

1 **Responses of arbuscular mycorrhizal fungi to long-term inorganic and**  
2 **organic nutrient addition in a lowland tropical forest**

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31  
32 **ABSTRACT**

33  
34 Improved understanding of the nutritional ecology of arbuscular mycorrhizal (AM) fungi is  
35 important in understanding how tropical forests maintain high productivity on low fertility  
36 soils. Relatively little is known about how AM fungi will respond to changes in nutrient  
37 inputs in tropical forests, which hampers our ability to assess how forest productivity will be  
38 influenced by anthropogenic change. Here, we assessed the influence of long-term inorganic  
39 and organic nutrient additions and nutrient depletion on AM fungi, using two adjacent

40 experiments in a lowland tropical forest in Panama. We characterised AM fungal  
41 communities in soil and roots using 454-pyrosequencing, and quantified AM fungal  
42 abundance using microscopy and a lipid biomarker. Phosphorus and nitrogen addition  
43 reduced the abundance of AM fungi to a similar extent, but affected community composition  
44 in different ways. Nutrient depletion had a pronounced effect on AM fungal community  
45 composition, affecting nearly as many OTUs as phosphorus addition. The addition of  
46 nutrients in organic form (leaf litter) had little effect on any AM fungal parameter. Soil AM  
47 fungal communities responded more strongly to changes in nutrient availability than  
48 communities in roots. This suggests that the ‘dual niches’ of AM fungi in soil versus roots are  
49 structured to different degrees by abiotic environmental filters, and biotic filters imposed by  
50 the plant host. Our findings indicate that AM fungal communities are fine-tuned to nutrient  
51 regimes, and support future studies aiming to link AM fungal community dynamics with  
52 ecosystem function.

53

## 54 INTRODUCTION

55

56 Arbuscular mycorrhizal (AM) fungi are an ancient, major group of plant symbionts that  
57 facilitate the uptake of limiting soil nutrients by plants in exchange for plant carbon (C)  
58 (Smith and Read, 2008). The majority of tropical trees—which make up 59% of global forest  
59 vegetation—depend on AM fungi (Dixon *et al.* 1994; Alexander and Lee, 2005; McGuire *et*  
60 *al.*, 2008; Averill *et al.*, 2014). This may help to explain how tropical forests account for  
61 nearly 40% of terrestrial net primary productivity, while occupying only 12% of the Earth’s  
62 land surface and frequently occurring on infertile soils (Townsend *et al.*, 2011; Camenzind *et*  
63 *al.*, 2017). Although most lowland tropical soils are strongly weathered and were thought to  
64 be P-limited, recent evidence suggests that multiple limiting nutrients interact to limit forest

65 productivity and function (Kaspari *et al.*, 2008; Wright *et al.*, 2011; Camenzind *et al.*, 2017).  
66 To anticipate future effects of anthropogenic change on tropical forest systems, an  
67 understanding of how nutrients limit forest productivity is required (Townsend *et al.*, 2011;  
68 Bonan *et al.*, 2012). However, AM fungi are severely understudied in tropical forests  
69 (Alexander and Selosse, 2009; Mohan *et al.*, 2014), and despite the well-established role for  
70 AM fungi in improving plant access to P (Smith and Read, 2008), their roles in lowland  
71 tropical forests remain unclear.

72  
73 There are two main mechanisms by which changes in nutrient availability could affect AM  
74 fungi. Nutrient addition may alleviate direct nutrient limitation of fungal growth, particularly  
75 where the background availability of nutrients is low. Conversely, nutrient addition could  
76 alter the symbiotic exchange of resources between plant and fungal partners, particularly  
77 where background nutrient availability is higher (Treseder and Allen, 2002; Johnson *et al.*,  
78 2010; Hodge *et al.*, 2010): AM fungi incur a substantial C cost to their plant partners (Smith  
79 and Read, 2008), and plants are therefore likely to reduce their C investment in AM fungi  
80 when nutrients are readily available (Johnson *et al.* 2010). Furthermore, plants may  
81 preferentially allocate C to AM fungal partners that supply required nutrients under more  
82 favourable ‘terms of trade’ (Bever *et al.*, 2009; Kiers *et al.*, 2011; Zheng *et al.*, 2014; Bever,  
83 2015).

84  
85 AM fungi are major actors in global C and nutrient cycles (Johnson *et al.* 2013; Rillig, 2004),  
86 and even small changes in the regulation of C flux into AM fungi could have a large global  
87 impact (Orwin *et al.*, 2011). This is particularly true of tropical forests, which are responsible  
88 for at least one third of terrestrial C flux (Cleveland and Townsend, 2006). The availability of  
89 nutrients regulates the allocation of plant C to AM fungi (Johnson, 2010), and the addition of

90 nutrients in inorganic or organic form can have quite different effects on nutrient pools in  
91 tropical forests and elicit markedly different responses from plants (Sayer *et al.*, 2012).  
92 However, few studies have compared the relative effects of inorganic and organic nutrient  
93 additions on AM fungal communities, and to our knowledge, no such studies have taken  
94 place outside temperate agricultural settings. This type of comparison is important because  
95 experimental inorganic and organic nutrient additions can reveal different aspects of AM  
96 fungal ecology. On the one hand, organic matter inputs are the primary route for the cycling  
97 of nutrients under natural conditions (Attiwill and Adams, 1993), and simulate the conditions  
98 under which the regulatory behaviours governing plant-AM fungal relations have evolved.  
99 By contrast, inorganic nutrient additions can highlight the role of specific limiting nutrients,  
100 and provide insight into possible ecosystem responses to anthropogenic nutrient deposition.  
101  
102 Two parallel, long-term field experiments in a lowland tropical forest in Panama provided a  
103 unique opportunity to unravel the relative importance of the form (organic versus inorganic),  
104 amount, and balance of nutrients (the bulk addition of litter versus single or paired inorganic  
105 nutrients) on AM fungal ecology. The Gigante Fertilisation Project (GFP) is a factorial NPK  
106 addition experiment that allowed us to evaluate AM fungal responses to the addition of  
107 inorganic nutrients alone or in factorial combination. The Gigante Litter Manipulation Project  
108 (GLMP) at the same site consists of control, litter addition, and litter removal treatments,  
109 which allowed us to evaluate AM fungal responses to both a doubling, and the removal of  
110 organic matter – a nutrient depletion treatment. Nutrient depletion is an important but rarely  
111 performed approach to understand nutrient limitation patterns in ecosystems (Sullivan *et al.*  
112 2014).

113

114 Together, these experiments allowed us both to investigate the primary nutrients driving  
115 plant-AM fungal interactions and assess the degree to which AM fungal communities are  
116 structured by resource-based environmental filters in both components of their ‘dual niche’:  
117 plant roots and soil (Valyi *et al.*, 2016). Specifically, we hypothesised:

118

119 1) Given the well-established role of AM fungi in plant P acquisition (Smith and Read,  
120 2008), the low availability of P in weathered lowland tropical soils (Vitousek, 1984),  
121 and the role of P in limiting tree distributions in this region (Condit *et al.*, 2013), P  
122 addition should cause the strongest changes in AM fungal abundance and community  
123 composition.

124 2) Nutrient addition should alter the ecological processes structuring AM fungal  
125 communities, leading to changes in the degree of relatedness (or phylogenetic  
126 dispersion) of AM fungal communities.

127 3) Given the different roles played by intra- and extra-radical AM fungal phases in  
128 acquiring C and nutrients respectively, AM fungal communities in the soil should be  
129 more sensitive to nutrient additions than those in roots.

130 4) The addition of single inorganic nutrients—which can create nutrient imbalances—  
131 should have a greater effect on AM fungal metrics than the simultaneous addition or  
132 removal of all nutrients with litter manipulation.

133

134

## 135 METHODS

136

### 137 **Site description and experimental design**

138 We sampled roots and soil in two parallel long-term experiments in a lowland tropical forest  
139 in Panama. The GFP was established in 1998, and had been running for 15 years at the time  
140 of sampling (Wright *et al.*, 2011). The GLMP was started in 2003, and had been running for  
141 nine years at the time of sampling (Sayer *et al.*, 2010).

142

143 We sampled from five treatments across the GFP (N, P, K, NP, and unfertilised controls).  
144 Each treatment was applied to four replicate 40 m × 40 m plots across the 38.4-ha study site.  
145 Annual doses are 125 kg N ha<sup>-1</sup> yr<sup>-1</sup> as coated urea, 50 kg P ha<sup>-1</sup> yr<sup>-1</sup> as triple superphosphate,  
146 and 50 kg K ha<sup>-1</sup> yr<sup>-1</sup> as potassium chloride (SI methods; Figure S1; Wright *et al.*, 2011).  
147 Phosphorus addition increased soil phosphate availability by 2800%; K-addition increased K  
148 availability by 91%; N-addition increased inorganic N availability by 120% and reduced pH  
149 from 5.25 to 4.47 (Mirabello *et al.*, 2013; Yavitt *et al.*, 2011; Turner *et al.*, 2013).

150

151 The GLMP consists of fifteen 45 m × 45 m plots. The leaf litter in five litter removal plots is  
152 raked up monthly (L-), distributed across five litter addition plots (L+), with five plots left as  
153 controls (Sayer and Tanner, 2010). Litter addition increased soil phosphate and calcium (Ca)  
154 availability by 47% and 57% respectively, and did not significantly alter inorganic N. Litter  
155 removal reduced soil P, inorganic N and Ca availability by 35%, 43%, and 53%, respectively.  
156 Neither litter treatment had significant effects on K (Sheldrake *et al.*, 2017a).

157

158 The GLMP litter addition and the GFP inorganic nutrient addition treatments supplied similar  
159 amounts of N and K to the plots as the inorganic N- and K-addition treatments (143 vs. 125  
160 kg N ha<sup>-1</sup> y<sup>-1</sup> and 39 vs. 50 kg K ha<sup>-1</sup> y<sup>-1</sup> for the GLMP and GFP, respectively). In contrast,  
161 the litter addition treatment added only 12% of the P added in the GFP (5.8 kg ha<sup>-1</sup> y<sup>-1</sup> vs. 50

162 kg ha<sup>-1</sup> y<sup>-1</sup>; Sayer *et al.* 2012), because greater inputs of inorganic P were necessary to  
163 overcome the P-sorption common to the soils at the study site.

164

## 165 **Sampling**

166 We sampled soil and roots from the four replicate N, P, K, NP and control plots in the GFP  
167 and from the five replicate L-, L+ and control plots in the GLMP (total of 35 plots) over two  
168 weeks in September 2012, at the peak of the growing season. In each plot, we collected 81  
169 soil samples (9 × 9 grid) at 0-10 cm depth, and composited them to make one sample per  
170 plot. To control for the effects of host identity on AM fungal parameters, we sampled roots  
171 from seedlings of seven of the most common tree species at the study site, harvesting 4-6  
172 seedlings per species per plot (*c.* 1300 seedlings in total; SI methods). In using seedlings, this  
173 study differs from previous studies at this site that used mixed root samples from cores  
174 (Wurzburger and Wright, 2015; Sheldrake *et al.*, 2017a). In this study, we do not provide an  
175 analysis of individual seedling species.

176

## 177 **AM fungal abundance**

178 We quantified colonisation of seedling roots by AM fungi using microscopy (staining with  
179 trypan blue), as described in Sheldrake *et al.* (2017b; SI methods); used the neutral lipid fatty  
180 acid (NLFA) 16:1 $\omega$ 5 as a biomarker for extra-radical AM fungal biomass in the soil (Olsson,  
181 1999; SI methods); and extracted and counted spores from the soil. We identified spores to  
182 family level using morphological characteristics, with reference to the International Culture  
183 Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi (INVAM;  
184 <https://invam.wvu.edu>; SI methods). The use of the biomarker lipid provides a root length-  
185 independent measure of net AM fungal abundance. Sheldrake *et al.* (2017b) previously  
186 published the colonisation and NLFA data from the GFP (N, P, NP, and C treatments).

187

## 188 **DNA extraction and sequencing**

189 Root and soil samples were individually pulverised in a homogeniser prior to DNA extraction  
190 (TissueLyser II, Qiagen). An equal mass of each root sample was pooled to make one  
191 composite sample per species per plot. We extracted DNA from pulverised roots and soil  
192 using MoBio PowerPlant and PowerSoil DNA isolation kits according to the manufacturer's  
193 instructions (MoBio Laboratories Inc., Carlsbad, CA, USA). We amplified the partial small  
194 subunit (SSU) region of 18S ribosomal DNA (*c.* 550 bp) with the universal eukaryotic primer  
195 NS41 (Simon *et al.*, 1992) and the AM fungal-specific primer AM1, which amplifies the  
196 major families of the Glomeromycota (Helgason *et al.*, 1998). Amplicon libraries were  
197 sequenced on an FLX Titanium system (Roche, Basel, Switzerland) at the Cambridge DNA  
198 Sequencing Facility (Department of Biochemistry, University of Cambridge, UK).

199

## 200 **Bioinformatic analysis**

201 Bioinformatic processing followed Sheldrake *et al.* (2017a; SI methods). Briefly, reads were  
202 removed from the dataset if they had > 1 error in the MID barcode sequence, > 2 errors in the  
203 forward primer, were shorter than 200 bp, or had an average quality score below 25 over any  
204 40 bp portion of the sequence. Clustering was performed using the algorithm Clustering 16S  
205 rRNA for Operational Taxonomic Unit (OTU) Prediction (CROP; Hao *et al.*, 2011).  
206 Sequence alignment was performed with the software MAFFT v7.149b (Kato *et al.*, 2002)  
207 and improved with MUSCLE (Edgar, 2004). We used the Basic Local Alignment Search  
208 Tool (BLAST; Altschul *et al.* 1990; minimum e-value  $10^{-30}$ ) on one representative sequence  
209 from each cluster iteratively against three databases in the following order of preference: i)  
210 sequences from Krüger *et al.* (2012); ii) all virtual taxa (VT) from the MaarjAM AM fungal  
211 sequence database ([www.maarjam.botany.ut.ee](http://www.maarjam.botany.ut.ee)); and iii) all 18S glomeromycotan sequences



212 from the SILVA database. Clusters were named based on matches to database entries at >  
213 97% similarity covering a minimum of 80% of the query sequence. Where clusters did not  
214 match a VT at > 97%, we assigned a name based on the highest VT match and phylogeny  
215 (eg. *Glomus\_OTU1*). Raw sequence data were deposited in the International Nucleotide  
216 Sequence Database Sequence Read Archive (accession no. SRP076949). Sequencing data  
217 from soil and seedlings in N, P, NP and control plots was previously published (Sheldrake *et*  
218 *al.* 2017b).

219

## 220 **Statistical analysis**

221 All statistical analyses were conducted in R, version 3.1.2 (R Development Core Team,  
222 2014).

223

224 We performed separate analyses for GFP and GLMP due to their different designs. The GFP  
225 includes four replicates per treatment in an incomplete block design, and ‘replicate’ was used  
226 in all models as a spatial blocking term to control for natural variation across the site (Wright  
227 *et al.*, 2011). For GFP data, we tested for N × P interactions (omitting the K treatment) using  
228 factorial models, and for the K treatment in a separate one-way model with a single  
229 ‘treatment’ term, using treatment contrasts to test the significance of K-addition relative to  
230 controls (we did not sample from all treatments so could not use the full factorial design). For  
231 the analysis of GLMP data, we built one-way models with a single ‘litter treatment’ term,  
232 using treatment contrasts to compare each treatment with controls. Each experiment had its  
233 own set of control plots. We calculated log response ratios and confidence intervals to allow  
234 visual comparison between experiments (Nakagawa and Cuthill, 2007). The SI presents  
235 figures showing the absolute value of variables.

236

237 To determine overall root AM fungal responses to treatments, we averaged across seedling  
238 species to calculate a pooled root response for each metric and plot. Three of the seedling  
239 species were absent from the litter removal treatment. To make results comparable between  
240 all treatments, we present only analyses based on the four remaining species. Unless  
241 otherwise indicated, analysis of all seven species led to the same conclusions.

242

### 243 *Analysis of AM fungal communities*

244 To account for variation in the number of sequences among samples, we used a variance  
245 stabilising (VS) transformation of the OTU table, implemented with the DESeq2 package  
246 (Anders and Huber, 2010). VS transformations use a mixture model framework based on the  
247 negative binomial distribution, and avoid the need for rarefaction, which fails to account for  
248 overdispersion, and can bias the results towards false positives (McMurdie and Holmes,  
249 2014; Hart *et al.*, 2015). We performed all subsequent analysis on the VS transformed OTU  
250 table, with root values calculated as the mean of individual seedling species, and using the  
251 copy number of DNA sequences as a measure of relative abundance of OTUs (SI methods).

252

253 To examine the effect of experimental treatments on AM fungal community composition, we  
254 used multivariate generalised linear models (M-GLMs) with negative binomial error  
255 structures using the mvabund package (Wang *et al.*, 2012), building separate models for root  
256 and soil communities. To compare the relative effects of treatment on root and soil  
257 communities, we built an M-GLM to test for the interaction between experimental treatment  
258 and ‘sample type’ (root or soil). We evaluated the degree to which individual OTUs were  
259 affected by litter manipulation using DESeq2 (Anders and Huber, 2010), which estimates the  
260 effect size of treatments relative to controls (as logarithmic fold change; SI methods).

261

262 We asked whether experimental treatments altered the degree of relatedness among taxa in  
263 AM fungal communities (or phylogenetic dispersion), using the Net Relatedness Index (NRI)  
264 as an index of community phylogenetic structure. Positive values of NRI indicate that taxa in  
265 a community are on average more closely related to each other than to members of the  
266 regional taxon pool (phylogenetically clustered), and negative values indicate that taxa in a  
267 community are less closely related (phylogenetically over-dispersed; Webb, 2000; SI  
268 methods).

269

#### 270 *Univariate analysis of AM fungal abundance and diversity*

271 We used linear models to analyse: i) the concentration of NLFA 16:1 $\omega$ 5 in the soil and, ii)  
272 the percentage of seedling root length colonised by AM fungi. We analysed spore counts  
273 using generalised linear models (GLMs) with Poisson errors (Venables and Ripley, 2002;  
274 Crawley, 2012). We built separate models for the total spore number and the number of  
275 spores in each family. Spatial blocking terms were included for the above analyses. We  
276 analysed the total number of AM fungal OTUs (richness), the proportional abundance of the  
277 most dominant taxon (predominance), and the NRI metric with linear mixed effects models  
278 (lme4 package; Bates *et al.*, 2015). The significance of fixed effects was assessed using  
279 likelihood ratio tests (LRT) and parametric bootstrapping. We modelled the relationship  
280 between occurrence frequency (the proportion of plots in which a given OTU is found) of  
281 AM fungal taxa in soil and root communities, using fixed dispersion beta regression (SI  
282 methods).

283

284 Additional details of all procedures and analyses are given in the SI methods.

285

## 286 RESULTS

287

## 288 **AM fungal abundance**

289 The amount of the AM fungal biomarker (NLFA 16:1 $\omega$ 5) in the top 10 cm of mineral soil  
290 was *c.* 30% lower with N-addition and *c.* 25% lower with P-addition ( $F_{1,9} = 11.2, P = 0.009$ ;  
291  $F_{1,15} = 6.3, P = 0.03$ , respectively; Figure 1b, S2). There was a significant overall effect of  
292 litter manipulation on the amount of NLFA 16:1 $\omega$ 5 in the soil, suggesting a trend towards a  
293 positive effect of litter addition and a negative effect of litter removal ( $F_{2,8} = 5.4, P = 0.03$ ,  
294 Figure 1b, Figure S2), but individual treatment contrasts were not statistically significant.  
295 Neither inorganic nutrient addition nor litter manipulation influenced the total number of AM  
296 fungal spores in the soil (Figure 2, S3). Across all treatments, we identified spores belonging  
297 to three families, Glomeraceae, Acaulosporaceae, and Gigasporaceae. The Glomeraceae  
298 constituted *c.* 90% of the total spore pool, Acaulosporaceae *c.* 10%, and Gigasporaceae *c.*  
299 0.4%. Separate analyses by family showed that Acaulosporaceae spores were more abundant  
300 in plots where N and P were added together (N+P) relative to the treatments where either  
301 nutrient was added alone (N  $\times$  P interaction,  $\chi^2 = 6.1, P = 0.01$ ; Figure 2, S3). There was no  
302 effect of inorganic nutrient addition on spores of the Glomeraceae or Gigasporaceae and no  
303 effect of litter manipulation on the number of spores from any family.

304

305 AM fungal colonisation of seedling roots was *c.* 18% lower with both N- and P-addition ( $F_{1,9}$   
306  $= 6.9, P = 0.03$ ;  $F_{1,9} = 7.2, P = 0.02$ , respectively; Figures 1a, S4) but was unaffected by litter  
307 manipulation. When the analysis was repeated with the additional three species (seven in  
308 total), there was a marginally significant N  $\times$  P interaction, whereby AM fungal colonisation  
309 of seedling roots was lower with the addition of N and P together compared to either N or P  
310 addition alone (N  $\times$  P interaction:  $F_{1,9} = 5.0, P = 0.05$ ).

311

312 K-addition had no significant effects on any of the metrics assessed in this study and is  
313 therefore not reported.

314

### 315 **AM fungal OTUs and sequencing**

316 Rarefaction curves for each sample indicated that sequencing intensity was sufficiently high  
317 to detect the majority of OTUs and that sampling effort was sufficient to capture AM fungal  
318 diversity across the sites (Figure S7). A total of 222 748 sequences were retained after quality  
319 control and clustered into 226 OTUs, of which 62 OTUs (corresponding to 22 069 sequence  
320 reads, 9.9% of total reads) matched either non-glomeromycotan taxa in the sequence  
321 databases or failed to match with any accessions in the database. OTUs remaining after  
322 blasting, filtering, merging, and trimming (exclusion of OTUs arising from only one sample  
323 or with a total of 5 reads or fewer), represented a total of 200 554 sequences. The number of  
324 OTUs and sequences per sample averaged 24 OTUs (range: 9 - 45) and 1146 sequences  
325 (range: 328 - 2117).

326

### 327 **AM fungal richness and predominance**

328 The total number of AM fungal OTUs (OTU richness) was *c.* 35% higher in soil than in roots  
329 in both the GFP and GLMP (LRT = 42.4,  $P < 0.001$  and LRT = 35.6,  $P < 0.001$  for GFP and  
330 GLMP, respectively). The mean number of OTUs was similar between experiments both for  
331 soil (GFP: 34 OTUs and GLMP: 35 OTUs) and root samples (GFP: 21 OTUs and GLMP 23).  
332 The occurrence frequency of AM fungal OTUs in soil and roots was strongly correlated (beta  
333 regression;  $\chi^2 = 196.3$ ,  $P < 0.001$ ; Figure S5), indicating that AM fungal OTUs that were  
334 common in soil communities also tended to be common in root communities. However, the  
335 proportional abundance of the most dominant taxon at a site (predominance) was higher in

336 root AM communities than soil communities (LRT = 42.0,  $P < 0.001$ ; Figure S6). The SI  
337 discussion provides a full description of sequencing results.  
338  
339 N-addition reduced OTU richness in both soil and roots (LRT = 17.9,  $P < 0.001$ , Figure 3a, b,  
340 S7), but the negative effect of N-addition on OTU richness was stronger in soil than in root  
341 samples (N  $\times$  'sample type' interaction; LRT = 6.9,  $P = 0.03$ ; Figure 3a, b, S8).  
342 Predominance in both sample types was *c.* 27% higher with N-addition (LRT = 17.9,  $P <$   
343  $0.001$ ; Figure 3c, d, S7). P-addition did not affect OTU richness or dominance; however, P-  
344 and N-addition together mitigated reductions caused by N-addition (N  $\times$  P interaction: LRT =  
345 8.1,  $P = 0.007$ ; Figure 3a, b). In the GLMP, there was a non-significant trend towards lower  
346 OTU richness in the soil in both litter manipulation treatments relative to controls  
347 ('treatment' term: LRT = 4.4,  $P = 0.17$ ; Figure 3a, b, S8). Predominance increased with litter  
348 addition in both soil and root communities (Full model LRT = 9.4,  $P = 0.02$ ; Figure 3c, d) but  
349 was unaffected by litter removal.

350

### 351 **AM fungal community composition**

352 Within all treatments, soil and root samples had distinct AM fungal community composition  
353 (treatment  $\times$  sample type interaction: Deviance = 960.3,  $P = 0.001$ ; Figure S9) and a greater  
354 number of OTUs were affected by nutrient manipulation in the soil than in roots (Figure 4  
355 and Table S1). There was a clear separation of AM fungal communities in plots with P-  
356 addition, regardless of sample type (soil: Deviance = 608.3,  $P < 0.001$ ; roots: Deviance =  
357 268.7,  $P = 0.002$ ; Figure 5a, b). In soil samples, the effect of P-addition on AM fungal  
358 community composition differed according to whether N was also added (N  $\times$  P interaction;  
359 Deviance = 254.8,  $P = 0.001$ ; Figure 5a, 4). A similar pattern was observed in root  
360 communities, although the N  $\times$  P interaction was only marginally significant (Deviance =

361 189.3,  $P = 0.06$ ; Figure 5b, 4) and there was no effect of N-addition alone. Litter removal  
362 altered AM fungal community composition in both soil and roots (soil: Deviance = 202.3,  $P$   
363  $< 0.001$ ; roots: Deviance = 181.3,  $P = 0.007$ ; Figure 5a, b), whereas litter addition only  
364 altered the composition of communities in soil (Deviance = 131.5,  $P = 0.01$ ; Figure 5a, b).  
365 Tables S2 and S3 present all OTUs significantly affected by experimental treatments.

366

367 AM fungal communities were no more phylogenetically clustered or dispersed than expected  
368 by chance (i.e. relative to simulated null communities), and there was no effect of any  
369 experimental treatment on the relatedness of taxa in AM fungal communities when the  
370 analysis was conducted with four seedling species. However, when the analysis was repeated  
371 with the additional three seedling species, N+P reduced the relatedness of taxa in AM fungal  
372 communities relative to treatments where they were added separately, across root and soil  
373 communities (N  $\times$  P interaction: LRT = 7.0,  $P = 0.01$ ; Figure S11).

374

## 375 DISCUSSION

376

### 377 **Primary nutrients driving plant-AM fungal relations**

378 We found support for our hypothesis that the addition of inorganic nutrients should have a  
379 stronger effect than litter addition. Both N- and P-addition reduced AM fungal abundance in  
380 roots and soil (colonisation and NLFA 16:1 $\omega$ 5, respectively) in a similar manner (Figure 1),  
381 whereas litter addition had no effect. Nutrient addition may affect AM fungi directly by  
382 alleviating fungal nutrient limitation (leading to an increase in fungal abundance), or  
383 indirectly, by altering plant C investment in their AM fungal symbionts. The second  
384 alternative may involve selection for AM fungi that provide more nutritional benefits or are  
385 better competitors for plant C, usually leading to a decrease in fungal abundance (Bennett and

386 Bever, 2009). Hence, the observed reductions in AM fungal abundance imply that plants  
387 reduced their investment in AM fungi as nutrients became more readily available following  
388 N- and P-addition, and suggest a role for AM fungi in both plant N and P acquisition under  
389 normal conditions (Johnson, 2010). That no nutrient treatment increased AM fungal  
390 abundance suggests that AM fungi at this site are not directly limited by nutrients apart from  
391 root-derived C (Treseder and Allen, 2002). These findings are consistent with the results of a  
392 global meta-analysis of AM fungal responses to N and P, which found that overall, N and P  
393 decreased AM fungal abundance, despite significant variability in AM fungal responses to N  
394 (Treseder, 2004).

395

396 Previous studies at this site have found strong evidence for plant K limitation, notably in root  
397 responses (Yavitt *et al.* 2011, Wright *et al.* 2011, Wurzburger and Wright, 2015). We  
398 observed no significant effects of K-addition on any AM fungal metric. This suggests that  
399 AM fungi do not play a role in plant K nutrition in this system, and/or that root C allocation  
400 to AM fungi does not vary as a function of plant K status.

401

402 Although N- and P-addition reduced AM fungal abundance by similar amounts (Figure 1),  
403 AM community parameters responded quite differently to N- versus P-addition. N-addition  
404 reduced OTU richness and increased predominance (Figure 3), whereas P-addition alone had  
405 no effect on OTU richness but when added with N (the N+P treatment), alleviated the  
406 reduction in OTU richness associated with N-addition (Figure 3). Furthermore, P-addition  
407 had much stronger effects on overall community composition than N-addition (Figure 5). The  
408 reduction in richness and increase in predominance following N-addition suggests that plants  
409 increasingly rely on a subset of AM fungi when P becomes more limiting. By contrast, when  
410 P limitation was reduced, AM fungi in soil and roots maintained their diversity despite the



411 decline in abundance, perhaps pointing to a role for the fungal partners in providing other  
412 nutrients or benefits to plants. These findings suggest that N- and P-additions affect plant-  
413 AM fungal relations in different ways and agree with a previous study at this site which  
414 suggested a strong effect of P-addition on plant-AM fungal relations without a concomitant  
415 effect of N-addition (Sheldrake *et al.*, 2017b).

416

417 We found that addition of N and P in combination reduced the relatedness of taxa in AM  
418 fungal communities relative to treatments where N and P were added separately. Taxa that  
419 share a common evolutionary history can also share traits and ecological functions (Maherali  
420 & Klironomos 2007; Powell *et al.* 2009). According to this principle – known as  
421 phylogenetic trait conservatism – an increase in phylogenetic dispersion suggests that AM  
422 fungal communities in N+P treatments experience increased competitive interactions among  
423 taxa, preventing closely related and functionally similar taxa (those sharing a common niche)  
424 from co-occurring. This possibility is consistent with a reduction in C supplied by plant hosts  
425 in response to N and P addition (as suggested by reduced AM fungal abundance), which  
426 would force AM fungi to compete for increasingly limited resources. However, the effect of  
427 N+P treatments on phylogenetic structure was weak and should be interpreted with caution.

428

429 Our results contrast with an earlier study at this site which found that AM fungal colonisation  
430 in mixed root cores decreased with N-addition but increased with P-addition (Wurzburger  
431 and Wright, 2015). Wurzburger and Wright (2015) used mixed cores, dominated by the roots  
432 of sun-exposed canopy adults, while we sampled roots from deeply shaded, understory  
433 seedlings. Given that photosynthetically fixed C represents the plant currency of symbiotic  
434 exchange, different degrees of light limitation could cause adult and seedling plants to adjust  
435 their investment in AM fungi in different ways in response to nutrient addition. However, in

436 the present study, AM fungal abundance in the soil (as indicated by the lipid biomarker)  
437 shows a similar response to nutrient addition as the AM fungal colonisation of seedling roots,  
438 suggesting that seedling colonisation levels reflect response of extra-radical AM fungal  
439 abundance to nutrient addition. We lack a good explanation for this discrepancy with our  
440 findings.

441

442 Interpretation of the effects of N-addition on AM fungal communities is complicated because  
443 ten years of N-addition reduced the pH from 5.25 to 4.47 (Turner *et al.*, 2013). However, the  
444 variety of AM fungal responses to reduced pH in the literature makes it difficult to determine  
445 which responses can be attributed to the decrease in pH. Low pH has been shown to reduce  
446 AMF spore production, colonisation and extra-radical hyphal growth (Daniels and Trappe,  
447 1980; Wang *et al.*, 1993; Clark, 1997; van Aarle *et al.*, 2002), and reduce AMF OTU richness  
448 (Kohout *et al.*, 2015). Accordingly, many of the effects of N that we observed in this study  
449 may be explained by a reduction in pH. Nonetheless, it is unlikely that pH entirely explains  
450 the observed effects of N on AM fungal community composition because: i) the N+P  
451 treatments clustered far more closely with P treatments than with N treatments (Figure 5),  
452 and ii) The addition of N+P did not reduce OTU richness, while the addition of N alone did.  
453 If lower soil pH explained the observed N effect, we would expect the AM fungal community  
454 in the N+P treatments (soil pH *c.* 4.8) to have a similar community composition and richness  
455 to N treatments (soil pH *c.* 4.5).

456

#### 457 **AM fungal responses in both components of their ‘dual niche’**

458 Soil and root communities differed from each other across all treatments (Figure S9). This  
459 may be because different AM fungal taxa have contrasting life history (Sýkorová *et al.*, 2007)  
460 or root-colonisation strategies (Dodd *et al.*, 2000; Hart and Reader, 2002), which can alter the

461 relative proportion of AM fungal taxa in intra- versus extra-radical phases (Clapp *et al.*,  
462 1995; Hempel *et al.*, 2007).

463

464 We hypothesised that AM fungal communities would be more sensitive to nutrient  
465 manipulation in soil than in roots because the intra- and extra-radical AM fungal phases play  
466 different roles in nutrient acquisition (the extra-radical phase obtaining nutrients from the  
467 substrate, and intra-radical phase obtaining fixed C from the plant). Our results support this,  
468 as most treatments (N-addition, P-addition, L+ and L-) altered AM fungal communities more  
469 strongly in the soil than roots (Figure 5). Similar effects of P-addition on AM fungi were  
470 reported in a recent study in maize fields (Liu *et al.*, 2016), and there is evidence that intra-  
471 and extra-radical phases are subject to different degrees of limitation depending on the  
472 relative availability of N, P and plant C (Hodge and Fitter, 2010). In this study, the greater  
473 sensitivity to experimental treatments of AM fungi in the soil suggests that extra-radical  
474 phases may be more sensitive to abiotic environmental filters, and intra-radical phases more  
475 sensitive to filters imposed by the plant host (such as preferential allocation; Werner *et al.*,  
476 2015). This intriguing possibility warrants further investigation.

477

478 Together with other studies performed at this site, our findings indicate that some treatments  
479 caused changes in overall plant belowground allocation (measured as fine root biomass)  
480 without appearing to affect plant allocation to AM fungi, and vice versa, suggesting a fine  
481 degree of control over C allocation to different belowground structures. K-addition reduced  
482 fine root biomass (Yavitt *et al.*, 2011, Wright *et al.*, 2011, Wurzbürger and Wright, 2015)  
483 while litter addition increased fine root biomass (Sayer *et al.*, 2006), with neither treatment  
484 affecting AM fungal abundance or communities (this study). By contrast, N- and P-addition  
485 reduced plant belowground allocation to fine roots (Wurzbürger and Wright, 2015), while

486 also reducing AM fungal abundance and altering AM fungal communities and increasing the  
487 sporulation of Acaulosporaceae when added together (this study).

488

### 489 **Inorganic versus organic nutrient addition**

490 As expected, the effects of litter manipulation on AM fungal abundance and community  
491 composition were generally not as strong as the effects of inorganic nutrient addition. This  
492 may be because inorganic treatments – particularly P – added a greater amount of fast-release  
493 nutrients than the litter addition treatment. As the amount of P added in the inorganic P-  
494 addition treatment was much greater than the amount added with litter, the potential influence  
495 of nutrient source is confounded by differences in nutrient amount. However, findings from  
496 other studies conducted at this site suggest that N and P added as litter were more available  
497 (in the case of N) or comparably available (in the case of P) to plants as the inorganic N and  
498 P added in the GFP. For example, litter N concentrations increased with litter addition but not  
499 with inorganic N-addition, despite the fact that both treatments supplied similar amounts of  
500 N. By contrast, although the litter addition treatment supplied only *c.* 12% of the P added as  
501 inorganic fertiliser, the estimated additional P-return with increased litterfall was very similar  
502 between litter addition and inorganic P treatments (*c.* 1.2 kg P ha<sup>-1</sup> y<sup>-1</sup> versus 1.4 kg P ha<sup>-1</sup>  
503 y<sup>-1</sup>; Sayer *et al.*, 2012). Consequently, if the effects of inorganic N- and P-addition were  
504 solely due to plants altering their C investment in AM fungi in response to requirements  
505 (according to the trade balance model; Johnson, 2010), we would expect to see comparably  
506 large effects of litter addition on AM fungal abundance and AM fungal communities in roots.

507

508 However, despite the large amounts of nutrients added with litter, AM fungal abundance in  
509 roots was unchanged (Figure 1a), and AM fungal abundance in soil tended to increase in

510 response to litter addition. These findings suggest that plants may have experienced the  
511 increases in N or P from organic versus inorganic sources in different ways.

512

513 We propose three possible reasons for the distinct responses of AM fungi to nutrients from  
514 organic versus inorganic sources. First, AM fungi are better than plant roots at acquiring  
515 nutrients from organic nutrient pools as opposed to inorganic pools, such that plant C  
516 allocation to their AM fungal associates were maintained despite the net increases in the  
517 amount of nutrients on the forest floor following litter addition. In other words, plants still  
518 needed AM fungi to fulfil the same nutritional function even though the supply of nutrients  
519 from organic matter had increased (Sheldrake *et al.*, 2017a). This possibility is also raised by  
520 Vargas *et al.* (2010), who reported increased AM fungal root colonisation in response to  
521 substantial organic matter inputs after a hurricane. A second possibility is that nutrient  
522 stoichiometry (as opposed to the absolute quantity of a nutrient) regulates plant-AM fungal  
523 relations (Azcón *et al.*, 2003; Blanke *et al.*, 2005; Johnson, 2010), and the addition of one or  
524 two inorganic nutrients, such as in the N-, P-, and N+P-addition plots, may have a larger  
525 effect on AM fungi than litter addition by creating greater nutrient imbalances (and thus  
526 potentially greater plant limitation and demand). Finally, AM fungi may have a ‘priming’  
527 effect, through which they stimulate other soil microbes in the rhizosphere involved in  
528 nutrient cycling via decomposition of organic matter (Herman *et al.*, 2012, Nuccio *et al.*,  
529 2013), providing a net benefit to plants, which could cause them to maintain C allocation to  
530 AM fungal symbionts.

531

532 The pronounced effect of litter removal on AM fungal community composition also suggests  
533 that nutrients from organic sources play an important role in AM fungal nutrition and  
534 function. Litter removal differs from all other treatments in that it involves the depletion

535 rather than the addition of nutrients but it affected nearly as many OTUs as P addition (Figure  
536 5), even though there was no effect on AM fungal abundance in roots, and only a marginal  
537 effect in soil (Figure 1). Interestingly, AM fungal community composition in roots in the  
538 litter removal treatment was similar to that in the N-addition treatment (Figure 5b) suggesting  
539 that both treatments exerted similar selective pressures on the AM fungal communities. We  
540 speculate that this could be due to an increase in plant demand for P in both treatments.  
541 Although the litter removal treatment reduced litter and foliar N concentrations, there was no  
542 reduction in the concentration of P in the litter (Sayer *et al.*, 2010; 2012), nor a reduction in  
543 seedling foliar P (Sheldrake *et al.*, *Unpublished Data*). This suggests that plants were able to  
544 maintain adequate P supply from alternative organic or inorganic sources in the soil,  
545 potentially due to a shift towards P-specialist AM fungal taxa (Sheldrake *et al.*, 2017a).

546

#### 547 **Concluding remarks**

548 We present a large, experimental dataset which helps to elucidate the roles of AM fungi in  
549 the lowland tropics, and provides a key reference for future studies hoping to link AM fungal  
550 community dynamics with symbiotic function, or integrate AM fungi into ecosystem models,  
551 notably those incorporating nutrient limitation (Townsend *et al.*, 2011). We show that P is  
552 the primary nutrient driving plant-AM fungal interactions in this lowland tropical forest,  
553 suggesting that AM fungi are a key mechanism by which tropical forests maintain  
554 productivity on low-P soils. Interestingly, while both N- and P-additions elicited reductions  
555 in AM fungal abundance, AM fungal communities showed a pronounced, yet distinct  
556 response to N- and P-addition. Our findings suggest that AM fungal interactions with plants  
557 are more sensitive to nutrient imbalances than to the bulk addition of nutrients with leaf litter,  
558 and suggest that plants depend on AM fungi to acquire nutrients from organic nutrient pools.  
559 The finding that soil and root communities differed in their responses to nutrient availability

560 provides evidence that the ‘dual niches’ of AM fungi are structured to different degrees by  
561 abiotic environmental filters and biotic filters imposed by the plant host, a possibility that  
562 warrants further testing. Future work should examine the functional significance of the  
563 observed shifts in AM fungal community and abundance in terms of both forest nutrition and  
564 C sequestration; the relative importance of AM fungi versus roots in nutrient uptake from  
565 different soil pools; and the mechanisms underlying shifts in plant-AM fungal relations in  
566 response to nutrient additions and altered nutrient stoichiometry.

567

#### 568 SUPPLEMENTARY INFORMATION

569

570 Supplementary information is available at ISME’s website.

571

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583

584

585 AUTHOR CONTRIBUTIONS

586

587 MS and SM designed the study. EVJT and EJS established the leaf litter manipulation  
588 experiment. SJW established the nutrient addition experiment. MS and DR performed the lab  
589 and field work. PAO supported the lipid analysis. NR conducted the bioinformatic analysis.  
590 MS conducted the statistical analysis and wrote the manuscript with input from all authors.

591

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## 797 FIGURE CAPTIONS

798

799 **Figure 1.** Effect of long-term inorganic and organic nutrient addition on AM fungal  
800 colonization in the roots of four seedling species (a) and on the concentration of the AM  
801 fungal lipid biomarker in the top 10 cm of forest soil (b). Significance was assessed using  
802 separate linear models for GLMP and GFP; significant effects are inset in panels above. Note  
803 that the overall effect of litter manipulation on NLFA 16:1 $\omega$ 5 was significant (ANOVA:  $F_{2,8}$   
804 = 5.4,  $P = 0.03$ ), although neither treatment significantly differed from controls. Values are  
805 log response ratios (not the predictions of the statistical models), and error bars represent  
806 95% confidence intervals obtained by bootstrapping with 9999 replicates. L- = litter removal;  
807 L+ = litter addition; N = nitrogen; P = phosphorus; NP = nitrogen + phosphorus; K =  
808 potassium.

809

810 **Figure 2.** Effect of long-term inorganic and organic nutrient addition on the abundance of  
811 AM fungal spores in the top 10 cm of forest soil. Significance was assessed using separate  
812 generalised linear models for GLMP and GFP; significant effects are inset in panels above.  
813 Values are log response ratios (not the predictions of the statistical models), and error bars  
814 represent 95% confidence intervals obtained by bootstrapping with 9999 replicates. L- = litter  
815 removal; L+ = litter addition; N = nitrogen; P = phosphorus; NP = nitrogen + phosphorus; K  
816 = potassium.

817

818 **Figure 3.** Effect of long-term inorganic and organic nutrient on the AM fungal OTU richness  
819 (a, b) and predominance (proportional abundance of the dominant AM fungal OTU; c, d) in

820 soil and root samples. Significance was assessed using separate linear models for GLMP and  
821 GFP; significant effects are inset in panels above (LRT = likelihood ratio test). Values are log  
822 response ratios (not the predictions of the statistical models), and error bars represent 95%  
823 confidence intervals obtained by bootstrapping with 9999 replicates. L- = litter removal; L+ =  
824 litter addition; N = nitrogen; P = phosphorus; NP = nitrogen + phosphorus; K = potassium.

825

826 **Figure 4.** Number of AM fungal OTUs significantly affected by long-term inorganic and  
827 organic nutrient addition. Significance was ascertained based on negative binomial Wald  
828 tests using standard maximum likelihood estimates for generalised linear models with *P*-  
829 values ( $\alpha = 0.05$ ) adjusted for multiple comparisons, as implemented in the DESeq2 package.  
830 L- = litter removal; L+ = litter addition; N = nitrogen; P = phosphorus; NP = nitrogen +  
831 phosphorus; K = potassium. Colours correspond to AM fungal genera.

832

833 **Figure 5.** Nonmetric multidimensional scaling (NMDS) ordination plot showing changes in  
834 AM fungal community composition in response to inorganic and organic nutrient-addition in  
835 soil (a) and root (b) samples in a lowland tropical forest in Panama. ‘Site’ scores are shown  
836 and ellipses describe 95% confidence limits. Ordinations are based on Bray-Curtis  
837 dissimilarity. Axes are scaled to half-change (HC) units, by which one HC unit describes a  
838 halving of community similarity. C1 = control treatment in GFP; C2 = control treatment in  
839 GLMP; L- = litter removal; L+ = litter addition; N = nitrogen; P = phosphorus; NP = nitrogen  
840 + phosphorus; K = potassium. Colours and symbol shapes correspond to different treatments.

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