Moderate phosphorus additions consistently affect community composition of arbuscular mycorrhizal fungi in tropical montane forests in southern Ecuador

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Summary

- Anthropogenic atmospheric deposition can increase nutrient supply in the most remote ecosystems, potentially affecting soil biodiversity. Arbuscular mycorrhizal fungal (AMF) communities rapidly respond to simulated soil eutrophication in tropical forests. Yet the limited spatio-temporal extent of such manipulations, together with the often unrealistically high fertilization rates employed, impedes generalization of such responses.
- We sequenced mixed root AMF communities within a seven year-long fully factorial nitrogen (N) and phosphorus (P) addition experiment, replicated at three tropical montane forests in southern Ecuador with differing environmental characteristics. We hypothesized: strong shifts in community composition and species richness after long-term fertilization, site- and clade-specific responses to N vs P additions depending on local soil fertility and clade life history traits respectively.
- Fertilization consistently shifted AMF community composition across sites, but only reduced richness of Glomeraceae. Compositional changes were mainly driven by increases in P supply while richness reductions were observed only after combined N and P additions.
- We conclude that moderate increases of N and P exert a mild but consistent effect on tropical AMF communities. To predict the consequences of these shifts, current results need to be supplemented with experiments that characterize local species-specific AMF functionality.

Introduction

Tropical Andean forests are centers of endemism and constitute the most biodiverse region of the world per unit area (Rahbek *et al.*, 2019b). Despite the large contribution of these forests to preserve Earth's biodiversity, many aspects of their ecology remain unresolved. Most notably, the role that soils –and soil dwelling organisms– play in shaping these ecosystems' response to global change drivers (Baez *et al.*, 2015; Hagedorn *et al.*, 2019). This is particularly relevant for tropical Andes, as montane forest soils store considerable amounts of carbon (C) (Girardin *et al.*, 2010; Moser *et al.*, 2011; Spracklen & Righelato, 2014), yet the drivers controlling C fluxes are shifting in this region. In the past two decades, the intensification of human activities in the neighboring Amazonian plains has fueled a moderate increment in the deposition rates of reactive nitrogen (N) (Wilcke *et al.*, 2013; Velescu *et al.*, 2016) and phosphorus (P) (Wilcke *et al.*, 2019) into the eastern Andes. Given that N and P are arguably the main soil elements regulating C cycling, and that their availability also affects soil microbes and the processes they drive (Camenzind *et al.*, 2018), understanding how tropical montane forests change in the face of ongoing soil eutrophication, requires a deeper understanding of how soil microbial communities respond to these disturbances.

Arbuscular mycorrhizal fungi (AMF) – a basal sub-phylum of mutualistic fungi (Glomeromycotina; Spatafora *et al.*, 2016) – form the most common type of mycorrhizal symbiosis worldwide (van der Heijden *et al.*, 2015), and are the dominant mutualists in Andean tropical forests (Kottke *et al.*, 2008; Smith & Read, 2008). AMF are ecologically relevant because they increase the uptake of P in exchange for plant derived C (Smith & Smith, 2012), and to a lesser extent the uptake of inorganic N (Hodge & Storer, 2015; Ushio *et al.*, 2017). Because of their prominent role in the flow of nutrients, assessing AMF community responses to shifting nutrient pulses might serve to establish a link between AMF diversity and ecosystem function (Rillig, 2004).

Based on what is currently known about the nutritional attributes of the symbiosis, several predictions on how AMF diversity may respond to increased N and P availability can be attempted. From a resource economy perspective (Johnson, 2010), when atmospheric deposition increases P supply beyond limitation, the benefit of the symbiosis is reduced (Johnson *et al.*,

2015). This may intensify competition between AMF taxa for plant derived C and for soil nutrients, as well as between the host and AMF for inorganic N. In both cases, a reduction in AMF diversity can be expected. Conversely, in cases when P supply is the most limiting resource (i.e. N supply increases beyond limitation), the benefit of the symbiosis is enhanced. In this case AMF diversity levels might be maintained. The situation is considerably more nuanced when hosts are N and P co-limited. In this scenario, the nutritional benefit of the symbiosis will still be required, yet weak competition between AMF taxa for resources (Powell & Rillig, 2018) might lead to shifts in community composition. This last prediction is congruent with the co-adaptation model (Johnson, 2010). This model predicts that over time, ambient nutrient status selects sets of plants and fungi that are able to co-exist and maximize the exchange of resources (Johnson *et al.*, 2010).

Quite importantly, all these predictions assume that each AMF taxon occupies a defined nutritional niche (Treseder & Allen, 2002). This assumption is underpinned by the fact that AMF isolates differ in the benefits they provide to plants (Koch *et al.*, 2017), and by different clades (e.g. families) differing in susceptibility to fertilization regimes (van der Heyde *et al.*, 2017; Treseder *et al.*, 2018; Roy *et al.*, 2019). Using classical abundance measures (e.g., root colonization, hyphal length), which are frequently used to assess fertilization effects, it is not possible to capture differences in responses of different AMF taxa to nutrient enrichment (Treseder, 2004). Information at such higher level of resolution can only be obtained by sequencing surveys. Yet the scarcity of surveys of this kind in tropical areas has been repeatedly noted in the literature (Cotton, 2018; Lilleskov *et al.*, 2019), particularly for the tropical Andes (Soteras *et al.*, 2019). We are aware of only two deep sequencing studies conducted at AMF dominated neo-tropical forests within the context of nutrient manipulation experiments (Camenzind *et al.*, 2014; Sheldrake *et al.*, 2018). These studies showed AMF diversity decreases when N is added alone or in combination with P, while community structure is affected mainly by the addition of P. These responses, however, appear to be modulated by the fertilization regime, the duration and dosage of the application, and whether AMF communities were characterized from DNA isolated from roots or soil.

Given that virtually all aspects of AMF ecology are understudied in the tropics, it is evident that important gaps in our understanding still remain. First, studies conducted on tropical AMF communities in the context of increased nutrient supply are geographically narrow. Given whole ecosystem manipulations are resource intensive, these can only be maintained over relatively small areas (Fayle *et al.*, 2015). Hence the majority of such experiments in the tropics have been established in mesic lowland forests that grow over P-deficient soils (Matson *et al.*, 1999; Mirmanto *et al.*, 1999; Kaspari *et al.*, 2008; Cusack *et al.*, 2011). In tropical montane forests, however, plants obtain most of their nutrients from thick layers of organic detritus of very heterogeneous nutritional condition (Tanner *et al.*, 1998; Wilcke *et al.*, 2002). This heterogeneity is thought to originate from the interaction of parent material of different age and composition (Hoorn *et al.*, 2010) with climate (i.e. thermal isoclines, cloud immersion, seasonal precipitation patterns, Rahbek *et al.*, 2019a) and topography (Tanner *et al.*, 1992; Werner & Homeier, 2015). In addition to the geographic bias, there is a temporal one. Up until now, assessments of tropical AMF communities within nutrient addition experiments have not been reproduced, thereby ignoring the temporal dimension of the disturbance (Zhang *et al.*, 2018). Finally, the majority of tropical nutrient manipulation experiments have set rates of mineral fertilization with the goal of assessing plant growth limitations (Tanner *et al.*, 1992; Mirmanto *et al.*, 1999; Kaspari *et al.*, 2008). These, however, often exceed the actual rates of atmospheric nutrient deposition that these regions experience (Cusack *et al.*, 2010).

In this paper, we assess the responses of tropical forest AMF communities to increased nutrient deposition in a more realistic scenario. We do so by surveying a seven year-long fully factorial nitrogen (N) and phosphorus (P) addition experiment in southern Ecuador (Homeier *et al.*, 2012). This experiment is fully replicated at three sites where P is the main limiting element for tree growth (Cárate-Tandalla *et al.*, 2018), but its availability, as well as that of mineralized N, is modulated by local environmental conditions (Martinson *et al.*, 2013). One of these sites was surveyed after two years of simulated atmospheric deposition (Camenzind *et al.*, 2014), indicating important short-term reductions in AMF species richness. Here we focus on assessing the long-term response and increasing the external validity of our results by including all three sites within the experiment. We hypothesized that: (1) there will be a decrease in AMF molecular diversity after fertilization in sites with greater P availability, (2) nutrient applications will shift AMF community composition, but these shifts will be mediated by ambient availability of nutrients at different sites, and (3) assuming AMF lineages differ in terms of nutrient use and exchange capacities, clade responses to nutrient applications will be also different. To the best of our knowledge, this constitutes the most encompassing assessment of nutrient addition effects on naturally occurring AMF dominated forests.

Materials and Methods

Study site

Experimental work occurred on three sites along the south eastern Andes of Ecuador. Sites are located at an average distance of 19 km and at an average elevation difference of 1000 m of each other, starting at *c.* 1000 m above sea level (m asl; Supporting Information Fig. S1). All sites are within protected areas and are covered by different forest types (Homeier *et al.*, 2013). The lowest site corresponds to pre-montane forest, the mid site to lower montane forests and the highest site to upper montane forest. Tree species turnover is complete between pre- and upper montane forests while fewer than five species are shared between lower montane and the other two forest types (Homeier *et al.*, 2013). Canopy openness and stand height are reduced, while fine root biomass sharply increases at the upper montane forest in relation to the other two forest classes (Moser *et al.*, 2011). From pre-

montane to upper montane forest, understorey vegetation becomes denser with decreasing canopy openness. This stratum is mainly composed of tree recruits, herbaceous monocots, ferns, and a few woody shrub species (J. Homeier, pers. comm).

Climate at the three sites is permanently humid and strongly influenced by the dominant easterlies coming from the Amazon. Radar and ground station data indicate high precipitation totals that increase towards the upper montane forest (2000–4500 mm yr^{-1}; Homeier *et al.*, 2010; Rollenbeck & Bendix, 2011). Precipitation patterns are weakly seasonal with a maximum usually distributed from April to July. Minima occur towards the end of the year (Sep-Dec), when the dominant easterlies briefly give way to westerlies coming from the Pacific Ocean (Oñate-Valdivieso *et al.*, 2018). Temperature regimes also shift between sites. Direct measurements of average daily temperature show a decrease from *c.* 19°C to *c.* 9°C between the pre- and upper montane sites (Moser *et al.*, 2007).

Soil physical and chemical characteristics also change between sites. Soils at the lower and upper montane sites are covered by 10–40 cm deep organic layers, have a propensity to water logging and a loamy mineral fraction (Wolf *et al.*, 2011; Werner & Homeier, 2015). At the pre-montane forest, soil texture becomes sandy, leading to a better drainage and the organic horizon depth is reduced close to 0 cm. Organic layers are generally acidic (pH range: 3–5) and suffer from chronic nutrient deficiencies. N and P availability tends to increase in the pre-montane forest relative to the lower and upper montane forests (Wolf *et al.*, 2011; Werner & Homeier, 2015). Despite of this, tree growth at all sites is predominantly limited by P availability (Graefe *et al.*, 2010; Cárate-Tandalla *et al.*, 2018).

Additional details of each site environmental characteristics can be found in Table S1.

Experimental design

A full factorial nutrient manipulation experiment started at each site in January 2008 (Homeier *et al.*, 2012; Fig. S1b). Since then, urea (50 kg of N ha^{-1} yr^{-1}) and monosodium phosphate (10 kg P ha^{-1} yr^{-1}) were applied manually every six months. These rates of application are moderate relative to the rates applied in similar experiments elsewhere (Liu *et al.*, 2015a; Sheldrake *et al.*, 2018) and correspond well to the annual rates of atmospheric deposition quantified at the lower montane forest site between years 2007–2012 (Velescu *et al.*, 2016; Wilcke *et al.*, 2019). Experimental factors are applied in a randomized block arrangement. That is, on each site there are four blocks of four plots each (16 plots per site, 48 plots total). Each block consists of three plots with different nutrient application regimes (+N, +P and +N+P) and one unfertilized plot (Ctrl). Ctrl plots were always located above fertilized plots to avoid fertilizer runoff. Fertilization regime was assigned randomly at the start of the experiment for the remaining plots. While randomization mitigates the effects of confounding sources of variability, blocking ensures greater homogeneity in environmental conditions between sets of plots. Plots are 400 m^2 and are at least 10 m apart to ensure independence of experimental units.

In August 2015, a 10 cm soil core (Ø 5 cm) was extracted from the organic layer of six sub-plots (4 m^2) within each treatment plot. Sub-plots were randomly established along two orthogonal transects. We sampled one core within each sub-plot. This yielded a total of 96 cores per site (Fig. 1). In order to standardize our sampling procedure, *c.* 20 fine root pieces of 1–2 cm length and < 2 mm diameter were separated from the organic layer of each soil core and subsequently preserved in 97% EtOH. Roots were favored over soil, because DNA extraction from the organic

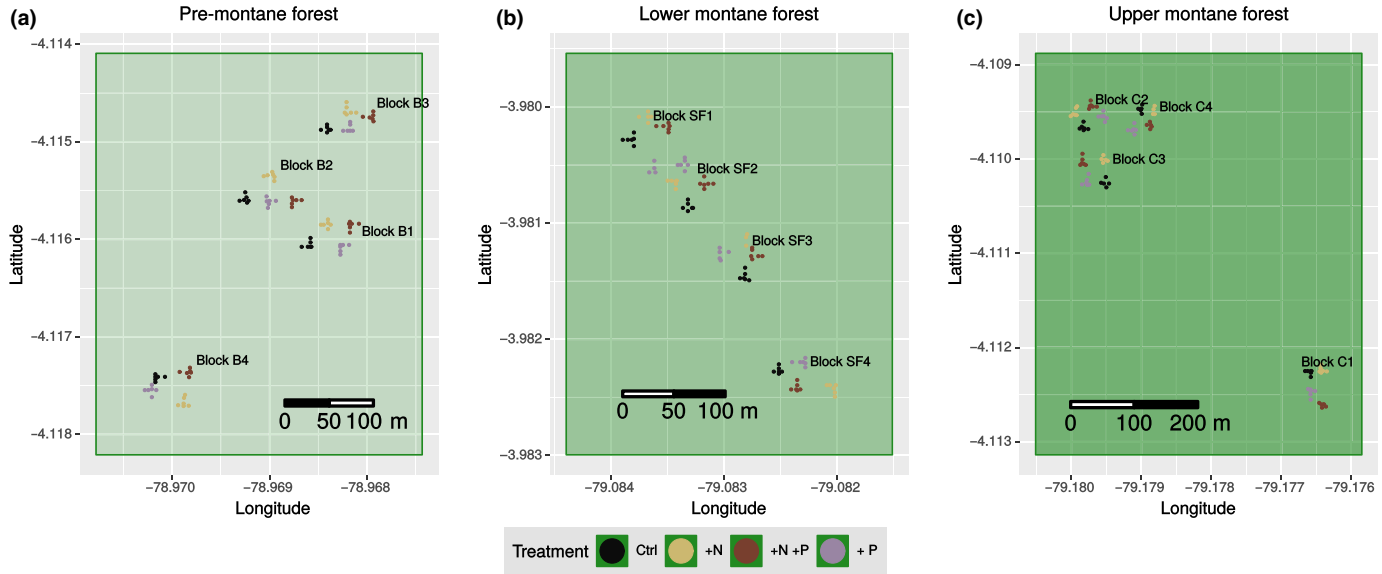

Fig. 1 Spatial distribution of cores collected for this study. Each cluster of six points of the same color represent cores within a plot according to their fertilization regime. Text indicates the relative position of blocks at each site, each encompassing four plots (i.e. 24 cores). Core position was allocated randomly using two orthogonal transects within each plot. Map coordinates are in decimal degrees and polygons in various shades of green intend to remind the reader that each site is different in terms of soil and plant community composition, forest structure and climate.

layer of these forests is cumbersome and hinders amplification. Samples were kept frozen upon their transport to the molecular ecology laboratories at the Institute for Biology of Freie Universität Berlin, where they were finally stored at −20°C.

DNA extraction, PCR amplification and sequencing

Roots from each of the samples were lyophilized overnight (Alpha 1-4 LDplus; Christ GmbH, Harz, Germany). Upon lyophilization, roots were pulverized by shaking a 2 mm metal bead along with roots in a 2 ml tube placed within a MM400 mill (2 min of 25 oscillations per second, Retsch GmbH., Hann, Germany). DNA was isolated from pulverized roots following the PowerSoil DNA isolation kit (MoBio Laboratories Inc., Carlsband, CA, USA) standard protocol. DNA extracts were stored at −20°C upon amplification. In order to minimize contamination, blank extracts were included, and all materials used were sterilized.

The genetic polymorphism within the nuclear rDNA operon was assessed adopting a nested PCR strategy. DNA extracts were amplified with a cocktail of Glomeromycotina specific primer sets developed by Krüger *et al.* (2009), in two consecutive PCR rounds. A third and final PCR round targeted a *c.* 400 bp fragment spanning the D1 and D2 variable domains of LSU with the LR2rev–LR3 primer set (Roy *et al.*, 2017). Details of the PCR conditions and amplicon library preparation can be found in the Methods S1. Amplicon libraries were sequenced in three separate reactions on an Illumina MiSeq platform using 2 × 250 paired-end chemistry at the Berlin Center for Genomics in Biodiversity Research (BeGenDiv).

Bioinformatic processing and taxonomic assignment

Paired-end reads were processed in USEARCH v.10 (Edgar, 2010). Reads from each site were processed separately for the merging, primer sequence removal and filtering steps. Reads that passed the filtering criteria were then combined in a single file for subsequent steps. MOTHUR (Schloss *et al.*, 2009) was employed to retain sequences with at least 375 bp and less than seven homopolymers. Sequences were clustered *de novo* into operational taxonomic units (OTUs) with UPARSE (Edgar, 2013), the minimum OTU cluster size was set to 8 and sequence similarity threshold to 97%. Chimera removal and clustering occurred simultaneously. Merged reads of each site were then mapped to OTUs to produce an OTU abundance table. Sequences representing these OTUs are deposited at the European Nucleotide Archive (ENA), under accession nos. LR656271–LR656682.

Phylotype taxonomic identity was assigned by aligning OTUs to Krüger *et al.* (2012) reference database using BLAST+ (Camacho *et al.*, 2009). Only the query sequences with alignment coverage ≥ 90% were retained. Following Martinez-García *et al.* (2015), an OTU was assigned to species level when the best hit was ≥ 97% identical to a reference sequence, to genus when identity was between 90–96%, and to family when identity was between 80–90%.

Environmental factors

One composite sample of the organic layer was created by aggregating and homogenizing six sub-plot samples extracted from each plot ($n = 48$). Air dried samples were then transported to the plant ecology laboratories at the University of Göttingen, Germany. Soil pH was determined by suspension of the sample in a KCl solution; organic soil C and N with a C/N analyzer (Vario EL III; Elementar, Hanau, Germany) and plant-available P with the resin-bag method (Amer *et al.*, 1955). Finally, all trees with a diameter at breast height ≥ 10 cm were identified to species level in order to calculate tree species richness per plot.

Statistical analyses

All statistical analyses were performed in R (v.3.4.3; R Core Team, 2017). Packages ADESPATIAL (Dray *et al.*, 2019), DESEQ2 (Love *et al.*, 2014), DPLYR (Wickham *et al.*, 2018), GGPLOT2 (Wickham, 2016), GGPUBR (Kassambara, 2018), LME4 (Bates *et al.*, 2015), LMERTEST (Kuznetsova *et al.*, 2017), MVABUND (Wang *et al.*, 2012), PHYLOSEQ (McMurdie & Holmes, 2013), RGDAL (Bivand *et al.*, 2019), SP (Bivand *et al.*, 2013) and VEGAN (Oksanen *et al.*, 2018) were employed. The commands used for the analyses can be found in Table S2.

Variability of environmental factors across sites and plots To visualize how environmental factors varied across plots and sites, variability was collapsed using a principal component analysis (PCA). Variables were scaled and centered and the two most informative axes were plotted.

Normalization of sequencing data As is typically observed in high throughput sequencing data, there was a high number of samples with few sequences and few samples with high number of counts (Fig. S2a). To account for the large differences in sequencing depth across samples, a variance stabilization transformation (VST) was applied (Love *et al.*, 2014). VST avoids rarefying to an arbitrary minimum sequencing depth while preserving the integrity of the data (McMurdie & Holmes, 2014; Sheldrake *et al.*, 2018). Applying VST normalized the density distribution of sequencing depth (Fig. S2b) while still allowing a sufficient coverage to characterize the diversity of AMF across samples (Fig. S3). Thus, the transformed table was used for all subsequent analyses.

AMF molecular diversity indices Following Morris *et al.* (2014), per sample AMF molecular diversity (hereafter referred as 'alpha diversity') was quantified by two indices: Hill number 0 (H_0) and 2 (H_2). H_0 and H_2 are generalized forms of popular diversity indices that facilitate comparisons across studies given they express taxonomic diversity in standardized units (Hill, 1973). H_0 equals richness (S) and expresses the number of OTUs per sample while H_2 equals to the inverse of Simpson's dominance index and expresses the effective number of 'abundant' OTUs per sample (Chao *et al.*, 2014). To visualize how alpha diversity partitioned between different families within

Glomeromycotina, H_0 and H_2 were also estimated by segregating OTU tables of the most represented families in our dataset (i.e. Acaulosporaceae, Glomeraceae and Gigasporaceae). To visualize AMF taxa turnover across sites, OTUs with relative abundance equal or greater to 1% were selected and their presence and relative abundance was plotted.

Effects of nutrient addition on AMF molecular diversity The response of AMF alpha diversity to fertilization was inferred through linear mixed effects models (LMMs; Bates *et al.*, 2015). To meet model assumptions, H_0 and H_2 estimates were square root transformed and specified as response variables. N and P were specified as fixed terms (i.e. $\text{sqrt}(H_0/H_2) \sim N \times P$). To account for the random variability imposed by the experimental design, a nested random term was specified (i.e. 1|Site/Block/Plot). Given that including all components of the random term led to model over-fitting (blocks contributed to explain 0 % of residual variability in H_0 and H_2, Table S3), the random structure of the models was re-specified as 1|Site/Plot. The full OTU dataset and the per-family OTU data sub-sets were fitted to this model structure. The difference from control in mean H_0/H_2 explained by the nutrient treatment regime, hereafter referred as the effect size, was used to infer the impact of nutrient addition on AMF alpha diversity. To visualize the magnitude and direction of these effect sizes and to provide a measure of uncertainty, 95% confidence intervals around effect sizes were estimated by refitting the model 1000 times with parametric bootstraps of the original data (Morris, 2002). In addition to this, we ascertained the effect of nutrient application regimes with classical null hypothesis significance testing by performing *t* tests. The null hypothesis was that the difference from control was not different from 0. Given that the current implementation of mixed models in LME4 package does not estimate *P*-values, these were determined via LMERTEST package (Kuznetsova *et al.*, 2017).

Effects of nutrient addition on AMF community composition The effects of fertilization on AMF community composition were examined with multivariate generalized linear models (MGLM; Wang *et al.*, 2012). MGLMs can handle multivariate response variables in which the variance is not constant (Warton *et al.*, 2012), which is the case here (Fig. S4). Given the compositional nature of the data (Gloor *et al.*, 2017), phylotype proportions cannot be considered to represent the abundance of AMF taxa in the environment. Consequently, to assess if fertilization elicits a change in AMF community composition, we focused on OTU occurrence data. Because our goal was to assess if the effect of each fertilization factor differed among sites – and since MGLMs cannot handle random effects, a separate model for each site was specified. Spatial dependencies in OTU presence within each site were accounted for by Moran eigenvectors maps (MEMs, Dray *et al.*, 2006). MEMs were estimated according to the method developed by Bauman *et al.* (2018b). This is both an estimation and selection procedure that yields a set of MEMs that optimally describe the spatial structures observed in biotic communities (Bauman *et al.*, 2018a). Thus, the selected MEMs were specified as predictors in each of the MGLMs (i.e. OTU occurrence ~ MEMs + N × P). The variance structure for all three models was specified as binomial. Finally, deviance tests were performed on each MGLM to measure the strength of nutrient addition effects on AMF community composition. If the sequential inclusion of explanatory terms significantly increased the fit of the data in relation to a reduced model, then such factor was considered to have a significant influence on OTU occurrence. In addition to this, distance based redundancy analysis plots based on Jaccard dissimilarity matrices were employed to visualize the effects of treatments on AMF community composition (RDA; Legendre & Anderson, 1999). One RDA per site was specified as a two-way model (N × P), including MEMs as conditional covariates.

Sensitivity analysis To test the robustness of our results and compare to previously observed short-term effects (Camenzind *et al.*, 2014), the whole dataset was re-analyzed with traditionally applied statistical procedures (i.e. rarefying to a common minimum depth and PERMANOVA; Anderson, 2001; Oksanen *et al.*, 2018). Details of these procedures are presented in the Methods S2.

Results

Taxonomic delineation and assignation

A total of 280 samples were amplified and generated 12 625 525 merged reads. Six samples with less than 10 reads each were discarded as they were considered defective and 503 495 unique sequences were retained after filtering. These sequences were clustered in 628 OTUs at 97% similarity, of which 65.6% (412 OTUs, 87.77% of reads) identified with known Glomeromycotina sequences. All Glomeromycotina OTUs were assigned to three orders and six families, but c. 75% of these reads could be assigned to a known genus.

Environmental variation and AMF community properties across sites

PCA of environmental factors indicated that environmental conditions in plots at the lower and upper montane forests were similar and differed from the conditions at the pre-montane forest site (Fig. 2a). In general, all experimental plots were characterized by low fertility and acidic soils. However, soils at the lower and upper montane plots had lower N and greater P availability than soils at the pre-montane site. In contrast, soils at the pre-montane site had higher pH and supported more diverse tree communities (Table S4). AMF OTU accumulation curves indicate that pre-montane plant communities hosted on average 126 more OTUs than lower and upper montane forests, which relative to each other reached a very similar number of OTUs (Fig. S5). Alpha diversity and relative abundance of reads of the most represented families within Glomeromycotina traced this pattern. While in the pre-montane plots OTUs assigned to Glomeraceae were more diverse and encompassed a greater proportion of reads than Acaulosporaceae, at both lower and upper montane sites OTUs

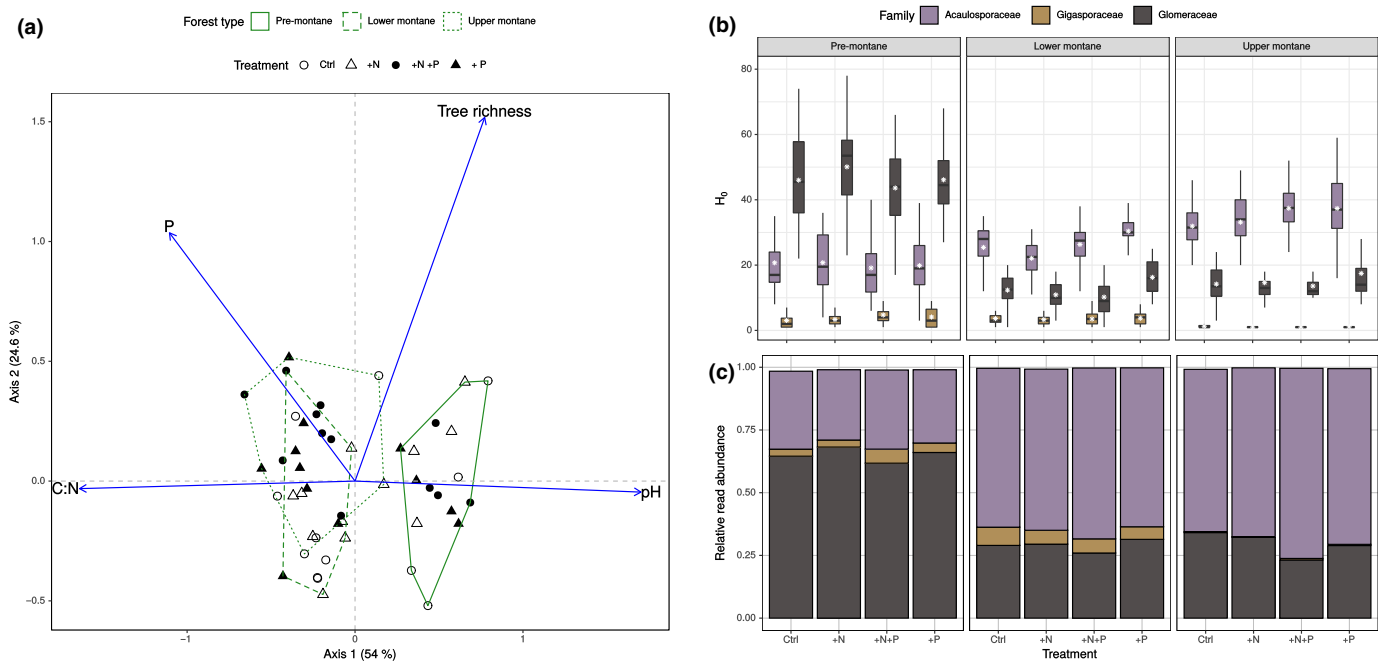

Fig. 2 Site variability in environmental parameters and relative abundance of phylotypes assigned to main clades within Glomeromycotina. (a) Principle component analysis (PCA) of soil organic layer parameters and tree species richness. Two axes were sufficient to capture 79.3% of within site variability in soil parameters ($n = 48$). Closed and open symbols represent homogenized soil samples according to fertilization regime and hulls enclose all samples within a site. (b) H_0 (Richness) of phylotypes and (c) proportion of reads assigned to the most represented families across sites. White stars within boxplots represent the mean while the mid-horizontal line represents the median.

assigned to Acaulosporaceae were more diverse and contributed with a greater proportion of reads than Glomeraceae (Fig. 2b,c). Turnover of the most represented OTUs within these families, however, was strong across sites. None of the aforementioned OTUs occurred in all three sites whereas *c.* 10% of these OTUs were shared between the lower montane and one of the other two sites (Fig. 3).

Effects of nutrient addition on AMF molecular diversity

Responses of diversity indices to fertilization regime were minimal and statistically insignificant when analyzing all sites together (Table S5). Closer inspection of effect sizes estimated for each site confirmed these differences were not biologically meaningful at any site (Fig. 4a). When the analysis was partitioned among families, no effect was observed with only one exception. Glomeraceae mean H_0 and H_2 decreased by 5.2 (± 2.4 SE) and 3.7 (± 1.7 SE) OTUs respectively ($P = 0.02$ and 0.02) as a response to the combined addition of N and P. The negative effect of the combined addition of N and P on Glomeraceae was consistent across sites (Fig. 4b).

Effects of nutrient addition on AMF community composition

Deviance tests indicate that nutrient addition consistently affected AMF community composition at every site (Table 1). Fertilization effects on community composition were dependent on the nutrient added and the ambient nutrient status at each site. While adding N did not elicit a shift in AMF community composition only in the pre-montane forest, adding P alone consistently elicited community shifts at all sites. Given that the most represented OTUs across sites are present in all fertilization regimes, the shifts detected by deviance tests are most likely driven by the appearance and disappearance of rare OTUs. Test results were robust to the inclusion of eigenvector maps, which also increased the fit of every model significantly. This suggests that, in addition to the fertilization effects, spatially structured factors also contribute to explain the observed variability in AMF community composition. RDAs on Jaccard dissimilarity index are congruent with this result, as these indicate that nutrient factors explained on average 3.87% variability in AMF community structure, while conditioned MEMs explained 15.5% (Fig. 5).

Sensitivity tests

Re-analysis of the dataset, using more traditionally employed statistical procedures, did not change results qualitatively (Tables S6 and S7). Rarefying to a minimum depth of 850 reads eliminated 3 AMF OTUs compared with VST. PERMANOVA on Jaccard distances found addition of P affects AMF community composition except for the upper montane forest site. In contrast the addition of N alone or in combination with P did not elicit shifts in AMF community composition.

Discussion

Our cross-site analysis indicates that tropical montane forests harbor highly diverse AMF communities that appear to be structured by site specific environmental conditions. We provide

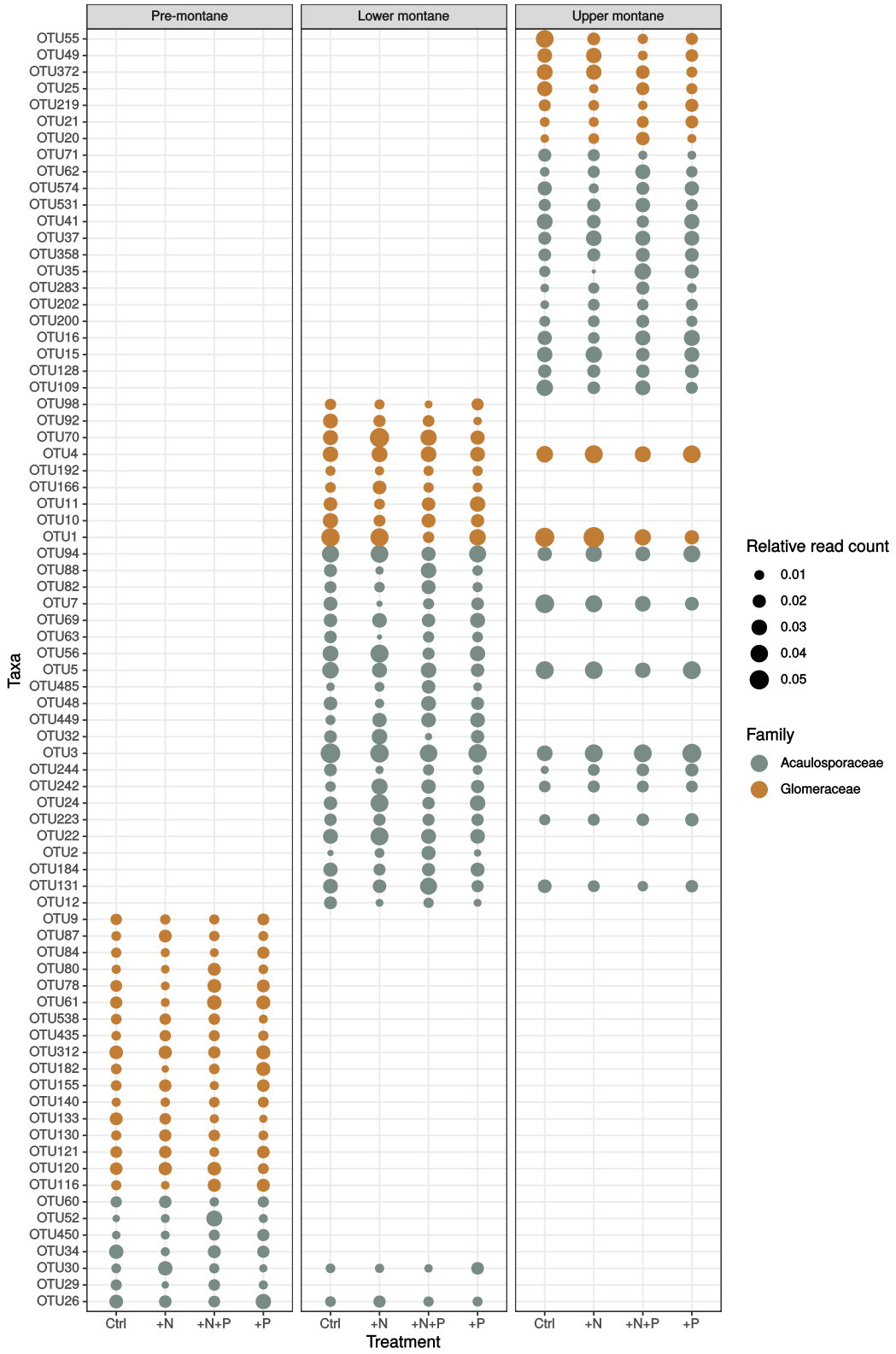

Fig. 3 Turnover of the most abundant operational taxonomic units (OTUs) across the three sites where the nutrient manipulation experiment took place. OTUs were selected if their relative abundance was greater than 1% of the total. Taxa are ordered by family to emphasize their turnover across sites.

evidence that indicates seven years of moderate N and P fertilization rates have affected AMF community composition but not richness, a finding consistent among sites. Nutrient effects are indeed mild, but remain clear even when spatial dependencies in AMF community composition are accounted for. Our results further suggest that fertilization effects depend on site ambient

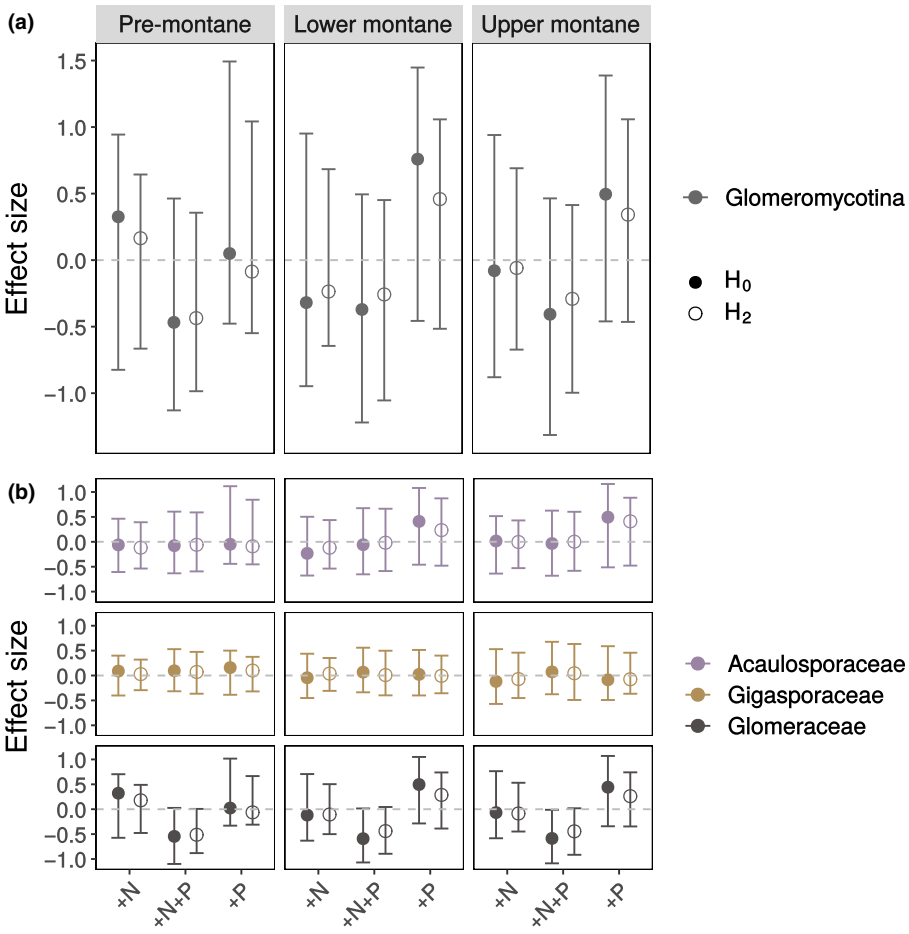

Fig. 4 Estimated differences in mean H_0 (Richness) and H_2 (1/Simpson's dominance) of fertilized plots in relation to controls at each experimental site. Overall differences at the sub-phylum level are presented in (a) while (b) presents differences at the family level. The magnitude of the differences is presented in the square-root scale. Open and closed symbols represent point estimates and whiskers represent their 95% confidence intervals estimated by refitting the model 1000 times with parametric bootstraps of the original data. A 0.5 increase or decrease represents a difference of *c.* 5 units.

Table 1 Deviance test results parameters describing how each predictor contributed to improve the fit of the observed data to the model. MEMs stand for Moran's Eigenvector maps.

Site	Model	df	Deviance	*P*
Pre-montane	*c.* 1	95		
	+ MEMs	86	4857.440	<0.001
	+ N	85	393.527	0.553
	+ P	84	593.784	<0.001
	+ N : P	83	520.412	<0.001
Lower montane	*c.* 1	88		
	+ MEMs	79	2862.472	<0.001
	+ N	78	260.035	0.030
	+ P	77	331.201	<0.001
	+ N : P	76	297.538	<0.001
Upper montane	*c.* 1	88		
	+ MEMs	80	2368.596	0.003
	+ N	79	329.737	0.003
	+ P	78	399.214	<0.001
	+ N : P	77	215.143	0.054

nutrient status, since N addition did not affect AMF communities in pre-montane forests, while P shifted community composition independently of soil nutrient status. Furthermore, the composition of the regional OTU pool was site specific and the response to fertilization was clade-specific, suggesting differences among AMF clades in terms of their adaptation to different nutrient conditions. Overall, our results indicate that the rate of atmospheric nutrient deposition experienced by these forests constitutes a modest, yet consistent disturbance for AMF communities.

Both the phylotype pool and mean richness in our study sites are one of the highest so far reported for AMF, yet still fall within the boundaries of previous global AMF diversity assessments (Kivlin *et al.*, 2011; Davison *et al.*, 2015). Our observations that there was a substantial turnover of AMF taxa at different sites are also congruent with recent literature that found a strong influence of elevation on AMF beta diversity (Geml *et al.*, 2014; Kivlin *et al.*, 2017; Haug *et al.*, 2019). Given that metabarcoding studies are not consistent in the strategies adopted to arrive at OTU definitions (Lekberg *et al.*, 2014; Hart *et al.*, 2015), and that elevation is a compound variable that usually involves a number of inter-related climatic, topographic and soil variables, it is not possible to generalize this pattern to other areas in the Andes. Nonetheless, recent reports of high AMF molecular diversity on both dry (Rodriguez-Echeverria *et al.*, 2017; Morgan & Egerton-Warburton, 2017) and wet (Bachelot *et al.*, 2017; García de León *et al.*, 2018) tropical lowland forests lend support to the idea that tropical Andean forests harbor highly diverse AMF communities. Acaulosporaceae higher abundance and richness at sites with acidic pH and low N availability is congruent with the characterization of members of this clade as stress tolerant (Oehl *et al.*,

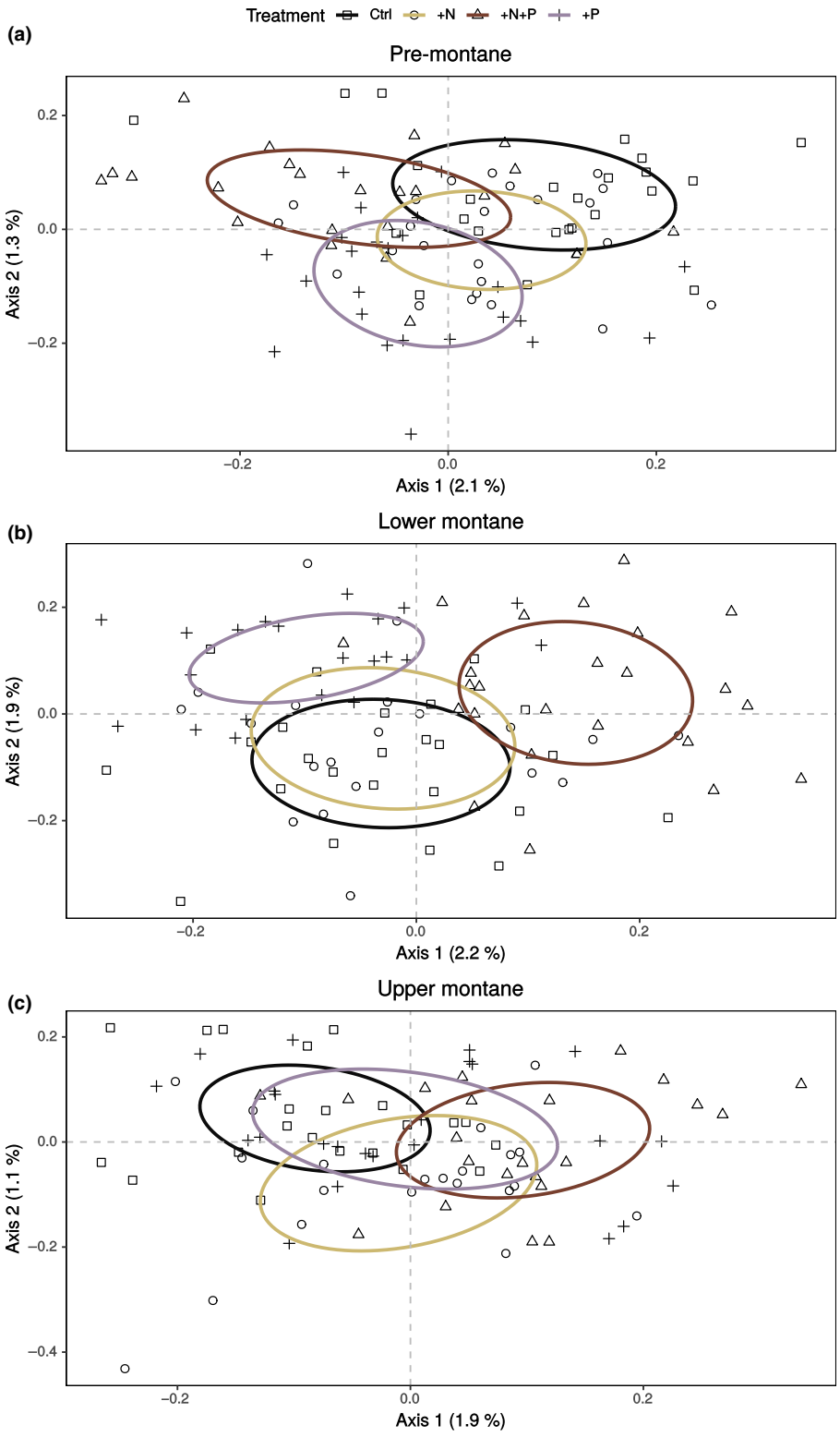

Fig. 5 Constrained ordination plots depicting the influence of nutrient addition on arbuscular mycorrhizal fungi (AMF) communities. Panels (a–c) present one ordination per site. Pairwise Jaccard distances were estimated from a normalized operational taxonomic units (OTU) table. Ellipses represent one standard deviation from group centroids. Two axes explained 4.3%, 4.5% and 3.9% variability in AMF community composition, after spatial dependencies were conditionally partialled out.

2009; Veresoglou *et al.*, 2013; Liu *et al.*, 2015b). By contrast, high abundance of Glomeraceae at the site with the lowest C/N ratio among the set is in line with the association of this clade with higher N availability (Treseder *et al.*, 2018).

We found little support for our first hypothesis that predicted an overall negative effect of fertilization on AMF alpha diversity, which included sites with a slightly higher P availability. These results deviate from the short-term responses reported during an earlier assessment at the lower montane forest site (Camenzind *et al.*, 2014). As re-analyzing our data with traditional statistical approaches yielded qualitatively similar results, it is unlikely our observations are caused by a technical bias. Rather, these results

could be attributed to temporal variability in the response of AMF communities to increased nutrient supply. Wide shifts in the response of AMF intraradical structures to fertilization over time have been observed in our study sites (Camenzind *et al.*, 2016). Alternatively, given that classic fertilization experiments have typically applied N and P at much higher rates (Egerton-Warburton *et al.*, 2007; Liu *et al.*, 2012; Sheldrake *et al.*, 2018), the rather moderate rate employed in this study could have allowed AMF communities to respond to the new nutrient condition without impacting taxonomic richness. Multiple examples of neutral responses of AMF richness as a function of fertilization dosage can be found in the literature (Alguacil *et al.*, 2010; Vályi *et al.*, 2015). Overall, these results support the notion that the intensity and duration of fertilization could be modulating the responses of AMF both in terms of abundance (Zhang *et al.*, 2018), alpha and beta diversity (Roy *et al.*, 2017).

In line with our second hypothesis, N and P addition did affect AMF community composition, with the effects of P addition the most consistent factor across sites. This is congruent with previous reports at both our study area and other tropical forests (Alguacil *et al.*, 2010; Camenzind *et al.*, 2014; Sheldrake *et al.*, 2018). It also suggests that AMF in this region are primarily involved in P for C transactions and that ambient nutrient status is important to consider when attempting to predict AMF root community responses to fertilization, as adding N alone did not affect AMF community composition as consistently as P. The addition of P may select for taxa with better ability to hoard P in order to maximize carbon gains from the host (Whiteside *et al.*, 2019). Our results also indicate that spatially structured ecological processes are influencing how AMF communities in these forests assemble. As this study was not designed to disentangle and quantify the relative importance of different ecological processes on AMF community composition, we can only speculate about this point. Previous studies have shown that at small to intermediate spatial scales, neutral and environmental drivers interact to determine the structure of AMF communities (Caruso *et al.*, 2012; Veresoglou *et al.*, 2019). In tropical forests, there is wide array of available hosts which are likely employing a variety of strategies to cope with nutrient limitations (Nasto *et al.*, 2014; Sayer & Banin, 2016; Baez & Homeier, 2018). Yet the degree to which individual tree species may influence the distribution and assemblage of AMF communities has yet to be firmly established in the tropics. For instance, a single AMF phylotype has been shown to associate with as many as 28 species of trees in one of our study sites (Haug *et al.*, 2013). What appears more likely, is that the composition of AMF communities inferred from mixed root samples is simultaneously reflecting the variability introduced by the host, fine scale edaphic factors, stochastic processes and priority effects. In order to identify the drivers behind these patterns, new field assessments that quantify environmental variation at smaller spatial scales are required.

We observed differential responses to fertilization of clades within Glomeromycotina in terms of taxonomic diversity, which lends some support to our third hypothesis. Differential trait expression (Chagnon *et al.*, 2013) might explain this contrasting response to some extent. Taxa within Acaulosporaceae are known to exhibit slow growth, both intra and extra radically (Hart & Reader, 2002). These traits have traditionally been associated with high carbon use efficiency. Following this logic, it is plausible that the negligible effect of fertilization on richness of this lineage is explained by their efficient use of carbon. By contrast, Glomeraceae consistent reduction in taxonomic diversity after N and P additions suggests that some members of this clade have greater carbon demands. As certain members of this clade tend to exhibit a fast colonization rate and greater investment in intra radical growth (Hart & Reader, 2005), it could be argued that they have a less efficient use of carbon and possibly provide less P for C benefit to the host (Pearson & Jakobsen, 1993). If this is so, nutrient addition might promote their down regulation by the host or their competitive exclusion by those taxa that indeed make a more efficient use of available C (Kiers *et al.*, 2011). Despite our observations fitting well with a differential trait expression framework, the highlighted traits might also vary at the species level (Maherali & Klironomos, 2012; Koch *et al.*, 2017). Since trait information only exist for a fraction of AMF isolates, at this stage we simply miss empirical information to clearly link AMF traits to nutrient requirements or function. This prevents us to unequivocally establish whether differential adaptations to nutrient supply are the basis for the patterns reported here.

In conclusion, AM fungal communities appear to have adjusted to moderate nutrient additions at all experimental sites by shifting their composition relative to control sites, while species richness remained stable. These changes are more subtle than predicted by studies using higher doses of experimental fertilization, yet its robustness and consistency clearly suggest that such responses to ongoing atmospheric deposition can also be expected across the tropical Andes. Regarding functional implications, selection of AMF clades that invest less in extra-radical mycelium might reduce C storage below ground and retention of surplus products of N mineralization (Baldos *et al.*, 2015; Velescu *et al.*, 2016). Changes in AMF community structure elicited by fertilization could also set feedback loops in motion (Bever *et al.*, 2012; Neuenkamp *et al.*, 2018). This could favor plants adapted to high nutrient availability and promote their dominance (Baez & Homeier, 2018). In the long-term, an increasing dominance of fewer plant hosts, the so called 'homogenization' of the mycorrhizal environment (Caruso *et al.*, 2012), could support less diverse AMF communities (Alguacil *et al.*, 2012; Liu *et al.*, 2012; Johnson *et al.*, 2015). In order to fully capture the functional consequences for these ecosystems we need to gain a better understanding of how AMF taxa functional roles differ in these diverse ecosystems, and on how fine scale structural heterogeneity shapes AMF communities in tropical forests. Future research needs to tackle how AMF community parameters vary at finer temporal, spatial and phylogenetic resolutions. Most importantly, complementary studies about AMF nutrient demands, host effects and feedbacks deserve further attention.

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Author contributions

JFD performed laboratory work, analyzed the data and wrote the manuscript. TC collected samples and assisted with data analysis. JR and SH assisted with bioinformatics and data analysis. JH designed the field experiment, collected samples and conducted environmental sample analysis. JPS assisted with data collection. MCR designed the study. TC, JR, SH, JH, JPS and MCR contributed with ideas and revised the manuscript.

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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 Study area, site location and experimental design schematic.

Fig. S2 Density distribution of sequencing depth across sequencing runs

Fig. S3 Per sample and per treatment rarefaction curves.

Fig. S4 Relationship between mean OTU presence and its variance.

Fig. S5 Taxon accumulation curves estimated from variance stabilized data.

Methods S1 Nested PCR conditions and library preparation.

Methods S2 Description of methods for data re-analysis or sensitivity tests.

Table S1 Experimental sites detailed environmental properties.

Table S2 Summary of the commands and packages used for statistical analysis.

Table S3 Contribution of each random term component to explain residual variability in H0 (Richness) and H2 (1/Simpson's dominance).

Table S4 Means and standard errors of environmental factors.

Table S5 Estimates and statistical tests derived from linear mixed effect models fitted with data normalized with variance stabilizing transformation.

Table S6 Estimates and statistical tests derived from linear mixed effect model fitted with data normalized by rarefying to 850 read minimum depth.

Table S7 Two-way PERMANOVA results based on Jaccard dissimilarity matrices estimated with data normalized by rarefying to 850 read minimum depth.

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