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LETTER

Phosphatase activity and nitrogen fixation reflect species differences, not nutrient trading or nutrient balance, across tropical rainforest trees

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Abstract

A fundamental biogeochemical paradox is that nitrogen-rich tropical forests contain abundant nitrogen-fixing trees, which support a globally significant tropical carbon sink. One explanation for this pattern holds that nitrogen-fixing trees can overcome phosphorus limitation in tropical forests by synthesizing phosphatase enzymes to acquire soil organic phosphorus, but empirical evidence remains scarce. We evaluated whether nitrogen fixation and phosphatase activity are linked across 97 trees from seven species, and tested two hypotheses for explaining investment in nutrient strategies: trading nitrogen-for-phosphorus or balancing nutrient demand. Both strategies varied across species but were not explained by nitrogen-for-phosphorus trading or nutrient balance. This indicates that (1) studies of these nutrient strategies require broad sampling within and across species, (2) factors other than nutrient trading must be invoked to resolve the paradox of tropical nitrogen fixation, and (3) nitrogen-fixing trees cannot provide a positive nitrogen-phosphorus-carbon feedback to alleviate nutrient limitation of the tropical carbon sink.

Keywords

Biodiversity, biogeochemical niche, biogeochemistry, nitrogen, nutrient acquisition, nutrient limitation, nutrient strategy, phosphorus, tropical carbon sink.

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INTRODUCTION

The observation that nutrients may limit the ability of tropical forests to serve as a sustained sink for atmospheric carbon dioxide underscores the importance of resolving the mechanisms and plant strategies that govern how tropical trees acquire nutrients (Wurzburger & Hedin 2016). Two strategies in particular – symbiotic nitrogen fixation and the synthesis of extracellular phosphatase enzymes – have received attention as they may be linked in a way that could: (1) allow trees to acquire nitrogen from the atmosphere via symbiosis with nitrogen-fixing bacteria (Jenny 1950; Vitousek & Howarth 1991) and/or phosphorus from the soil by hydrolyzing otherwise biologically unavailable organic phosphorus (Harrison & Pearce 1979; Duff *et al*. 1994; Treseder & Vitousek 2001), and (2) explain why nitrogen-fixing trees are common in tropical forests despite the predominance of nitrogen-rich, phosphorus-poor soils (Houlton *et al*. 2008). (A third acquisition strategy, mycorrhizal phosphorus uptake, falls outside the scope of this study; Baribault *et al*. 2011.)

Recent studies have indicated that the capacities to produce phosphatases and fix nitrogen may be associated – and perhaps even mechanistically linked – in nitrogen-fixing tree species. High rates of phosphatase activity can occur in soils associated with nitrogen-fixing trees (Houlton *et al*. 2008) and on the root surfaces of some nitrogen-fixing tree species in the field (Nasto *et al*. 2014; Png *et al*. 2017) or in greenhouse conditions (Olde Venterink 2011; Nasto *et al*. 2017). Nasto *et al*. (2017) further showed that phosphatase activity can increase as a function of increased fixation.

It is unclear, however, whether these patterns reflect a link between fixation and phosphatase production that is widespread across nitrogen-fixing species. Alternatively, the ability to fix nitrogen may allow for higher biomass growth rates and thus also trigger phosphatase production. This distinction matters for resolving whether nitrogen fixers have a unique ability to trade fixed nitrogen for enhanced competitive success (relative to non-fixers) in low phosphorus and high nitrogen soils. Such a mechanism can be central for resolving the distribution of fixers across soil conditions in tropical forests (Houlton *et al*. 2008).

Two alternative hypotheses could explain why trees would vary their investment in nitrogen fixation and phosphatase activity. The *nutrient trading hypothesis* proposes that fixation and phosphatase activity are closely linked, with fixers able to use fixed nitrogen to invest in nitrogen-rich phosphatase enzymes. This mechanism would give fixers the unique competitive advantage of acquiring phosphorus from organic

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compounds in phosphorus-poor soils (Houlton *et al.* 2008) and can thus explain why fixation has evolved within phosphorus-poor tropical forests despite the apparent abundance of nitrogen. Three predictions (Fig. 1) are required to be met to support the hypothesis: prediction 1 – for fixers to have a competitive advantage and thus become more abundant in phosphorus-poor tropical forests, phosphatase activity must be higher in fixers than non-fixers. Prediction 2 – for these traits to be linked mechanistically as a result of natural selection over evolutionary time, individual plants must possess a positive relationship between phosphatase activity and the rate of fixation. And, prediction 3 – for fixation to underpin the production of phosphatase enzymes rather than soil nitrogen, fixation rates must be insensitive to the nitrogen balance of the plant.

Support for the nutrient trading hypothesis has derived from observations of a subset (but not all) of the predictions and has been indirect (as noted above) or demonstrated across a range of phosphorus substrates (Nasto *et al.* 2017). Two studies did not detect a link between fixation rate and phosphatase activity (Batterman *et al.* 2013a; Nasto *et al.* 2014).

The *nutrient balance hypothesis* proposes that trees adjust their nutrient acquisition strategies to satisfy any plant nutrient demand in excess of soil supply (Fig. 1; e.g. Gutschick 1981; Bloom *et al.* 1985; Treseder & Vitousek 2001). This perspective identifies the tree growth rate as a fundamental control on strategies of nutrient acquisition. In conditions of high biomass growth rate where both nitrogen and phosphorus demand are high relative to supply, fixation could correct a deficiency in soil nitrogen for plants capable of fixation, while investment in phosphatase activity could correct a phosphorus deficiency for both fixers and non-fixers. The nutrient strategies would respond to either nitrogen limitation or phosphorus limitation, and therefore both strategies would not both be up-regulated at the same time (unless plants were co-limited, discussed below). Thus, the nutrient balance hypothesis contrasts with the nutrient-trading hypothesis, requiring no positive relationship between phosphatase activity and nitrogen fixation (prediction A; Fig. 1). The nutrient balance hypothesis would instead require prediction B – increased phosphatase and fixation activities with increasing plant demand (or decreasing soil supply) of phosphorus and nitrogen, respectively. These nitrogen and phosphorus acquisition strategies would be sensitive to the stoichiometric ratios of nitrogen and phosphorus, consistent with the type of limitation, with increased fixation with decreasing levels of soil available nitrogen relative to phosphorus (N:P) and increased phosphatase activity with increasing soil available N:P (prediction C).

No study has explicitly examined the nutrient balance hypothesis for both fixation and phosphatase, although there is indication that fixers can adjust fixation to both nitrogen and phosphorus supply (Barron *et al.* 2011; Batterman *et al.* 2013a; Menge *et al.* 2017) and that plants can alter phosphatase activity to match soil phosphorus supply (Treseder & Vitousek 2001; Olde Venterink 2011; Turner & Wright 2014; Zalamea *et al.* 2016).

It is possible that nitrogen fixers display both high fixation and high root phosphatase activity, simply because biomass

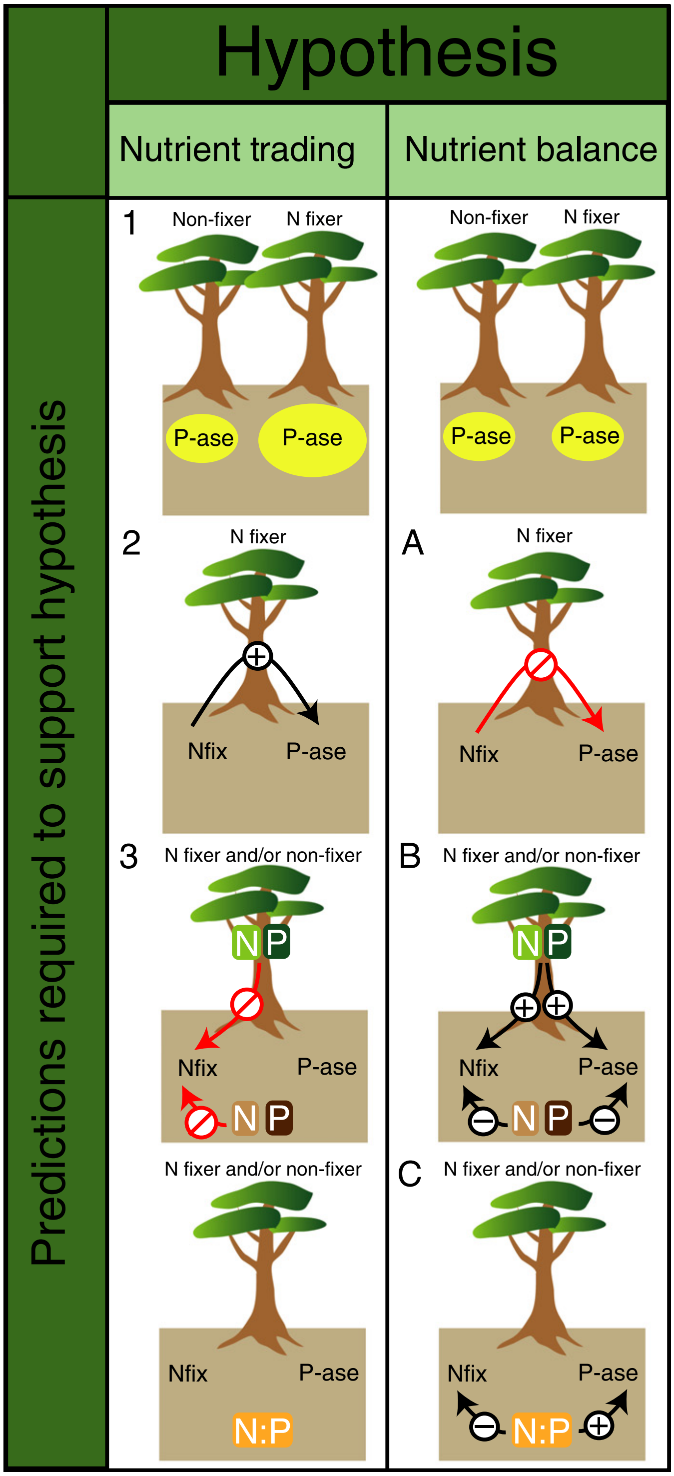

Figure 1 Conceptual diagram of the nutrient trading and nutrient balance hypotheses and the predictions for nitrogen fixation (‘Nfix’) and phosphatase activity (‘P-ase’) required to be met to support each hypothesis. Trees represent nitrogen fixers or non-fixers, box represents soil and yellow circles represent phosphatase activity. Arrows indicate the direction of relationships, and circles indicate the nature of the predicted relationship, including a positive (+), negative (–) or lack (Ø) of relationship. Absence of arrows indicates no specific relationship is predicted for the hypothesis. Numbers or letters signal the predictions required for each hypothesis, while their absence indicates that the relationship or absence of relationship is not required for the hypothesis.

growth causes demand for *both* nitrogen and phosphorus and plants are co-limited (Vitousek & Howarth 1991; Binkley *et al.* 2003). The co-occurrence of high fixation and high root phosphatase activity thus does not necessarily imply a nitrogen-for-phosphorus trading strategy (i.e. nutrient trading hypothesis), but rather could reflect high growth demand for *both* nitrogen and phosphorus (consistent with the nutrient balance hypothesis). A key consideration is whether species within a given functional group – fixer or non-fixer – show a consistent response, or behave individually. Each group is diverse, with species that may have evolved to function in different ways, thereby filling distinct functional niches. We should therefore expect some inherent variability across species within each group, and an examination of the degree to which species vary within versus across groups would resolve whether fixer species have a uniquely and coherently evolved strategy. Indeed, three studies have demonstrated that fixation rates differ at the species level within the fixer functional group (Batterman *et al.* 2013a; Wurzburger & Hedin 2016; Nasto *et al.* 2017). Analogously, two studies found that legume fixers display species-specific differences in phosphatase activity with some species having similar activity to non-fixer species (Olde Venterink 2011; Nasto *et al.* 2017). Finally, a recent global synthesis indicates that belowground traits are particularly phylogenetically conserved, suggesting we may expect a degree of similarity across closely related fixer species and difference from less closely related non-fixer species (Valverdes-Barrantes *et al.* 2017).

However, no study has systematically and simultaneously examined either hypothesis for phosphatase and fixation activity across a large number of individual trees from several tropical fixer and non-fixer species grown in a natural landscape.

We here use our conceptual framework developed above (Fig. 1) to evaluate two hypotheses for the expression of phosphatase and fixation activity in tropical trees. Although a thorough evaluation would require dozens of species and individuals to evaluate inter versus intra-specific variation within and across functional groups, we provide a first test by measuring the nutrient acquisition strategies in 6–13 young trees in each of four common species of fixers and three common species of non-fixers (total 97 trees), grown across soil nutrient gradients in a reforestation experiment in Panama. Our common garden approach allowed us to control both abiotic and biotic factors. Because phosphatase activity and fixation rates can vary across individual trees and because we sought to resolve these variables within versus between species, we designed our study to have high within-species replication. After 3 years of growth, we measured phosphatase activity on roots, nitrogen fixation activity, soil nitrogen and phosphorus availability, and plant nitrogen and phosphorus demand. Our findings suggest that species differ widely in both phosphatase and nitrogen fixation activity, but that plants do not appear to adjust these strategies to meet their nutrient balance. Furthermore, we found no positive relationship between fixation and phosphatase activity within individual trees, implying that tropical nitrogen fixers may not have evolved a nitrogen-for-phosphorus trading strategy to overcome phosphorus constraints and that a unique access to phosphorus by fixers may not resolve the paradox of tropical nitrogen fixation.

MATERIALS AND METHODS

Site description and experimental design

We conducted our experiment at the Agua Salud Project experimental plantation, Panama (Mayoral *et al.* 2017), where rainfall is 2700 mm, temperature ranges daily from 23 to 32 °C (Ogden *et al.* 2013), and soils are closely related Oxisols and Inceptisols (Mayoral *et al.* 2017; Turner, Baillie & Hall, unpublished data). Our 97 trees comprised part of a reforestation experiment established in 2008 in which 100 000 seedlings were planted in treeless pastures (van Breugel & Hall 2008; Mayoral *et al.* 2017). In this experiment using a natural environment setting we controlled for a large number of factors, including competition, land use history, soil physical and chemical properties, slope, light and climate (see Supplemental Information for further details).

Nutrient acquisition strategies

During the August-September wet season of 2011 when physiological activity was highest, we quantified root phosphatase activity and nitrogen fixation on 6–13 trees from each of four tree species capable of nitrogen fixation (*Inga punctata* Willd., Caesalpinioideae; *Gliricidia sepium* (Jacq.) Kunth ex Walp., Papilionoideae; *Erythrina fusca* Lour., Papilionoideae; *Dalbergia retusa* Hemsl., Papilionoideae; Legume Phylogeny Working Group 2017) and three tree species without the ability to fix nitrogen (*Tabebuia rosea* (Bertol.) DC., Bignoniaceae; *Terminalia amazonia* (J.F. Gmel.) Exell, Combretaceae; *Pachira quinata* (Jacq.) W.S. Alverson, Malvaceae) (Batterman et al. 2018). See Table 1 and Table S1 for exact numbers of trees and species for each measurement.

Phosphatase activity was measured on five replicate fine root samples from the base of 6–10 trees per species. Phosphomonoesterase activity was measured on each root sample using *para*-nitrophenyl phosphate (*p*NP) as an analogue substrate for phosphomonoesterase (see Supplemental Information for details), and calculated as the mean across samples for each tree, expressed in μmol *p*NP g^{-1} dry mass roots h^{-1}.

We determined per-area nitrogen fixation rate for each tree by multiplying per-area nodule biomass by per-nodule biomass nitrogen fixation rate, determined by acetylene reduction assay (ARA) and isotopically labelled ^{15}N incubations. To quantify per-area nodule biomass, we excavated active nodules at the base of each tree (Supporting Information). Nitrogen fixation activity was measured on nodules using ARA on a subset of 11–13 trees for each of our four species (following Batterman *et al.* 2013a) and $^{15}N_2$ incubations on a further subset of 4–5 trees per species (following Shearer & Kohl 1988 and Andrews *et al.* 2011) to estimate a C_2H_4:N_2 conversion ratio (Supporting Information; Tables S1 and S2).

Demand and supply of nutrients

We measured the aboveground nutrient demand from aboveground biomass growth and tissue nutrient content. Leaf and wood growth rates were determined from basal diameter measurements in 2009 and 2011 using locally derived allometric

Table 1 Mean, minimum, maximum and number of samples (n) of phosphatase and fixation activities across functional groups and species. For the functional group summary across species, we calculated the mean of species from the geometric mean from individual trees for each species

	Phosphatase activity (μmol pNP g^{-1} dry weight root h^{-1})				Fixation rate (μmol N$_2$ m^{-2} h^{-1})			
	Geometric mean	Min	Max	*n*	Geometric mean	Min	Max	*n*
N$_2$ fixers (across species)	44.7			4	9.6			4
N$_2$ fixers (across individuals)	39.2			28	3.0			48
Dalbergia retusa	25.1	15.2	42.3	10	33.5	0.1	532.5	12
Erythrina fusca	39.3	12.4	103.0	6	0.5	0.003	68.1	13
Gliricidia sepium	66.0	31.1	124.2	6	2.2	0.1	469.1	11
Inga punctata	48.5	29.5	77.2	6	2.3	0.1	36.4	12
Non-fixers (across species)	17.5			3				
Non-fixers (across individuals)	17.0			18				
Pachira quinata	22.3	16.8	36.4	6				
Tabebuia rosea	12.2	7.3	16.6	6				
Terminalia amazonia	18.0	10.0	27.7	6				

equations (Sinacore *et al.* 2017), and the tissue-specific nitrogen and phosphorus demand was determined by multiplying growth rates by species-specific nutrient contents from the literature (Supporting Information).

We quantified the availability of soil nutrients to each tree by measuring nitrate, ammonium and Bray-1 extractable phosphorus in a soil core from the top 10 cm at the base of each tree (Supporting Information). N:P was considered as the ratio of available nitrogen and Bray phosphorus.

Statistical analyses

We used analysis of variance (ANOVA) to determine whether functional groups differed in phosphatase activity, including species as a nested random variable. We then used ANOVA and Tukey post-hoc test to identify specific differences in phosphatase and fixation activity. We further used multiple linear regression with the independent continuous explanatory variable of interest (nitrogen demand, phosphorus demand, soil available nitrogen, soil available phosphorus, N:P or nitrogen fixation rate) plus species as a fixed variable to test effects on phosphatase and fixation activity and to examine any relationship between fixation and phosphatase; we also evaluated any variable-by-species interaction. All analyses were performed in R (R Core Team version 3.1.1 2014-07-10; Supporting Information).

RESULTS

We found that plant nutrient acquisition strategies, plant nutrient demand and soil nutrients varied widely across trees and species in our study sites (Table S3). Here we discuss nitrogen fixation rates, but we found similar results when considering nodule biomass as an alternative measure of nitrogen fixation potential (Supporting Information).

Species-specific variation in nutrient strategies

We first considered whether functional groups differed in phosphatase activity and if species showed consistent nutrient acquisition strategies within a functional group, or if there was significant variation across species. We found some evidence that trees capable of nitrogen fixation differ in phosphatase activity from non-fixing trees (ANOVA with functional type as a fixed variable and species as a nested random variable for $n = 6$–10 trees for each of our seven species: $F_{(1,5)} = 7.6$, $P < 0.05$). On average, phosphatase activity was more than two times higher in nitrogen-fixing than non-fixing species (mean of 44.7 μmol *p*NP g^{-1} dry weight root h^{-1} for fixer species vs. 17.5 μmol *p*NP g^{-1} dry weight root h^{-1} for non-fixers; Table 1). A similar range of difference was found whether considering the mean of either all species or all trees within a functional group (Table 1).

However, this difference in phosphatase activity between fixers and non-fixers appeared to be driven by one fixer species (*Gliricidia sepium)*, which possessed high phosphatase activity. When we excluded this species, the difference disappeared (ANOVA with functional type as a fixed variable and species as a nested random variable for $n = 6$–10 individuals for each of six species: $F_{(1,4)} = 6.5$, $p > 0.05$). Furthermore, we found substantial variation across individuals within species, and across all species within and across the functional groups such that species differed significantly in both phosphatase and fixation activity (Fig. 2, Table 1; ANOVA for phosphatase activity: $F_{(6,39)} = 10.49$, $P < 0.0001$, $n =6$–10 trees per species; ANOVA for fixation rate: $F_{(3,44)} = 4.91$, $P < 0.01$, $n = 11$–13 trees per species). The fixer species that possessed high phosphatase activity (*G. sepium*) differed significantly from another fixer species, *Dalbergia retusa*, as well as the non-fixer species. Three fixer species (*Inga*, *Erythrina* and *Dalbergia*) were similar to most of the non-fixer species (see Fig. 2a). Fixation rates also varied within and across species within the fixer functional group, with *Dalbergia* fixing at more than 14 times the rate of other species (34 vs. 0.5–2.3 μmol N$_2$ m^{-2} h^{-1}, respectively; ANOVA: $P < 0.01$, $F_{(3,44)} = 4.91$, $n = 11$–13 trees per species; Fig. 2b). This result does not support the existence of a functional link between fixation and phosphatase activity, as described below.

Evaluating the nutrient trading hypothesis

We examined whether our data followed the predictions (Fig. 1) for the hypothesis that phosphatase activity was enhanced by nutrient trading within the fixer functional group. While we found support for our prediction 1 – that nitrogen fixers have higher phosphatase activity, this result nevertheless depended on a single high-phosphatase but low fixation species, as described above (Fig. 2a). In addition, we found no support for prediction 2, in that phosphatase activity did not increase with fixation rate at the species or individual level (Fig. 3; linear regression: $t = 0.49$, d.f. = 21, $P > 0.1$). *Dalbergia retusa* fixed at a rate 14 times higher than the other fixer species, but had phosphatase levels that were similar to the non-fixing species.

Instead, our results point to a different determinant for soil phosphatase activity. High phosphatase activity was only found when fixation rates were low, consistent with phosphorus limitation of growth and fixation rather than fixation rates determining the ability of plants to overcome phosphorus limitation. However, prediction 3 – that fixation rate should be insensitive to the plant nitrogen balance – was supported in our data (Fig. 4d; linear regression: $t = 0.53$, d.f. = 46, $P > 0.1$ for $n = 11$–13 trees for each of 4 species). For all statistical tests, we included species as a random variable; neither species nor its interaction with fixation (in prediction 2) significantly changed these results.

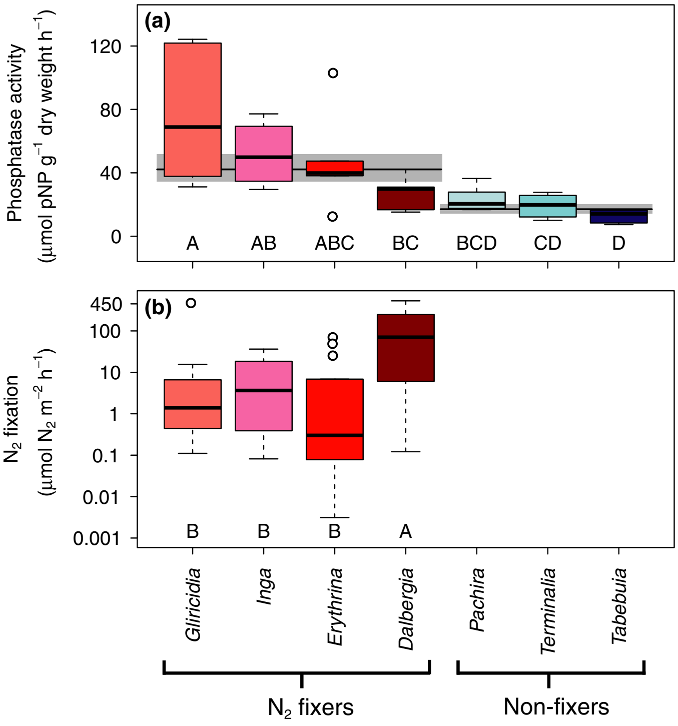

Figure 2 Phosphatase activity (a; µmol pNP g^{-1} dry weight root h^{-1}) and nitrogen fixation activity (b; µmol N$_2$ m^{-2} h^{-1}) of nitrogen-fixing (reds) and non-fixing (blues) tree species in a Panamanian tropical moist forest. Boxes represent the median, first and third quartiles, lower and upper whiskers and outliers for $n = 28$ nitrogen-fixing and $n = 18$ non-fixing trees for phosphatase activity and $n = 48$ trees for nitrogen fixation. ANOVAS on log-transformed data indicates a significant difference between species for phosphatase: $P < 0.0001$, $F_{(6,39)} = 10.49$, $n = 6$–10 trees per species; and, a significant difference between species for fixation: $P < 0.01$, $F_{(3,44)} = 4.91$, $n = 11$–13 trees per species. Significant differences between species determined with a Tukey *post-hoc* test are noted with letters. Black lines and grey shading in the background of (a) represent the mean ± standard error of the mean of species-level phosphatase activity, which was calculated as the geometric mean for individuals of fixers and non-fixers. Colours for each species match Figs 3 and 4.

Evaluating the nutrient balance hypothesis

For our second hypothesis, that plants adjust nutrient strategies to accommodate the plant nutrient balance, our results supported prediction A (Fig. 1): we found no clear relationship between phosphatase activity and fixation (Fig. 3). Furthermore, 15% of fixers lacked detectable nodule biomass, consistent with facultative fixation. In contrast to prediction B, however, (1) phosphatase activity did not increase with increasing phosphorus demand (Fig. 4a; linear regression: $t = 0.40$, d.f. = 44, $P > 0.1$, $n = 6$–10 trees each for 7 species) or decrease with increasing soil phosphorus availability (Fig. 4b; linear regression: $t = -0.68$, d.f. = 44, $P > 0.1$, $n = 6$–10 trees each for 7 species), and (2) nitrogen fixation activity did not increase with increasing nitrogen demand (Fig. 4d; linear regression: $t = 1.48$, d.f. = 46, $P > 0.1$, $n = 11$–13 trees each for 4 species) or decrease with increasing soil nitrogen availability (Fig. 4e; linear regression: $t = 0.04$, d.f. = 46, $p > 0.1$, $n = 11$–13 trees each for 4 species). We found a similar result – that neither nutrient strategy could be explained by plant demand – even when controlling for available soil nutrients (multiple linear regression of phosphatase activity with plant phosphorus demand and soil phosphorus availability or nitrogen fixation with plant nitrogen demand and soil nitrogen availability: $P > 0.1$).

We found some support for prediction C (Fig. 1) – that fixation decreased with increasing soil available N:P (Fig. 4f; linear regression: $t = -2.41$, d.f. = 46, $P < 0.05$, $n = 11$–13 trees each for 4 species) – in support of the idea that fixation is responsive to differences in soil nitrogen levels, although phosphatase activity did not correlate with available N:P (Fig. 4c; linear regression: $t = 0.61$, d.f. = 44, $P > 0.1$, $n = 6$–10 trees each for 7 species).

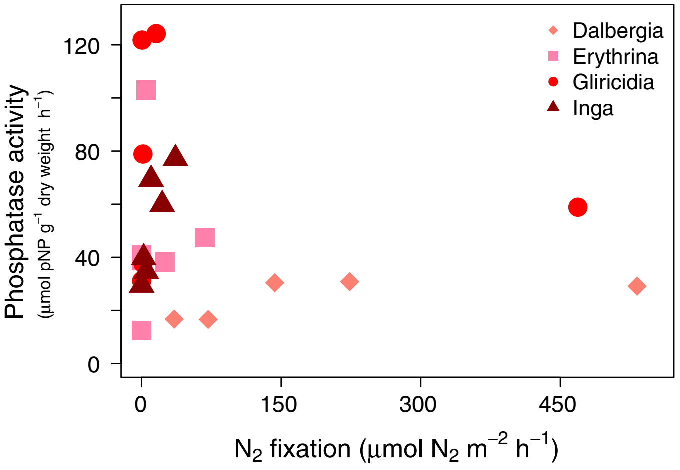

Figure 3 Evaluation of the nutrient trading hypothesis: relationship between phosphatase activity on roots (µmol pNP g^{-1} dry weight root h^{-1}) and nitrogen fixation activity (µmol N$_2$ m^{-2} h^{-1}). Linear regression indicates no significant relationship between N$_2$ fixation activity and phosphatase activity or the interaction of fixation and species ($P > 0.1$). Each point represents activity on a tree from one of four species, as indicated by colours and symbols in the legend.

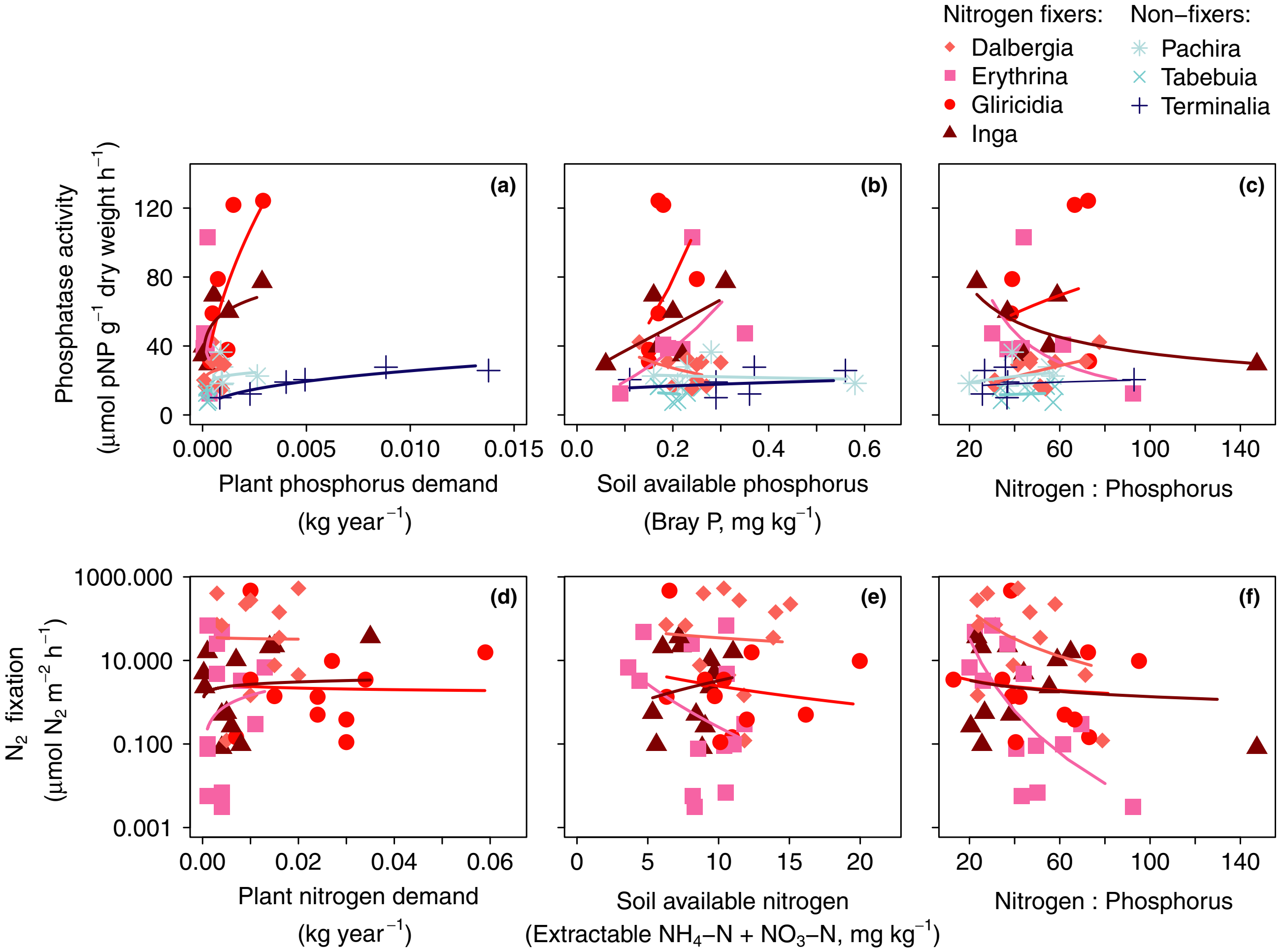

Figure 4 Evaluation of the nutrient balance hypothesis: Relationships between nutrient acquisition strategies root phosphatase activity (µmol pNP g^{-1} dry weight root h^{-1}; a–c) and nitrogen fixation activity (µmol N_2 m^{-2} h^{-1}; (d–f) and plant nutrient demand (phosphorus or nitrogen; kg y^{-1}), soil available Bray extractable inorganic phosphorus (mg P kg^{-1}; b), soil available nitrogen (nitrate + ammonium; mg N kg^{-1}; e), and soil available N:P (c and f). Each point represents a tree that is either a fixer (reds) or non-fixer (blues), with the colour and shape representing individual species (see figure legend). Linear regression indicates no relationships were significant ($P > 0.1$), with the exception of a negative effect of soil N:P on fixation rate ($t = -2.41$, d.f. = 46, $P < 0.05$). Lines represent untransformed linear regressions of transformed data. Most regression lines were not significant for species, with the exception of *Erythrina*, which had significant slopes with available P and N:P versus phosphatase activity (see Table S4 for statistical results).

The interaction of species and the nutrient-related explanatory variables was not significant ($P > 0.1$) across trees in any of these models, but we did find non-significant trends in one species responding to nutrients. *Erythrina fusca* trees displayed a marginally positive increase in phosphatase activity in response to either available nitrogen or phosphorus, and a decrease in response to increasing available soil N:P ratios (Table S4; Fig. 4). In contrast, nitrogen fixation rate did not respond to differences in soil nutrients for individuals of any species (Table S4; Fig. 4).

DISCUSSION

By studying 6–13 trees each of four nitrogen-fixing and three non-fixing species in the lowland moist tropical forests of Panama, we examined (1) whether nitrogen-fixing trees have higher phosphatase activity than non-fixers, and (2) which mechanisms govern tree investment in two important nutrient acquisition strategies: nitrogen fixation and root phosphatase activity. Our results do not support the idea that phosphatase is categorically higher in nitrogen fixers compared to non-fixers at either individual or species levels. One species of fixer displayed significantly higher phosphatase activity than the other fixer and non-fixer species, suggesting that species may have evolved distinct strategies. In addition, neither the nutrient trading nor the nutrient balance hypotheses could explain the observed high variation in strategies that we observed within and across individual plants, species and functional groups. Most broadly, our results are consistent with a model of high species-specific variability in nutrient acquisition strategies, independent of functional group.

Mechanisms promoting nutrient acquisition strategies

Nutrient trading hypothesis

We found little evidence that the tropical trees examined in this study use nitrogen fixation to increase activity of phosphatase enzymes and thus their phosphorus supply, as expected in the first prediction of the nutrient trading hypothesis. Our extensive sampling within species revealed that individuals and species differed significantly in investment in phosphatase, and one species of fixer had particularly high fixation activity. While nitrogen fixers displayed on average more than twice the phosphatase activity on their roots than non-fixers, across either individual trees or species, the statistical significance of these differences disappeared when we removed this species. We therefore cannot conclude that phosphatase activity primarily differs due to the fixation status of an individual tree or species.

We considered whether sampling bias could have caused this result. While a larger selection of fixing and non-fixing species would be desirable, such comparison would be logistically difficult to accomplish in the experimental forest conditions that we used. Nevertheless, we can quantitatively infer that phosphatase activity is highly variable across species within each functional group, and that, in the case of our combination of species, we observe no consistent difference save for one high-phosphatase fixer species.

We also found no support for the second prediction, in that phosphatase activity did not increase with fixation activity. Finally, our findings were consistent with the third prediction – fixation rates did not change across nitrogen availability or demand; however, tropical fixers did not always fix nitrogen, corresponding to a facultative fixation strategy, discussed below, that may be sensitive to soil nitrogen in some instances. When considered together, our findings therefore do not support the idea that nutrient trading is a major mechanism in our phosphorus-poor tropical forests.

Our result is supported by two recent studies that also examined, but found no evidence for, a positive relationship between fixation rate and phosphatase activity (Batterman *et al.* 2013a; Nasto *et al.* 2014). However, they differ from inferences made from previous studies that used different methods. First, a greenhouse study of two *Inga* species reported a positive correlation between fixation rate per unit of nodule mass (instead of per unit soil area) and phosphatase activity per unit soil volume across different types of soil phosphorus substrates (Nasto *et al.* 2017). In addition, phosphatase activity has been reported to be higher in either the soil (Houlton *et al.* 2008) or roots (Olde Venterink 2011; Nasto *et al.* 2014, 2017; Png *et al.* 2017) of plant species capable of fixation. It is less clear, however, whether these observations indicate a systematic effect caused by an intrinsic difference between fixer and non-fixer species, or a sampling effect that depends on the exact species considered.

Nutrient balance hypothesis

Our evaluation of the nutrient balance hypothesis indicated that, across our trees, nutrient balance was not a major factor determining plant investment in nutrient strategies. Phosphatase activity did not decrease with increasing phosphorus availability, and fixation activity did not decrease with increasing nitrogen availability, in contrast to prediction B. There was some indication that phosphatase activity may increase with phosphorus availability, although this relationship was only statistically significant in one species, *Erythrina fusca*. Such a positive relationship contrasts with prediction B, but could emerge if phosphatase activity increases in response to higher substrate availability. Furthermore, we found a significant but weak negative effect of soil available N:P on fixation across all trees (prediction C), consistent with the idea that fixation rates should be higher when nitrogen availability is low relative to phosphorus (Wurzburger & Hedin 2016).

These findings differ from previous studies that pointed to soil nutrients as the major influence on both fixation (McHargue 1999; Barron *et al.* 2011; Batterman *et al.* 2013b; Sheffer *et al.* 2015) and phosphatase activity (Treseder & Vitousek 2001; Marklein & Houlton 2012; Zalamea *et al.* 2016). However, they support the idea that species differ in their ability to regulate fixation in response to nutrients in Mediterranean (Menge *et al.* 2015) and tropical forest ecosystems (Wurzburger & Hedin 2016). Despite the lack of response to nitrogen balance, our observation that not all trees were actively fixing would be expected by a facultative fixation strategy (Barron *et al.* 2011; Batterman *et al.* 2013a,b; Bauters *et al.* 2016), in which individual trees do not always fix nitrogen at the same rate.

Our lack of support for the nutrient balance hypothesis might be due to the phosphorus-poor soils (<0.7 mg kg^{-1}) in our study sites relative to soils in other tropical forests in the region (0.2-24.6 mg kg^{-1}, mean 2.2 mg kg^{-1}; Turner *et al.* 2018 or elsewhere (e.g. Clinebell *et al.* 1995; Quesada *et al.* 2010; Nasto *et al.* 2014). Generally low phosphorus may have caused our trees to routinely invest in phosphatase activity, even when their measured soil phosphorus was relatively high. However, based on laboratory studies (Olde Venterink 2011; Batterman *et al.* 2013a) we would expect trees to most strongly produce phosphatase enzymes in soils with lowest available phosphorus, but most of our species did not show high phosphatase activities on their roots (compared to e.g. Zalamea *et al.* 2016).

Alternatively, our trees may all have had relatively high nitrogen demand since they were small (< 20 cm DBH) and growing rapidly in high light conditions. Our observation that the majority of fixers examined (85%) were actively fixing supports this interpretation. However, the early period of growth when trees have the highest nitrogen demand should best reveal sensitivity of the nutrient strategies to variations in nutrient balance. Our experiment should therefore be adequate for testing the response in nutrient acquisition across our species and soil conditions.

A final possibility is that our metrics and experimental setup may not have been able to fully capture the plant nutrient balance. Our measures of aboveground biomass nutrient demand are relatively precise, but, however unlikely, it may be that belowground biomass investment is not proportional to aboveground investment, thus introducing an unmeasured bias.

Do tree species function as either nitrogen fixer or non-fixer functional groups?

For the tree species and soil nutrient gradient examined here, phosphatase activity varied most strongly across species, even within the functional groups. Trees also differed in fixation rates across species, with some individuals not actively fixing nitrogen above our detection limit and with one species, *Dalbergia retusa*, displaying significantly higher fixation rates than other species. These findings indicate that treating either fixers or non-fixers as a single homogeneous functional group would mask interspecific differences in both phosphatase and fixation activity.

Our results instead reinforce the idea that individual species – whether classified as fixers or non-fixers – possess species-specific strategies for phosphorus acquisition and nitrogen fixation (in the case of the fixer group). These species differences could be explained if species have evolved to fill distinct biogeochemical niches, with some specializing on high nutrient conditions and others on low nutrient conditions and with investment in phosphatase activity or fixation appropriate to the soil nutrients for which the species are competitive (e.g. Condit *et al.* 2013). Species may also differ in the degree of plasticity in adjusting their nutrient strategies, causing non-consistent responses in either phosphatase activity or nitrogen fixation as a function of changing growth and soil nutrient conditions. A third explanation could be that some species invest in alternative strategies to phosphatase activity, such as mycorrhizal colonization (Nasto *et al.* 2017) or higher root biomass, to acquire phosphorus when in conditions of phosphorus limitation. Finally, species could have phylogenetically conserved nutrient strategies based on their evolutionary history (e.g. such as in leaf traits; Givnish 1987; Fyllas *et al.* 2009; Goldsmith *et al.* 2016). Indeed, a recent review suggests that belowground traits may be particularly conserved across the phylogeny relative to aboveground traits (Valverde-Barrantes *et al.* 2015; 2017).

The finding of high species-specific variation supports findings from previous studies that have examined the relative contribution of environment versus species identity to the variation in traits. Species identity rather than any measured environmental condition determined much of the variation in fixation activity and mycorrhizal colonization in nitrogen-fixing trees in a nearby Panamanian forest (Wurzburger & Hedin 2016), and fixation strategies across Mediterranean fixer species (Menge *et al.* 2015). Gei & Powers (2013) found that species identity mattered more for tropical tree effects on soil properties than whether a tree belonged to the fixer or non-fixer functional group.

Future work should examine a broader selection of species representing both fixer and non-fixer functional groups with sufficient replication of individual trees (e.g. minimum 6–10) per species to more definitively resolve whether the appropriate model is one of broad functional group differences or of species-specific niche differentiation in the case of nutrient acquisition strategies.

Implications for numerical models

Our findings provide two guidelines for the incorporation of these critical nutrient strategies into conceptual and numerical models. First, our results indicate that, because of the clear difference between nitrogen fixers and non-fixers in the ability to acquire nitrogen from fixation and similarity of both tree types to use phosphatase, models (e.g. Wang *et al.* 2007; Goll *et al.* 2012; Wieder *et al.* 2015) should include fixer and non-fixer plant functional types that can explicitly acquire nitrogen and phosphorus through the fixation and phosphatase nutrient strategies. Second, our findings of high variation in nutrient strategies in both individual trees and across species indicate that models should not, however, generalize the positive feedback between the fixation and phosphatase traits across the entire fixer functional group. Our results suggest that, at least for our species, the fixation-for-phosphatase nutrient trading mechanism does not act to allow fixers to remain competitive relative to non-fixers in high nitrogen, low-phosphorus soils. Excluding a fixation-for-phosphatase trading feedback in models is critical because such a feedback could erroneously lead to a perpetual alleviation of both nitrogen and phosphorus limitation and a continuous carbon sink in tropical forests. Given that this feedback is necessary for some models (e.g. Houlton *et al.* 2008) to explain the otherwise counter-intuitive persistence of fixers in high nitrogen low phosphorus tropical forest soils, another mechanism must explain the paradoxical abundance of nitrogen-fixing trees in tropical forests.

CONCLUSIONS

Our study examined two hypotheses of plant investment in nutrient strategies. We found no clear support for the nutrient trading hypothesis, including that we could not identify a positive feedback between fixation and phosphatase activity. Furthermore, we found weak to no support for the nutrient balance hypothesis, when analyzed across individual trees or species. Our analysis provides evidence of differences in plasticity among species in phosphatase activity and nitrogen fixation, reinforcing the idea that a homogeneous functional group of ‘fixers’ and ‘non-fixers’ is not appropriate to characterize the functional role of species within tropical tree communities. Our conceptual framework should therefore be tested across a wide range of fixer and non-fixer species. The lack of evidence for the nutrient trading mechanism does not support the proposal that higher ability to acquire phosphorus by nitrogen-fixing trees can explain the paradox of abundant nitrogen-fixing trees in nitrogen-rich tropical forests. Most broadly, our results indicate that there may be substantial limits to the acquisition of new nitrogen and phosphorus by tropical trees as nutrient demand increases with rising atmospheric CO_2.

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AUTHORSHIP

SB, LH, JH and BT developed the ideas; SB, JH, BT and MvB designed the study; JH and MvB set up the experiment; KLW, PS, SB and BT collected data; SB analyzed the data and wrote the manuscript; BT, LH, MvB and JH provided comments on the manuscript.

DATA ACCESSIBILITY STATEMENT

Data is archived at the Environmental Information Data Centre (http://eidc.ceh.ac.uk), with DOI 10.5285/3578bae2-4b88-4b2e-93e1-6965dfe1348c.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

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

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