the response of N fixed to light availability in *P. macroloba* seedlings interacted the N fertiliser treatment ($P = 0.012$; Table S2). In *P. macroloba*, N fixed was suppressed under the N fertiliser additions, especially for seedlings growing under the gap-shade treatment. N fixed in *S. microstachyum* did not have a strong relationship with light ($P = 0.79$), and N fixed was 9-fold to 1.4-fold lower in

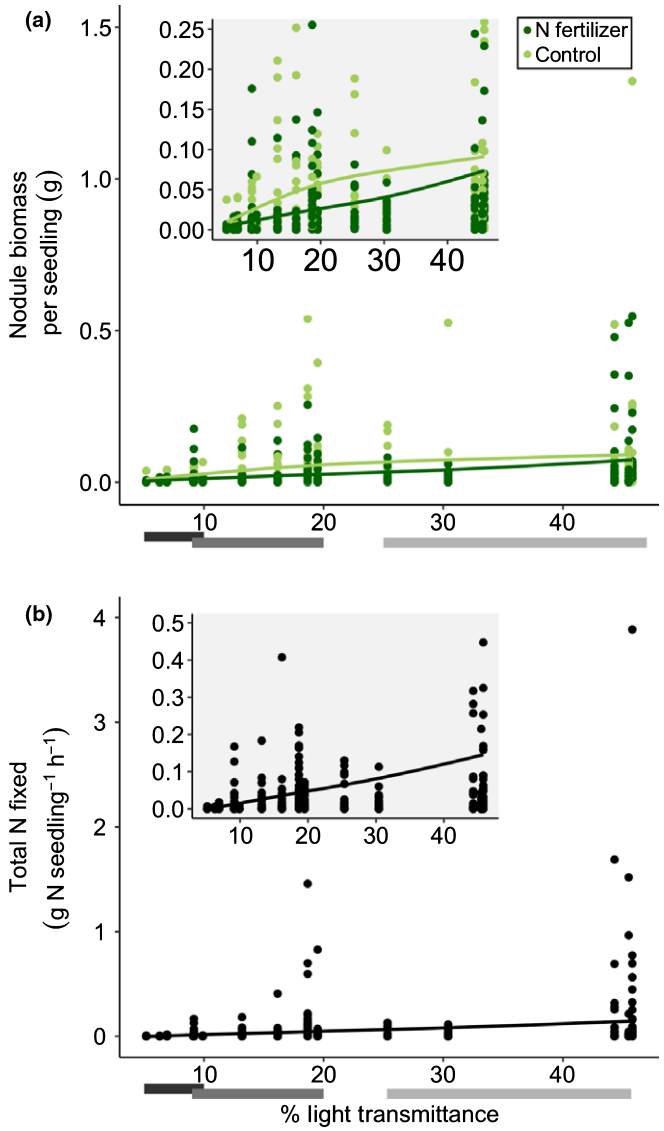

Fig. 3 Light intensity effect on nodule biomass per seedling expressed in grams (a) and total nitrogen (N) fixed expressed in grams N seedling^{-1} h^{-1} (b) for all species pooled. For nodule biomass (a), dark green circles and lines denote seedlings that received N fertiliser and light green circles and lines denote seedlings that received no N fertiliser. All circles and lines in (b) are black, as N fertiliser had no significant effect on total N fixed. Trendlines presented are significant ($P < 0.05$) and were produced using GAMLSS. Horizontal grey bars below the x-axes indicate the light intensities (% Light transmittance) associated with each of the three light treatments (grey colour scale indicates understory, gap-shade and gap-full light treatments from dark to light, respectively) and insets provide an expanded view of y-axes at lower values.

S. microstachyum compared with *P. macroloba* and *Z. longifolia*, respectively.

Seedlings from all three species grew more under high light intensities (Fig. 2a). Therefore, we used nodule allocation (g nodule g^{-1} seedling), N fixed per g seedling (g N g^{-1} seedling h^{-1}) and N fixed per g nodule (g N g^{-1} nodule h^{-1}) to test for treatment effects on SNF while controlling for seedling and/or nodule biomass. Nitrogen fertiliser additions led to lower nodule allocation ($P < 0.001$; Table 1; Fig. 4) for all species pooled and two

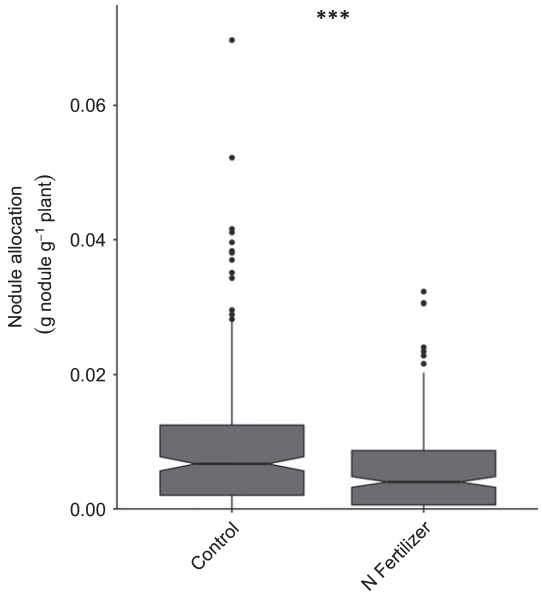

Fig. 4 Nitrogen (N) fertilisation treatment effect on nodule allocation expressed in grams nodule gram^{-1} seedling for all species pooled. Boxes represent the interquartile range, the line is the median, and the whiskers are the lowest or highest value within 1.5-fold the interquartile range. All points are beyond 1.5-fold the interquartile range and asterisks indicate $P < 0.05$.

species individually (*P. macroloba*, $P < 0.001$; *Z. longifolia*, $P = 0.015$; Table S2). N fixed per g seedling was only significantly affected in one species (*P. macroloba*, $P = 0.027$) by light availability (Table S2). Nitrogen fixed per g nodule (g N g^{-1} nodule h^{-1}) was not influenced by the experimental treatments in any of these three species (Table S3), but was positively correlated with nodule biomass (Spearman's correlation test; $r_s = 0.37$, $P < 0.001$).

Discussion

Despite the natural heterogeneity in light, soil nutrients, soil moisture and soil microbial symbionts captured in our experimental sites, we found significantly higher SNF with increases in light availability and suppressed SNF with N fertiliser addition. SNF rates have been observed to decrease as secondary forests transition into mature tropical forests (Batterman *et al.*, 2013a; Davidson *et al.*, 2018; Taylor *et al.*, 2019; but see Winbourne *et al.*, 2018), but this work suggested that SNF is upregulated in mature forest canopy gaps. Here we report experimental evidence to support the hypothesis, generated by shadehouse and observational studies, that conditions in canopy gaps promoted substantially higher rates of SNF compared with those in the surrounding mature tropical forests, and that this difference is mostly attributable to increased light availability.

Light is a large driver of gap effects on symbiotic nitrogen fixation

Light availability had strong, positive effects on total seedling biomass, nodule biomass and total N fixed (Figs 2, 3), and little to

no effect on SNF variables that were normalised by seedling and/or nodule biomass (nodule allocation, N fixed per g seedling, N fixed per g nodule; Tables S2, S3). This suggests that SNF was indirectly limited by light, and that the seedlings that grew largest tended to have the highest nodule biomass and SNF rates. The increased capacity to capture and allocate carbon to N-fixing bacteria in seedlings growing under high light conditions could explain the positive relationship we observed between SNF and light availability. However, it is also possible that, as light availability increases and the seedlings grow, so too does the N demand. SNF may be upregulated to meet this N demand if the soil N supply is not sufficient. We cannot disentangle these two possibilities, but our results suggested the later. If SNF increased only as a result of more carbon allocation to N-fixing bacteria, we would expect to see a positive relationship between light availability and SNF variables normalised by plant biomass, but we do not.

Despite no strong light effect on normalised SNF variables, we still observed a positive effect of increased light on non-normalised SNF variables (nodule biomass and N fixed; Fig. 3) when controlling for environment type (canopy gap vs understory). We examined this relationship for each species individually to determine if there were species-specific responses. *P. macroloba* had, based on arithmetic means, five-fold higher nodule biomass, yet both *P. macroloba* and *Z. longifolia* exhibited significant light or light–N fertiliser addition interaction effects on nodule biomass or N fixed. Nitrogen fertiliser addition was the only experimental treatment to have a significant (negative) effect on nodule biomass and N fixed for *S. microstachyum*, suggesting the strength of a light effect (if any) is likely to be species specific. This is in line with previous studies that demonstrated that the taxonomic identity of legume species had a significant effect on SNF rates and which abiotic conditions more strongly regulated SNF (Batterman *et al.*, 2013a, 2018; Wurzburger & Hedin, 2016; McCulloch *et al.*, 2021). Furthermore, within and between the ranges of light availability found in the canopy gap light treatments (gap-full light or gap-shade) that these response variables increased with light, suggesting that light is at least in part driving the upregulation of SNF in canopy gaps (Table 1).

Soil nutrient and moisture effects on symbiotic nitrogen fixation

Soil N and P availability can also influence SNF rates in tropical forests (Batterman *et al.*, 2013b; Nasto *et al.*, 2014, 2017; Taylor & Menge, 2018; Trierweiler *et al.*, 2018) and canopy gap dynamics influence nutrient cycling (Vitousek & Denslow, 1986; Denslow *et al.*, 1998). How long these disturbances influence soil nutrient availability within a gap and what effect this has on SNF are not well understood. A previous observational study in successional plots near La Selva found no correlation between nodulation and soil N availability in *P. macroloba* seedlings of undetermined ages (Taylor & Menge, 2018) and we found similar results in our study. Contrary to our expectations that soil N availability would inhibit SNF, we find that site-level soil N availability had a significant positive relationship with N fixed and N fixed per g seedling for one species (*S. microstachyum*; Table S2).

In addition to the hypothesis that a spatial mismatch between N supply and demand may explain the paradoxically high SNF rates in the Neotropics (Hedin *et al.*, 2009), it has also been proposed that legumes use the additional N from SNF to acquire P (Houlton *et al.*, 2008). Our study supports this line of thinking, as soil P availability had a negative relationship with nodule biomass (Table 1; Fig. S2). This relationship was strongest in *P. macroloba*, but *Z. longifolia* also showed a negative relationship between nodule biomass and soil P availability (Table S2). However, caution should be taken when extrapolating these results beyond these species. Other SNF response variables also had an overall negative relationship with soil P availability, but like nodule biomass, these results were not consistent across species. Therefore, the negative relationship of N fixation to soil P availability is not readily generalised (Table S2). That said, higher SNF associated with relatively lower soil P availability is consistent with previous findings that suggested that investment in SNF in the Neotropics could be a P acquisition strategy (Houlton *et al.*, 2008; Nasto *et al.*, 2014). It is important to note that other studies have not found a strong relationship between SNF and P acquisition strategies (Batterman *et al.*, 2018; Soper *et al.*, 2018a).

Soil moisture, however, had a significant positive effect on all reported response variables except for a few cases in *S. microstachyum* (Tables 1, S2, S3). This suggests that water availability may be another important component in SNF regulation that requires further exploration (McCulloch *et al.*, 2021). Specifically, the importance of soil moisture in canopy gap SNF regulation may be related to the overall size of the canopy gap. We found significantly drier soil moisture conditions in the canopy gap sites compared with understory sites, which was counter to that previously reported at La Selva (Vitousek & Denslow, 1986; Denslow *et al.*, 1998; Ostertag, 1998). However, our experiment used canopy gaps found in a substantially larger disturbed area (*c.* 60 000 m^2) than the canopy gaps used by other studies (9–611 m^2). The canopy gaps found within this large disturbed area may differ from canopy gaps that form from a single treefall, as there was likely to be more low angle light availability in our relatively larger canopy gaps. This may explain some of the differences in abiotic conditions between our group and other groups' findings. Important SNF regulators (soil moisture, soil temperature, light availability, and soil nutrient dynamics) are likely to vary with canopy gap size (Denslow *et al.*, 1998), and therefore the size of the canopy gap may influence the strength of the canopy gap effect.

These data suggested that, in the field, light may be a stronger regulator of SNF compared with soil N, at least in seedlings. Although, canopy trees by definition are in high light conditions, adult trees bordering canopy gaps experience even higher light availability (Stark *et al.*, 2012). Therefore, our results suggested the upregulation of nodule formation in canopy trees bordering gaps, which has been documented by others (Barron *et al.*, 2011; Wurzburger & Hedin, 2016), may be driven, at least in part, by high light availability.

Furthermore, there are several other important SNF regulators that vary between canopy gaps and intact forests that contribute

to SNF upregulation. For example, temperature is positively correlated with SNF rates (Prevost *et al.*, 1987; Houlton *et al.*, 2008). Canopy gaps can experience higher temperatures compared with the understory of intact forests, especially at midday when light availability is also high (Miller *et al.*, 2007). As temperature and light are correlated in these settings, further study is needed to disentangle how these two abiotic conditions interact to affect SNF in neotropical canopy gaps.

While light appears to be the most important variable driving overall SNF, we did observe downregulation of SNF with N fertiliser addition. Unlike the indirect light availability effect on SNF, we find that N fertiliser addition did affect the relative allocation to SNF in this study (Fig. 4). Nodule allocation was significantly lower in seedlings that received N fertiliser compared with those that did not (Fig. 4). Nitrogen fertiliser addition also decreased nodule biomass and N fixed in *P. macroloba* and *Z. longifolia* (Table S2). This result offered further evidence that SNF can be downregulated in response to high soil N (Batterman *et al.*, 2013b), but this downregulation may not be as strong under high light conditions (Taylor & Menge, 2018). Interestingly, we did not find a strong N fertiliser addition effect on plant biomass for any of the study species (Fig. 2a), although there was a marginally significant biomass increase with N fertiliser addition in *P. macroloba*. This suggests that either SNF was sufficient to fuel plant growth or that seedling growth was not limited by soil N availability of this site. Overall, SNF (non-normalised variables) appeared to be mostly decoupled from soil N availability, whereas N fertiliser addition suppressed SNF at the individual seedling scale (normalised variables).

It is possible that applying the N fertiliser treatment twice was not sufficient to significantly increase local soil N availability over the course of the experiment, as N can be leached out during rain events (Schreeg & Porder, 2014). Although we did not measure N availability over time at these sites, N fertiliser addition did have a significant effect on several of the response variables (total seedling biomass, nodule biomass and nodule allocation). Therefore, N fertiliser addition was sufficient to elicit a response, but potential N losses may have weakened the strength of these responses. It also is important to note that this study was conducted on soils that were relatively fertile compared with other lowland tropical forest soils (Sollins *et al.*, 1994; Powers *et al.*, 2005b; Porder *et al.*, 2006) and this difference may limit our scope of inference. However, Chou *et al.*, 2018 found co-limitation of light and soil nutrients to sapling growth in N-fixing species (but there were species-specific differences reported). This suggests that, despite relatively high soil N availability, N limitation to seedling or sapling growth is possible at La Selva, and this should favour SNF upregulation.

Nitrogen fixed per gram nodule (g N g^{-1} nodule h^{-1}) had no significant relationship with the experimental treatments and was not significantly different among species. This suggests that SNF upregulation is not realised by increases in N fixed per gram of nodule, but rather through a higher investment in nodule biomass. Variation in the amount of N fixed per gram of nodule may not be regulated by the experimental treatments or site variables measured in this experiment, and instead by other biotic (e.g. nodule bacteria community) or abiotic (e.g. temperature) conditions. Although we find a significant relationship between nodule biomass and N fixed per g nodule, the correlation value is only 0.36 and therefore we do not think that nodule biomass can be used as a sufficient proxy for SNF rates (Winbourne *et al.*, 2018b).

Conclusions

This field-based manipulative experiment documents the relative importance of light and soil N availability on SNF in the Neotropics. It provides experimental evidence for the hypothesis that naturally occurring canopy gaps may serve as SNF hotspots across the landscape, and that light, not soil N availability, is likely to be the major stimulus for this SNF upregulation. These results were generally consistent across the three species in this study. SNF upregulation by light was driven by increases in plant biomass, which lead to increases in nodule biomass and N fixed, rather than an increased relative allocation of seedling resources to SNF under these high light conditions. Interestingly, only the N fertiliser treatment negatively influenced the relative allocation to SNF in this experiment. This suggests that soil N availability may be important for SNF regulation on the individual scale, but increases in light availability contributed to significantly higher SNF in canopy gaps environments compared with intact forests through light effects on seedling growth. If such upregulation is pervasive in tropical N-fixing legumes, naturally occurring canopy gaps could produce spatial heterogeneity in N inputs to neotropical forests. This canopy gap effect on SNF may be of increasing importance for understanding neotropical biogeochemical cycling, as tree mortality rates, and therefore treefall gaps, have significantly increased in recent decades.

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Author contributions

LAM and SP designed the study. LAM performed the research and analysed the data. LAM wrote the first draft of the manuscript and both authors wrote the final manuscript.

Data availability

The data produced in this study are publicly available on Figshare (https://figshare.com/articles/dataset/SNF_CanopyGap_

LightExperiment/12605273; https://doi.org/10.6084/m9.figshare.12605273). The code produced to make the figures and for data analysis is archived on Github (https://github.com/lmcculloch/LaSelva_AMF_2020) and published on Zenodo (https://doi.org/10.5281/zenodo.4813034).

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Light intensities (% light transmittance) associated with each experimental light treatment.

Fig. S2 Bivariate relationships between nodule biomass per seedling and phosphate availability (mg P cm^{-1} d) and soil moisture (% VWC).

Table S1 Mean values for site variation.

Table S2 GAMLSS summary statistics for total biomass (g), nodule biomass (g), nodule allocation (g nodule g^{-1} seedling), N fixed (g N $seedling^{-1}$ h^{-1}) and N fixed per g seedling (g N g^{-1} seedling h^{-1}) for each species.

Table S3 GAMLSS summary statistics for N fixed g^{-1} nodule (g N g^{-1} nodule h^{-1}) for the species pooled.

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